Cross-Presentation by Dendritic Cells of Tumor Antigen Expressed in Apoptotic Recombinant Canarypox Virus-Infected Dendritic Cells

Iris Motta, Fabrice André, Annick Lim, James Tartaglia, William I. Cox, Laurence Zitvogel, Eric Angevin and Philippe Kourilsky

*J Immunol* 2001; 167:1795-1802; doi: 10.4049/jimmunol.167.3.1795

http://www.jimmunol.org/content/167/3/1795

References

This article cites 43 articles, 29 of which you can access for free at:

http://www.jimmunol.org/content/167/3/1795.full#ref-list-1

Why *The JI*? Submit online.

- Rapid Reviews! 30 days* from submission to initial decision
- No Triage! Every submission reviewed by practicing scientists
- Fast Publication! 4 weeks from acceptance to publication

*average

Subscription

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Cross-Presentation by Dendritic Cells of Tumor Antigen Expressed in Apoptotic Recombinant Canarypox Virus-Infected Dendritic Cells

Iris Motta, Fabrice André, Annick Lim, James Tartaglia, William I. Cox, Laurence Zitvogel, Eric Angevin, and Philippe Kourilsky

We have investigated the possible usefulness of recombinant canarypox virus (ALVAC) encoding the melanoma-associated Ag, Melan-A/MART-1 (MART-1), in cancer immunotherapy, using a dendritic cell (DC)-based approach. ALVAC MART-1-infected DC express, and are able to process and present, the Ag coded by the viral vector. One consistent feature of infection by ALVAC is that these viruses induce apoptosis, and we show cross-presentation of Ag when uninfected DC are cocultured with ALVAC MART-1-infected DC. Uptake of apoptotic virally infected DC by uninfected DC and subsequent expression of tumor Ag in the latter were verified by flow cytometry analysis, image cytometry, and confocal microscopy. Functional activity was monitored in vitro by the stimulation of a MART-1-specific cytotoxic T cell clone. Heightened efficiency in Ag presentation is evidenced in the 2- to 3-fold increase in IFN-γ production by the T cell clone, as compared with the ALVAC-infected DC alone. Cocultures of ALVAC MART-1-infected and uninfected DC are able to induce MART-1-specific T cell immune responses, as assessed by HLA class I/peptide tetramer binding, IFN-γ ELISPOT assays, and cytotoxicity tests. Overall, our data indicate that DC infected with recombinant canarypox viruses may represent an efficient presentation platform for tumor Ags, which can be exploited in clinical studies. The Journal of Immunology, 2001, 167: 1795–1802.

Much attention has recently been focused on dendritic cells (DC) as extremely efficient tools for eliciting anti-tumor immunity. The development of protocols for generating large numbers of DC from murine bone marrow cultures (1) or from human peripheral blood monocytes (2) and CD34+ precursors (3) has allowed investigations using DC-based approaches to induce protective tumor immunity. Peptide-pulsed DC have been shown to afford the generation of potent anti-tumor CTL responses and tumor resistance in vaccinated mice (4–6). Significantly, their efficacy has been reported also for weakly immunogenic tumor models (5, 7). More recently, mature DC have been shown to be potent natural cell adjuvants for vaccination in healthy volunteers. Both CD4+ and CD8+ Ag-specific responses were enhanced by immunization with Ag-pulsed DC and not with either Ag or DC alone (8).

Although these results provide evidence for the beneficial effect of an adoptive transfer of Ag-loaded DC, the development of more effective means of delivering tumor Ags to DC remains a main issue. Recent studies have emphasized protocols that not only would allow sustained and efficient CTL responses but also would circumvent the need for prior knowledge of relevant MHC haplotypes or tumor peptide sequences. Various strategies have been reported, including the loading of DC with soluble tumor protein Ags, tumor-derived RNA, unfractionated acid-eluted peptides from the MHC class I molecules of tumor cells, tumor cell lysates, or the transfection of DC with plasmid DNA encoding tumor-associated Ags (9). Products of DC and tumor cell fusions (10, 11) or cocultures (11) were found to be immunogenic and induce antitumor immunity. One other highly efficient method of obtaining tumor Ag-expressing DC is via the infection of the latter by viral vectors. DC transduced with recombinant adenovirus (12–16), retrovirus (17), or vaccinia virus (18, 19) vectors have proved to be effective in inducing CTL responses and protection against tumors.

