Immune characterization of clinical grade-dendritic cells generated from cancer patients and genetically modified by an ALVAC vector carrying MAGE minigenes

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Gene delivery into dendritic cells (DC) is most efficiently achieved by viral vectors. Recombinant canarypox viruses (ALVAC) were validated safe and efficient in humans. We aimed firstly to evaluate DC transduction by ALVAC vectors, then to investigate if such infection induced or not the maturation of the DC, and finally to assess the efficiency of ALVAC-MAGE-transduced DC to activate specific CTL clones. Clinical grade DC from melanoma patients were generated from blood monocytes and infected with a recombinant ALVAC virus encoding either a marker gene (EGFP) or the MAGE-1-MAGE-3 minigenes. According to the patient-donor, $22 \pm 16\%$ of immature DC were successfully transduced. Flow cytometry analysis of surface markers expressed on DC after ALVAC infection did not reveal a mature phenotype. Moreover, ALVAC transduction did not interfere with the capacity of the DC to further mature under poly:IC stimulation. But most importantly, our results demonstrated that DC from HLA-A1 patient-donors infected with the recombinant ALVAC MAGE-1-MAGE-3 minigenes virus were capable of activating a MAGE 3/A1 CTL clone more efficiently than same DC loaded with MAGE 3/A1 peptide, as shown by increased IFN- γ secretion. These results could be the basis for the development of a new clinical strategy in melanoma patient's immunotherapy.

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Targeting identified tumor-associated antigens (TAA) to dendritic cells (DC) has become the basis of various vaccination strategies in order to obtain an immune control of tumor growth. DC are considered the most potent antigen-presenting cells (APC) due to their ability to induce strong T-cell responses, even of naive T cells. Recent clinical trials using DC loaded with MHC class-I-specific synthetic peptides or with whole tumor cell lysate preparations^{1–4} have demonstrated that DC-based active immunization has no major toxicity in the vaccinated patients, but results in tumor regression in only a minority of them. Indeed tumor-specific T-cell responses were induced and detected *in vitro* but, unfortunately, they were often only transient. The overall results of these trials revealed the need for improved DC-based vaccines before they become an optimal commonly used modality for the treatment of cancer.

Transferring TAA genes into DC might have a number of potential advantages over other methods of antigen delivery. Transduced DC may be able to constitutively express and process TAA to produce long-term antigen presentation *in vivo*. Moreover, they may be able to present multiple identified or yet undefined MHC class I and II epitopes without allelic HLA restriction and so efficiently stimulate antitumor responses which possibly involve CD8⁺ cytotoxic and CD4⁺ helper T cells.⁵ Other advantage is the possibility of adding sequences coding for various immunomodulators (like cytokines or costimulatory molecules) that could enhance the immune response. Several methods of gene transfer into DC have been reported using either physical methods including liposome complexes or electroporation^{6,7} or viral vectors.^{8–12} The transduction of human monocytes-derived DC seems to be more efficient by using viral vectors. DC transduced with recombinant adenovirus,^{7,11,13–15} retrovirus,¹⁶ or vaccinia virus^{10,17} have proved to be effective in inducing CTL responses and protection against tumors. However, some of these viral vectors can disturb the function of DC as APC.^{18–21} ALVAC recombinant virus were derived from a highly attenuated canarypox virus that does not replicate in mammalian cells, the production

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of infectious progeny virus being limited to avian species.^{22,23} They are appealing for gene therapy for several reasons. Firstly, they are able to infect a wide variety of cell types with high efficiencies of infection.²⁴ In addition, abortive replication of ALVAC vectors in infected mammalian cells does not preclude expression of the inserted foreign genes.²⁵ Finally, the safety and immunogenicity of ALVAC vectors have been validated in humans within diverse infectious diseases trials or cancer trials.^{26–28} Besides, successful identification of new antigens of the melanoma antigen (MAGE) family,^{29,30} more precisely the ones encoded by the MAGE-A1 gene resulted from the *in vitro* stimulation of blood-derived DC from normal donors with an ALVAC recombinant virus containing the entire MAGE-A1 gene.³¹ The same study coincidentally reported that the procedure was efficient for the activation of anti-MAGE CTL precursors, allowing the isolation of permanent anti-MAGE CTL clones.