Our interest is centered on recombinants of an attenuated Avipoxvirus, canarypox, and their capacity to transduce human immature DC in attempts to determine their potential usefulness in cancer immunotherapy. The choice of this vector, referred to as ALVAC, stems from the facts that 1) like vaccinia virus, ALVAC can be engineered to express multiple foreign genes and 2) ALVAC is host range-restricted for replication, in that production of infectious progeny virus is limited to avian species. Nevertheless, abortive replication of ALVAC does not preclude expression of the inserted foreign genes in infected mammalian cells (20, 21). Furthermore, evidence that canarypox-based recombinants are safe, immunogenic, and protective against viral infections is documented in numerous studies (22). We have determined that human immature DC are sensitive to infection by recombinant ALVAC vectors and in particular by ALVAC encoding the melanoma-associated Ag, Melan-A/MART-1 (MART-1). One noteworthy and consistently observed feature of infection by ALVAC is that it leads to apoptotic cell death in human immature DC. The capture by DC of apoptotic...
influenza virus-infected monocytes with subsequent presentation to CD8+ T cells of virus Ag epitopes has been recently documented (23). Advantage was then taken of the apoptosis induced by such recombinant ALVAC vectors, and we show that cocultures of ALVAC MART-1-infected and uninfected DC exhibit an increased capacity to stimulate in vitro MART-1-specific responses, as compared with the virally infected DC alone. Evidence is presented that a cross-presentation of tumor Ag via the uptake of apoptotic virally infected DC by uninfected DC underlies this heightened stimulatory activity.

Materials and Methods

Culture medium

All cultures were maintained in complete FCS 10 medium comprised of RPMI 1640 with 2 mM t-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 50 μM 2-ME, 100 μg/ml streptomycin, and 100 U/ml penicillin supplemented with 10% FCS (all obtained from Life Technologies, Grand Island, NY).

Preparation of DC

PBMCs from HLA-A2+ (HLA-A2)-positive or -negative donors were used to derive DC. HLA-A2 expression was determined using the mAbs BB7.2 (HB-82; American Type Culture Collection, Manassas, VA) and MA2.1 (a gift of F. Lemmerman, Institut Pasteur, Paris, France), which recognize HLA-A2 and HLA-A2* and HLA-B1*7, respectively. Staining with these mAbs was followed by PE-conjugated goat F(ab')2 anti-mouse IgG (Caltag, South San Francisco, CA).

DC were prepared as described (2, 24). PBMCs were allowed to adhere for 2 h at 37°C, 5% CO2 in a humidified atmosphere. The adherent cells were then cultured for 7 days in complete FCS 10 medium containing 800 U/ml of recombinant human (rh) GM-CSF (PeproTech, Rocky Hill, NJ) and 10% FCS 10/ml rhIL-4 (PromoCell, Heidelberg, Germany). Cytokines were added every 2 or 3 days from day 0. Unless otherwise stated, experiments were performed using DC derived with this procedure, which typically yields immature DC (2, 24). To induce maturation, day 5–7 DC were cultured with LPS (2 μg/ml; Sigma, St. Louis, MO) and TNF-α (100 U/ml; PeproTech) for 48 h. Maturation was evidenced by the expression of high levels of CD83, CD86, and HLA-DR. Monocytes were purified from PBMCs by negative selection, using a kit from Miltenyi Biotec (Paris, France), according to the manufacturer’s instructions. Approximately 98% of the enriched population are CD14+.

Cell lines and clones

The HLA-A2+ MART-1-specific clone LT12 was derived from a melanoma patient (25) and provided by F. Faure (Institut Curie, Paris, France). The T2 (HLA-A2/β2-m), tumor FON (HLA-A2/MART-1 melanoma cell line; Institut G. Roussy), and A2 Mel+ (an HLA-A2+/MART-1+ melanoma cell line; Institut G. Roussy) cell lines were used as target cells in cytotoxicity assays. Clone LT12 was cultured in the complete FCS 10 medium supplemented with 200 U/ml rhIL-2 (PeproTech).

Canarypox virus vectors and infection of DC

Parental ALVAC, recombinant ALVAC containing the Lac-Z gene (ALVAC-β, life) (vCP 326), and ALVAC vectors carrying DNA sequences coding for MART-1 (vCP 1467) or melanoma gp100 (vCP 1465) were developed at and provided by Virogenetics (Troy, NY).

Viral vectors were added to the DC (105 cells/ml) in RPMI 1640 supplemented with 2% FCS at a multiplicity of infection (moi) of 10 or 30. The infection was conducted at 37°C for 1 h, following which the infected DC were washed twice in complete FCS 10 medium. Infected DC were recultured for 3–18 h, as specified.

Detection of MART-1 expression and apoptosis in virally infected DC

ALVAC MART-1-infected DC were fixed in 1% paraformaldehyde and labeled with anti-MART-1 mouse IgG1 mAb (clone A103; Novoceastra, Newcastle, U.K.) followed by FITC-conjugated goat F(ab')2 anti-mouse IgG Ab (Caltag) in PBS + 1% BSA + 0.1% saponin. Negative controls included parental ALVAC or ALVAC-melanoma gp100-infected DC.

Apoptotic cell death was determined using the Annexin-V-Fluos staining kit (Roche Diagnostics, Meylan, France) and propidium iodide. Staining was performed according to the manufacturer’s instructions. All cytoluminometric analyses were performed on FACScan, using CellQuest software (BD Biosciences, Mountain View, CA).

Image cytometry and confocal microscopy

DC were infected with ALVAC MART-1 (moi = 10) for 10 h, labeled red with PKH26-GL, and then cocultured for 4 h with uninfected DC (ratio infected to uninfected DC = 1:1) that had been stained green with PKH67-GL. Both fluorescent cell linkers were obtained from Sigma. Cells were analyzed by FACSScan. The double-positive cells were sorted using a Coulter Epics Altra cell sorter (Coulter, Miami, FL). Sorted DC were placed in Lab-Tek chambered coverglasses (Nunc, Naperville, IL), then visualized by image cytometry and confocal microscopy on the ACAS 570 (Meridian, Okemos, MI). For the detection of MART-1, DC were infected with parental ALVAC or ALVAC MART-1 and cocultured 1 h with uninfected DC labeled green with PKH67-GL. DC were recovered, fixed with 1% paraformaldehyde, and stained for MART-1, following the procedure for intracellular staining outlined above. Anti-MART-1 Ab was revealed with PE-conjugated goat F(ab')2 anti-mouse IgG. Double-positive cells were sorted, placed in Lab-Tek chambered coverglasses, and examined in image cytometry.

Activation of the MART-1-specific T cell clone LT12

Five thousand LT12 cells were cultured, along with stimulating DC, in 96-well round-bottom plates (Costar) in a final volume of 200 μl complete FCS 10 medium. Triplicates were set up for each group. Twenty-four hours later, supernatants were collected and assayed for IFN-γ, using the human IFN-γ ELISA kit from Bender MedSystems (Camarillo, CA). Anti-IFN-γ Ab was revealed with streptavidin-peroxidase and 3,3',5,5'-tetramethylbenzidine substrate system (Sigma). Unless otherwise specified, data are presented as picograms of IFN-γ released/2.5 × 105 cells/ml/24 h.

In vitro induction of MART-1-specific T cell responses

Cultures were set up in 96-well round-bottom plates (Costar) in RPMI 1640 medium supplemented with 10% human AB serum, 2 mM t-glutamine, 1 mM sodium pyruvate, 50 μg/ml streptomycin, 50 μM penicillin (medium AB), 50 U/ml rhIL-2, and 2% T cell growth factor medium. DC were infected with ALVAC MART-1 or ALVAC-β-gal (moi = 30) and cultured for 12 h before the addition of 6 × 105 uninfected DC. The ratio of uninfected to infected DC was 1:3. PBMCs (3 × 105) were then plated with the stimulating autologous DC. Medium and cytokines were renewed every 2 days. On day 7, cells were restimulated with uninfected and ALVAC-infected DC, as indicated earlier. Assays for MART-1-specific responses were performed 8 days later.

HLA-A2/pEptide tetramer complexes

PE-labeled HLA-A2/pE peptide tetramer complexes were synthesized as described by Altman et al. (26). HLA-A2/MART-1(26–35)A27L peptide (ELA GIGILTV) and HLA-A2/influenza matrix Flu-MA 5 8–6 6 peptide (GILG9) were conjugated to biotinylated HLA-A2+ DC and FITC. DC were infected with ALVAC MART-1 (moi = 10) and cocultured with uninfected DC for 4 h. Tetramer complexes were synthesized by Altman et al. (26). HLA-A2/MART-126 –35 A27L peptide (ELA GIGILTV) and HLA-A2/influenza matrix Flu-MA 5 8–6 6 peptide (GILG9) were conjugated to biotinylated HLA-A2+ DC and FITC. DC were infected with ALVAC MART-1 (moi = 10) and cocultured with uninfected DC for 4 h. Tetramer complexes were synthesized by Altman et al. (26). HLA-A2/MART-126 –35 A27L peptide (ELA GIGILTV) and HLA-A2/influenza matrix Flu-MA 5 8–6 6 peptide (GILG9) were conjugated to biotinylated HLA-A2+ DC and FITC.