The aims of this study were firstly to evaluate the efficiency of ALVAC recombinant vectors to transduce clinical grade DC derived from cancer patients, especially melanoma patients, then to investigate if such infection could induce or not induce the maturation of the DC, and finally to assess comparatively *in vitro* the potency of ALVAC-MAGE-transduced DC *versus* MAGE peptide-pulsed DC to activate specific CTL clones.

Materials and methods

DC generation

Clinical grade DC were generated in a closed system (Cell Culture Container PL2417, Nexell Therapeutics, Irvine, CA) from peripheral blood monocytes of melanoma patients included in DC vaccination clinical protocols as previously described.⁴ Briefly, peripheral blood mononuclear cells (PBMC) were obtained by leukapheresis on a COBE SPECTRA separator (Cobe, Denver, CO). The monocytes-enriched fraction, resulting from a modified-adherence step in the culture bag, was seeded for 7 days in a serum-free medium (X-VIVO 20, Bio-Whittaker, Walkersville, MD) supplemented with 800 U/ml GM-CSF (Leukomax, Novartis, Basel, Switzerland) and 1000 U/ml IL-4 (Schering-Plough, Kenilworth, NJ). DC purity was checked and evaluated by morphological analysis (May Grunwald Giemsa staining) and flow cytometry using specific antibodies directed against DC markers (CD1a and HLA-DR), monocyte marker (CD14), T-cell marker (CD3), B-cell marker (CD19) and NK markers (CD16+56). According to patient donor, DC yield reached $70 \pm 16\%$ with a minimum of 45% and a maximum of 95%. Unless otherwise stated, experiments were performed using DC derived with this procedure, which typically gives immature DC. They were utilized either fresh or were kept frozen at -80°C , and were thawed the day of the experiment. To generate mature DC, nonadherent immature DC were cultured before or after infection by ALVAC vectors, during 48 or 72 hours,

in the presence of LPS (1 or 5 $\mu\text{g/ml}$; Sigma-Aldrich, Bornem, Belgium) or poly I:C (20 $\mu\text{g/ml}$, Roche Diagnostics, Brussels, Belgium), respectively.

Recombinant ALVAC viruses and infection of dendritic cells

The recombinant canarypox ALVAC-EGFP (vCP1540) and ALVAC-MAGE-1-MAGE-3 CTL epitopes (minigenes) (vCP1469a) were developed by Pasteur-Merieux Connaught (Lyon, France) and produced by Virogenetics Corporation (Troy, USA). Viral stocks contained different amounts of plaque-forming units (PFU): 1.9×10^9 and 3.0×10^9 PFU/ml respectively. Viral vectors were added to the DC (2×10^6 cells/ml X-VIVO 20 medium) at various multiplicity of infection (MOI 5, 10, 15, 20, 30). The MOI was calculated as a number of PFU/cell, considering the total number of cells in the sample, independently of the real number of DC. The infection was conducted at 37°C for different times (1, 2, 6 hours) following which the infected DC were washed twice in X-VIVO 20 medium. Infected DC were then recultured for 24 hours or more when specified. Efficiency of gene transduction was assessed by measuring EGFP expression by flow cytometry within an FSC/SSC region focused on DC, as testified by the fact that at least 90% of cells in the gate expressed the CD1a and HLA-DR surface markers. The DC mortality was evaluated by propidium iodide (PI) staining.

DC phenotype

The phenotype of ALVAC-infected dendritic cells was analysed by flow cytometry using different phycoerythrin (PE)-conjugated antibodies specific of characteristic surface antigens: CD1a, HLA-DR, CD40, CD80, CD83, CD86. Briefly, cells were incubated with antibodies for 30 minutes at 4°C , then washed twice and analysed by flow cytometry using a FACScan (Becton Dickinson) and the Cell Quest software.