ELISPOT assay for IFN-γ-producing cells

PBMCs (104) and 105 peptide-pulsed autologous DC in 200 μl medium AB were plated into ELISPOT plates (Millipore S.A., Molsheim, France) precoated overnight with 10 μg/ml of a primary anti-IFN-γ mAb (MABTECH, Stockholm, Sweden). DC were pulsed for 2 h with 4 μg/ml of either MART-126–35 (A2GIGILTV) or MAGE-3 (FLWGRPALV) peptide. All peptides were obtained from NeoSystem (Strasbourg, France). Following a 24-h incubation, the cells were removed from the ELISPOT plates and a second biotinylated anti-IFN-γ mAb (MABTECH) was added to the wells at 1 μg/ml. Spots were revealed with streptavidin alkaline phosphatase conjugate (MABTECH). All cultures were set up in triplicate. MART-1-specific IFN-γ-producing cells represent the difference between the mean number of spots in cultures stimulated by peptide MART-1-pulsed DC and the mean number of spots in cultures stimulated by peptide MAGE-3-pulsed DC.

Assessment of CTL reactivity

Cytolytic activity was determined in a 3-h 51 Cr release assay. All target cells (T2, tumor FON, and A2 Mel+) were labeled with 100 μCi of 51 Cr.
Canarypox virus-driven intracellular expression of MART-1 was determined by FACS analysis in immature DC infected with the recombinant viral vector ALVAC MART-1. Negative controls included DC infected with parental ALVAC or ALVAC melanoma gp100. Maximal expression was observed at ~10 h postinfection, when 40–50% of the DC are MART-1 

Expression of tumor Ag MART-1 and apoptosis in DC infected with recombinant canarypox viruses

Canarypox virus-driven intracellular expression of MART-1 was determined by FACS analysis in immature DC infected with the recombinant viral vector ALVAC MART-1. Negative controls included DC infected with parental ALVAC or ALVAC melanoma gp100. Maximal expression was observed at ~10 h postinfection, when 40–50% of the DC are MART-1 

Results

Expression of tumor Ag MART-1 and apoptosis in DC infected with recombinant canarypox viruses

Canarypox virus-driven intracellular expression of MART-1 was determined by FACS analysis in immature DC infected with the recombinant viral vector ALVAC MART-1. Negative controls included DC infected with parental ALVAC or ALVAC melanoma gp100. Maximal expression was observed at ~10 h postinfection, when 40–50% of the DC are MART-1 

![Figure 1](image)

**FIGURE 1.** a, Kinetics of MART-1 expression in ALVAC-infected DC. DC were infected with parental ALVAC or ALVAC melanoma gp100 or ALVAC MART-1 vectors at an moi of 10 for 1 h at 37°C. DC were then washed, recultured for the indicated periods of time, fixed, and stained for intracellular expression of MART-1 with anti-MART-1 mAb followed by FITC-conjugated goat F(ab'2) anti-mouse IgG Ab. Data represent the mean percentage ± SD of DC expressing MART-1 in at least two independent infections for each time point. b, Representative FACS analysis of ALVAC MART-1-infected DC, 14 h postinfection, with ALVAC melanoma gp100-infected DC (broken line) as negative control. c, ALVAC vectors induce apoptosis in infected DC. DC were left untreated (left) or infected ( moi = 10) with ALVAC MART-1 (right). All DC were recultured for 13 h before labeling with Annexin V-FITC and propidium iodide.

is shown in Fig. 1b. By 24 h, most infected DC express MART-1, albeit weakly, in terms of mean fluorescence intensity. At 48 h postinfection, MART-1 could no longer be detected (data not shown).

Aside from recombinant gene expression, cell death appears to be one other consequence of infection by ALVAC vectors. ALVAC-infected DC undergo apoptosis, as evidenced by their binding Annexin V to a much greater extent than uninfected DC (Fig. 1c). Annexin V+ cells represented, on the average, 58 ± 6 and 69 ± 2% of DC infected at a moi of 10 (five experiments) and 30 (three experiments), respectively. A greater proportion of Annexin V+/PI+ DC was observed for a moi of 30 than for a moi of 10 (47 ± 3 vs 33 ± 1%).

ALVAC-MART-1-infected immature DC are able to process and present the tumor Ag, as evidenced by their ability to stimulate the HLA-A2-restricted, MART-1-specific clone LT12 to produce IFN-γ. In contrast, mature DC are unable to stimulate clone LT12 (Fig. 2). This is consistent with our observation that mature DC are resistant to infection by the ALVAC vectors, as indicated by the lack of expression of MART-1 in those DC, upon infection with ALVAC MART-1 (data not shown).

**Uninfected DC phagocytose ALVAC-infected DC**

Cocultures of labeled ALVAC MART-1-infected (red) and uninfected immature DC (green) were set up as indicated in Materials and Methods and incubated for 4 h either at 37°C or at 4°C. Cells were then analyzed by flow cytometry. Almost all (93%) of the uninfected DC were double-positive in 37°C cocultures (Fig. 3a). Image cytometry and confocal microscopy of the sorted double-positive DC show internalization of the infected cells in uninfected DC (Fig. 3b). Comparatively, in cocultures incubated at 4°C, only 5% of the uninfected DC were double-positive, indicating a blockade of the uptake of the ALVAC-infected cells at low temperature (Fig. 3a). For the expression of MART-1, uninfected DC (labeled green) were incubated with Parental ALVAC or ALVAC MART-1-infected DC (unlabeled) for 1 h at 37°C. Cells were fixed, then labeled with an anti-MART-1 mAb, followed by PE-conjugated goat F(ab')2 anti-mouse IgG. Flow cytometry analysis shows that 2% of the uninfected DC were double-positive when cocultured...
with ALVAC MART-1-infected DC, whereas none were detected in the cultures of uninfected and parental ALVAC-infected DC (data not shown). Sorted double-positive cells were then visualized by image cytometry (Fig. 3). Expression of MART-1 in the uninfected DC confirms the uptake by the latter of ALVAC MART-1-infected DC.

FIGURE 3. Phagocytosis of ALVAC MART-1-infected DC by uninfected DC. a, DC infected with ALVAC MART-1 (moi = 10), then labeled red with the PKH26-GL fluorescent linker. Ten hours later, they were cocultured at a ratio of 1:1 with uninfected DC (stained green with PKH67-GL) for 4 h at 37°C or 4°C. FACS analysis was performed as indicated in Materials and Methods. b, Double-positive cells from the above cocultures sorted and examined in image cytometry. Visualization on confocal microscopy of one double-positive cell is shown on the right. c, DC infected with parental ALVAC or ALVAC MART-1 (moi = 10). Cocultures with uninfected DC (labeled green with PKH67-GL) were set up as indicated in a. DC were recovered following a 1-h incubation at 37°C, fixed in 1% paraformaldehyde, and intracellular staining for MART-1 was performed as described in Materials and Methods. Double-positive cells were observed in the cocultures of uninfected DC with ALVAC MART-1-infected DC and not in those with parental ALVAC-infected DC. Image cytometry of the sorted double-positive DC is shown.

Close contact between uninfected and infected DC appears to be required for maximal activation of LT12 cells. No stimulation was observed in cultures in which the two DC populations were separated by a 0.45-µm membrane. This also excludes transfer of free Ag to uninfected DC (Fig. 5).

Whether monocytes or mature DC are efficient in cross-presentation was also assessed. HLA-A2+ immature DC infected with parental ALVAC or with ALVAC MART-1 (moi = 10) were cultured and then mixed with uninfected HLA-A2+ monocytes, immature or mature DC, using the same protocol as specified above. LT12 cells were added 3 h later, and the stimulatory activity of the APC was measured as IFN-γ production by LT12 cells in 24-h supernatants. As seen in Fig. 6, ALVAC MART-1-infected immature DC cocultured with uninfected monocytes or mature DC failed to activate, whereas cocultures of these DC with uninfected immature DC were able to stimulate an IFN-γ response from LT12 cells (Fig. 6).