CTL activation assays

DC generated from HLA-A1 cancer patients were infected with an ALVAC-MAGE-1-MAGE-3 (minigenes) recombinant vector at different MOI (5, 10, 20) for 1 or 2 hours, then washed and distributed in round-bottomed 96 wells plates at 3×10^4 cells/well/50 μl . A total of 10,000 cells of the CTL clone 82/30, specific for the MAGE1-A1 epitope or of the CTL-clone 20/38 specific for the MAGE3-A1 epitope (kind gift from P van der Bruggen, Ludwig Institute, Brussels) were then added to ALVAC-transduced DC. After 24 hours stimulation at 37°C in presence of recombinant IL-2 (25 U/ml), culture supernatants were harvested and tested for IFN- γ content by ELISA. DC pulsed with relevant MAGE1-A1 or MAGE3-A1 peptides were also used in parallel as stimulators. Pulsing was carried out by incubating 2×10^6 cells/ml for 2 hours at 37°C with 50 $\mu\text{g/ml}$ peptide as previously described.⁴ We used as negative controls for CTL activation nontransduced DC or ALVAC-EGFP-

transduced DC and as positive control DC cocultured with the MEL-Z2 melanoma cell line.

Cytokine determination

Determination of IL-12 p40 production, IFN- γ production or IL-10 production was done in culture supernatants using ELISA kits purchased from Biosource Europe (Fleurus, Belgium) following the manufacturer's instructions.

Statistics

Statistical analysis were performed using the unpaired *t*-test with 95% confidence interval.

Results

Optimization of DC-ALVAC infection protocol

We first determined the optimal ALVAC infection conditions for melanoma patients-derived immature DC generated *in vitro* from adherent blood monocytes by 7 days culture in X-VIVO 20 medium supplemented with GM-CSF and IL-4 as described in Toungouz *et al.*⁴ We therefore checked the viability/mortality and the expression of a marker transgene (EGFP, enhanced green fluorescent protein) after infection with an ALVAC recombinant virus carrying the EGFP gene, as function of MOI and duration of infection. The cells (2×10^6 cells/ml X-VIVO 20 medium) were submitted to infection at MOI 5, 10, 15, 20, 30 PFU/cell for 1, 2 or 6 hours, washed, recultured for another 20 hours in X-VIVO 20 medium in presence of IL-4 and GM-CSF and then tested by flow cytometry for EGFP expression. As a positive control of infection, we infected 2×10^6 cells/1 ml complete RPMI 1640 supplemented with 10% FCS with ALVAC-EGFP at MOI 30 for 2 hours as done in Chaux *et al.*³¹ Data from pilot experiments (not shown) demonstrated that the DC EGFP expression already reached a plateau after 1 hour infection at MOI 10. The percentage of cells transduced was not augmented by the use of higher MOI (20 or 30) or longer infection duration (2 or 6 hours). On the contrary, the DC mortality which was limited and similar for the DC kept nontreated in culture or infected with ALVAC vector at MOI 10 for 1 hour, increased to worse with higher MOI (30) and longer infection duration (6 hours). Therefore, the infection protocol chosen for further studies was thus 1 hour infection at MOI 10.

ALVAC vectors successfully infect immature DC without changing their phenotype

We thereafter tested the variability in cancer patients-derived DC transduction by ALVAC. As shown in Figure 1, 5–60% (mean $\pm$ SD: 22.5 ± 16.1) of immature DC generated from cancer patients were successfully infected by an ALVAC recombinant vector carrying the EGFP gene. Variability in EGFP expression was observed

Figure 1 Variability in ALVAC DC transduction. Totally, 2×10^6 immature DC from different cancer patients ($n = 10$) were infected by an ALVAC-EGFP recombinant virus at an MOI 10 for 1 hour. EGFP expression was monitored by flow cytometry after 24 hours reculture in X-VIVO 20 medium within gated DC.