The comparative stimulatory activity of ALVAC-infected DC alone, of cocultures of ALVAC-infected and uninfected DC, and of peptide-pulsed DC is shown in Fig. 4. The efficacy of ALVAC MART-1-infected HLA-A2+ DC alone in stimulating LT12 cells is quite comparable to that of DC pulsed with 4 µg/ml peptide MART-127-35. Coculturing ALVAC MART-1-infected DC with uninfected DC leads to greater stimulatory activity than that observed with ALVAC MART-1-infected DC alone (Fig. 4). Statistical analyses by the Student t test of data from three experiments show that, compared with ALVAC MART-1-infected DC alone or the peptide-pulsed DC, the 2- to 3-fold increase in IFN-γ release in cultures of LT12 stimulated with both infected and uninfected DC is significant (p < 0.001). Similar results were obtained with DC infected with the ALVAC vectors at a moi of 30 (data not shown).

The uptake of apoptotic ALVAC-infected DC by immature DC results in efficient cross-presentation of tumor Ag MART-1

We then sought to determine whether the apoptotic ALVAC-infected DC population could serve as a source of Ag for uninfected DC with ensuing cross-presentation and stimulatory activity. HLA-A2+ DC were infected with parental ALVAC or with ALVAC MART-1 (moi = 10) and cultured for 10 h before being mixed with uninfected autologous DC at a ratio of 1:1. Three hours later, clone LT12 cells were added. Activation was assessed as IFN-γ release in 24-h supernatants.

FIGURE 4. Comparative ability of tumor Ag presentation by ALVAC-infected DC in the presence or absence of uninfected DC. Immature DC derived from an HLA-A2+ donor were infected with parental ALVAC or ALVAC MART-1 (moi = 10). Ten hours postinfection, cocultures of infected DC with autologous uninfected DC were set up at a ratio of 1:1 (1250:1250). Five thousand MART-1-specific clone LT12 cells were added after a 3-h coculture of DC. Stimulation with MART-127-35 peptide-pulsed DC (4 µg/ml/106 DC) was included. Activation was assessed as IFN-γ released at 24 h. Data are presented as indicated in Fig. 2. One of three experiments with similar results is shown.
Induction of MART-1-specific responses in PBMCs stimulated with cocultures of ALVAC-infected and uninfected immature DC

PBMCs from several HLA-A2+ normal donors and melanoma patients were primed in vitro with cocultures of ALVAC MART-1-infected and autologous uninfected DC. MART-1-specific responses were assessed by HLA-A2/peptide tetramer binding, IFN-γ ELISPOT assays, and cytotoxicity tests.

The frequency of MART-1-specific CD8+ T cells, as evaluated by staining with HLA-A2/peptide MART-1 tetrameric complexes, was determined before (day 0) and after two in vitro stimulations (day 15) for donors NV1, MEL 1, and MEL 2 (Table I). Compared with unstimulated PBMCs, a significant increase (17- to 90-fold) in MART-1-specific CD8+ T cells was observed following in vitro stimulation (Table I). MART-1-specific IFN-γ-producing cells, as determined by ELISPOT assay, were also elicited, when the in vitro primed cells were activated with peptide-pulsed DC during the assay (Table I). Cytotoxic activity against the HLA-A2+ MART-1 tumor FON cells (donor MEL 1), peptide MART-1-pulsed T2 cells (donors MEL 1, MEL 2, and NV1) was also detected on day 15 (Table I). For donor MEL 1, cytotoxicity against tumor FON cells was significantly higher in ALVAC MART-1-infected than in ALVAC β-gal-stimulated cultures (p < 0.05), thus indicating specificity of priming. In addition, sorted MART-1-specific CD8+ cells derived from in vitro primed cultures of donor NV 2 had cytotoxic activity against tumor FON cells but did not lyse an HLA-A2+ MART-1 melanoma cell line.

The efficiency of cocultures of ALVAC MART-1-infected and uninfected DC in their capacity to induce MART-1-specific CD8+ cells was compared with that of ALVAC MART-1-infected DC alone, for PBMCs derived from a melanoma patient (MEL 3), and a normal donor (NV2). The number of MART-1-specific CD8+ cells was determined by HLA-A2/peptide MART-1 tetramer binding at day 15 (7 days after a restimulation on day 8) for donors MEL 3 and NV2 and also at day 30 (1 wk after a third stimulation) for donor NV2. As shown in Fig. 7, a significantly greater proportion of MART-1-specific CD8+ cells was detected in PBMCs stimulated with cocultures of ALVAC MART-1-infected and uninfected DC than in those primed with ALVAC MART-1-infected alone. Specificity of priming was evidenced with staining with HLA-A2/peptide FluMA58-66 tetramers. In addition, for donor NV2, the percentage of MART-1-specific CD8+ cells was similar whether the priming was performed with ALVAC MART-1-infected DC alone or with parental ALVAC-infected DC in the presence or absence of uninfected DC (data not shown). An expansion of the MART-1-specific CD8+ population was observed after a third cycle of stimulation, either by direct presentation or cross-presentation of the MART-1 Ag (day 30, donor NV2, Fig. 7). Taken together, our data demonstrate the efficient induction of relevant MART-1-specific responses, using uninfected DC cocultured with DC expressing virally driven tumor MART-1 Ag.