Figure 2 Infection of immature DC by ALVAC recombinant virus does not change their phenotype. A total of 2×10^6 DC were infected by an ALVAC-EGFP vector at an MOI 10 for 1 hour. Analysis of surface markers expressed on DC was performed by flow cytometry using a two-step gating procedure: the first focusing DC within an FSC/SSC region, the second targeting EGFP-positive DC. Control D0 are noninfected DC at the day they were infected. Control D1 are noninfected DC left in culture for 24 hours as done with the ALVAC D1-infected DC. Results are pooled data from five different experiments. *Not statistically significant: $P > .05$ versus Ctrl D1.

according to the patient-donor ($n = 10$) or repetitive experiments within same patient-donor (donor D/D').

We also checked if infection by ALVAC recombinant virus resulted in DC maturation. Expression of classical surface markers on DC after infection with ALVAC-EGFP was tested by flow cytometry using a two-step gating procedure: the first focusing DC within a FSC/SSC region, as testified by the expression of the CD1a and HLA-DR markers; the second targeting EGFP positive DC. Pooled data from five different donors did not reveal a mature phenotype when compared to control non-infected DC tested at the same day (Fig 2). Indeed, even if slight increases in the expression of DR and CD86 molecules were detectable, those were not statistically significant ($P = .08$ and $.22$, respectively). Moreover, the CD83 molecule whose expression is characteristic of a

Figure 3 (a) Maturation of DC in X-VIVO 20 medium by LPS. In all, 2×10^6 DC were cultured in the presence of LPS for 48 hours. Surface markers expressed on DC were analysed by flow cytometry. CTRL D0, CTRL D2, LPS D2. Results are from one representative experiment out of three. (b) Maturation of DC in X-VIVO 20 medium by poly I:C. In total, 2×10^6 DC were cultured in the presence of poly I:C for 72 hours. Surface markers expressed on DC were analysed by flow cytometry. CTRL D0, CTRL D3, poly I:C D3. Results are from one representative experiment out of three.

mature DC phenotype was never expressed. Consistently, as illustrated in Figure 5, no or low IL-12 secretion was detected in DC culture supernatants after ALVAC infection.

Immature DC infected by ALVAC vectors are still able to mature

DC maturation is a crucial step in inducing adaptive immunity. By controlling as a prerequisite the capacity of immature DC generated in serum-free X-VIVO 20 medium to mature under stimulation by different agents, we have observed that no maturation was induced when DC were treated by LPS, even for 48 hours at $5 \mu\text{g/ml}$ (Fig 3a). However, a 72-hour incubation of same DC with poly I:C at $20 \mu\text{g/ml}$ resulted in maturation as reflected by the increased expression of the costimulatory markers and the slight expression of the CD83 (Fig 3b).

We thereafter checked if it was still possible to induce the maturation of DC previously infected by ALVAC recombinant virus. Flow cytometry analysis was conducted using the two-step gating procedure, as described previously. Results from one representative experiment (Fig 4) showed an upregulation of the costimulatory markers DR, CD40, CD80, CD86 as well as the slight

Figure 4 Immature DC infected by ALVAC vectors are still able to mature. Totally, 2×10^6 DC were infected by an ALVAC-EGFP recombinant virus at an MOI 10 for 1 hour. They were then cultured in the presence of poly I:C for another 72 hours (ALVAC + poly I:C D4) or left in culture for same time (ALVAC D4). Surface markers expressed on DC were controlled by flow cytometry using the same two-step gating procedure as described in Figure 2. Results are from one representative experiment out of three.

upregulation of the CD83 marker. Immature ALVAC-infected DC thus still kept the capacity to mature in response to the addition of poly I:C. We also observed by using an ALVAC-EGFP recombinant virus that poly I:C matured DC were infected with an about 10% lower efficacy than immature ones (data not shown).