FIGURE 5. Close contact between ALVAC-infected and uninfected DC is required for stimulatory activity. DC from an HLA-A2+ donor were infected with parental ALVAC or ALVAC MART-1, as indicated in Fig. 4. Ten hours postinfection, 1.5 × 10^5 DC were placed in either a 24-well plate (lower well) or a transwell (0.45-μm membrane; Costar). The transwells were inserted into the 24-well plate and, where indicated, uninfected DC were added (1:1 ratio with respect to infected DC). Three hours later, 6 × 10^5 clone LT12 cells were distributed in all insert wells. Data are presented as picograms per milliliter of IFN-γ released/6 × 10^5 cells/ml/24 h (mean ± SD of duplicate cultures). One of two experiments with similar results is shown.

FIGURE 6. Immature DC, but not mature DC or monocytes HLA-A2+, are efficient in cross-presentation. HLA-A2+ immature DC were infected with parental ALVAC or ALVAC MART-1 vectors (moi = 10) and then cocultured with uninfected immature or mature DC or monocytes, as indicated in Fig. 4. MART-1-specific clone LT12 cells (5000) were added to the APC cocultures. IFN-γ release in 24-h supernatants was measured by ELISA. Data are presented as in Fig. 2. One of two experiments with similar results is shown.
Discussion

This study was undertaken to evaluate the possible use of ALVAC vectors encoding tumor-associated Ags in a DC-based approach to cancer immunotherapy. One feature that makes ALVAC vectors attractive is their inability to productively replicate in nonavian species, thereby eliminating the safety concerns that may hinder the use of other viral recombinants (22). In addition, efficacy of recombinant ALVAC vectors in inducing in vivo and/or in vitro Ag-specific CTL responses has been reported for HIV-1 (27, 28), MAGE-1 (29, 30), and CEA, the human carcinoembryonic Ag (31). ALVAC expressing p53 was shown to protect mice against a lethal tumor challenge (32, 33).

We show in this report that human immature DC infected by recombinant ALVAC encoding MART-1 express and present the melanoma-associated Ag, as evidenced by their capacity to stimulate in vitro an HLA-A2\(^+\), MART-1-specific CTL clone. Their efficiency is equivalent to that of DC pulsed with MART-1\(^{27-35}\) peptide.

In addition to recombinant gene expression, infection with ALVAC vectors leads to apoptotic cell death of infected DC. Consistent with their known high efficiency in phagocytosis (34, 35), uninfected immature DC are able to phagocyte the apoptotic ALVAC-infected DC. Phagocytic uptake of apoptotic ALVAC-infected DC by uninfected DC was visually confirmed by image cytometry and confocal microscopy. Importantly, expression of the Ag MART-1 could also be detected in DC that were cultured with the apoptotic ALVAC-MART-1-infected cells for 1 h at 37°C.

Cross-presentation of Ag derived from the apoptotic virally infected DC, the antigenic material can be derived from either allogeneic or syngeneic apoptotic ALVAC-infected DC. Interestingly, in our system, only immature DC are able to cross-present Ag. Mature DC or monocytes fail to do so. As shown in this report, two weekly stimulations with DC pulsed with virally induced apoptotic cells were able to elicit MART-1-specific responses in PBMCs derived from five volunteers.

To our knowledge, ours is the first demonstration of a cross-presentation of Ag, using human immature DC as the source of both apoptotic antigenic “meal” and APCs. Findings similar to ours have used human DC pulsed with either apoptotic influenza virus-infected monocytes (23, 36) or an irradiated MAGE-3-expressing cell line (37).

It is noteworthy that stimulation of LT12 cells with cocultures of HLA-A2\(^+\) apoptotic ALVAC MART-1-infected DC and autologous uninfected DC resulted in a 2- to 3-fold increase in IFN-γ release when compared with that produced by LT12 cells activated by the virally infected DC alone or peptide-pulsed DC. Higher efficacy of cocultures of ALVAC MART-1-infected and uninfected DC was also observed in the ability to induce MART-1-specific CD8\(^+\) cells. The fact that the system relies, on the one hand, on virally induced Ag expression and thereby on a direct presentation of the latter and, on the other hand, on virally induced apoptosis and cross-presentation of Ag following the uptake by uninfected DC of the apoptotic cells may account for the higher efficiency observed in eliciting Ag-specific CTL responses. However, it is not excluded that infection with ALVAC vectors and the phagocytosis of apoptotic cells result in an increased synthesis and a longer half-life of HLA class I molecules in DC. Influenza virus infection has been reported to induce maturation in human DC along with an up-regulation of MHC class I and II, adhesion, and costimulatory molecules (38). Infection by canarypox virus vectors leads to a maturation of the infected DC population, based on the expression of CD83, as well as to an up-regulation of CD86, CD40, and CD80 (data not shown). DC that have internalized apoptotic cells have been shown, in a murine system, to also undergo maturation and up-regulate expression of MHC class II Ags, CD40, and CD86 (39).

Generation in vivo of MHC class I-restricted CTL by cross-priming has been shown to be dependent on CD4\(^+\) helper T cells, and both CTL and helper epitopes need be recognized on the same APCs (40). Protective antitumoral immunity also requires vaccination with tumor-specific T helper and CTL epitopes (41). Using viral vectors with genes encoding the whole tumor Ag makes unnecessary the need for prior knowledge of relevant MHC haplotypes or tumor peptide sequences and may allow for the activation of both CD4\(^+\) and CD8\(^+\) T cells. In effect, direct immunization with several ALVAC vectors has been shown to induce both humoral immunity and CTL responses, implying that these vectors can target both CD4\(^+\) and CD8\(^+\) T cells (22).

---

**Table I. MART-1 specific responses in PBMCs stimulated in vitro with ALVAC-infected and uninfected DC**

<table>
<thead>
<tr>
<th>Donor</th>
<th>PBMCs Stimulated with Uninfected DC</th>
<th>% MART-1-Specific CD8(^+)/CD8(^+) Cells(^a)</th>
<th>IFN-γ Spots/10(^6) PBMCs(^b)</th>
<th>Maximal % Specific Lysis (E:T Ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEL 1</td>
<td>ALVAC β-gal-infected DC</td>
<td>0.07 64</td>
<td>0.07 0.3</td>
<td>0.07 0.3</td>
</tr>
<tr>
<td></td>
<td>ALVAC MART-1-Infected DC</td>
<td>0.07 5.5</td>
<td>0.07 5.5</td>
<td>0.07 5.5</td>
</tr>
<tr>
<td>MEL 2</td>
<td>ALVAC MART-1-Infected DC</td>
<td>0.1 65</td>
<td>0.1 65</td>
<td>0.1 65</td>
</tr>
<tr>
<td>NV 1</td>
<td>ALVAC MART-1-Infected DC</td>
<td>0.01 65</td>
<td>0.01 65</td>
<td>0.01 65</td>
</tr>
<tr>
<td>NV 2</td>
<td>ALVAC MART-1-Infected DC</td>
<td>ND 65</td>
<td>ND 65</td>
<td>ND 65</td>
</tr>
</tbody>
</table>

---

\(^a\) Percentage of HLA-A2/peptide MART-1 tetramer\(^+\) cells as determined by flow cytometry analysis.

\(^b\) IFN-γ-producing cells were enumerated on day 15 in PBMCs stimulated with peptide MAGE-3- or peptide MART-1-pulsed DC during the assay. Mean SD of three cultures are presented.

\(^c\) MART-1-specific CD8\(^+\) cells were sorted and used as effectors.
with autologous uninfected immature DC at a 1:1 ratio. PBMCs (2 × 10^5) were then placed with the stimulating autologous DC. PBMCs were stimulated from an HLA-A2

Acknowledgments

Overall, our data may bear some relevance in the choice of strategies for in vivo priming by recombinant canarypox virus vectors. Immunization protocols that target the DC system may prove to be advantageous, despite the cytopathic effects of the viruses. ALVAC-infected apoptotic DC may recruit and be internalized by dendritic cells transduced with adenovirus-encoding endogenous tumor-associated antigens. J. Immunol. 163:699.

References