DC infected by ALVAC vector and matured with poly I:C secrete IL12

All DC culture supernatants from the various maturation and ALVAC transduction experiments were tested by ELISA for IL12 p40 production. Data from Figure 5 show a highly increased IL-12 secretion by DC treated with poly I:C (mean $\pm$ SD: 3141 ± 3562) as compared to the low amount of IL-12 produced by immature noninfected DC (mean $\pm$ SD: 67 ± 37) or by ALVAC-transduced DC (mean $\pm$ SD: 40 ± 0). Furthermore when DC were infected by ALVAC prior to poly I:C treatment, they still secreted high amounts of IL-12 p40 (mean $\pm$ SD: 2176 ± 2512). Besides, IL-10 secretion by the DC was negligible ($<10 \text{ pg/ml}$) in basal condition and not modulated after ALVAC infection (data not shown).

Enhanced activation of a MAGE 3/A1 CTL clone by DC transduced by ALVAC-MAGE-1-MAGE-3 minigenes-vector

We finally evaluated comparatively the potency of ALVAC-transduced DC *versus* peptide-pulsed DC to activate specifically CTL clones. Two different CTL clones restricted respectively for MAGE 1/A1 (data not shown) or MAGE 3/A1 tumor antigens were tested under stimulation by DC from HLA-A1 patient-donors transduced by an ALVAC vector carrying MAGE-1/A1 and MAGE-3/A1 minigenes. DC pulsed with relevant MAGE1-A1 (data not shown) or MAGE3-A1 peptides were also used in parallel as stimulators. We used as

Figure 5 IL-12 secretion. In all, 2×10^6 DC were cultured in various conditions: (●), Control $n=10$; (■), DC infected by ALVAC-EGFP vector at an MOI 10 for 1 hour, $n=8$; (▲), DC cultured in the presence of poly I:C for 72 hours, $n=5$; (▼), DC infected by ALVAC-EGFP vector at an MOI 10 for 1 hour and then matured in the presence of poly I:C for 72 hours, $n=3$. All culture supernatants were tested by ELISA for IL12 p40 content (means $\pm$ SD).

negative controls for CTL activation nontransduced DC or ALVAC-EGFP-transduced DC from same donor, and as positive control, DC cocultured with the MEL-Z2 melanoma cell line. Results presented in Figure 6a–c demonstrated that DC from HLA-A1 patient-donors transduced with ALVAC vectors carrying MAGE 1-MAGE 3 minigenes were capable of activating a MAGE 3/A1 CTL clone more efficiently than same DC loaded with MAGE 3/A1 peptide, as shown by increased IFN- γ secretion. Quite interestingly, clear enhanced CTL activation was also obtained by stimulation with a DC population that had been infected by the ALVAC-MAGE-1-MAGE-3 minigenes vector with a low efficiency of transduction (as measured at same time following infection of same DC with the ALVAC-EGFP viral vector), that is, score of 5% for donor S.

Discussion

The aim of this study was to characterize carefully the transduction of DC derived from melanoma patients by an ALVAC vector as a prerequisite for future clinical vaccination trials using DC genetically modified to present products of MAGE 1 and MAGE 3 minigenes. We thus firstly optimized the infection procedure of clinical grade DC by ALVAC recombinant virus and then showed that ALVAC vectors were capable of infecting immature DC without inducing their maturation. However, ALVAC transduction did not interfere with the

Figure 6 (a–c) DC transduced with an ALVAC vector carrying MAGE-1-MAGE-3 minigenes highly activate a MAGE 3/A1-specific CTL clone. DC generated from three different HLA-A1 cancer patients (B, L and S) were infected with an ALVAC-MAGE-1 MAGE-3 minigenes vector at different MOI (5, 10, 20) for 1 or 2 hours and then used at 3×10^4 cells/well/50 μ l to stimulate 10^4 cells/well/50 μ l of a MAGE 3-A1 CTL clone. After 24-hour stimulation at 37°C in the presence of recombinant IL-2 (25 U/ml), culture supernatants were harvested and tested for IFN- γ content by ELISA. DC pulsed with relevant MAGE 3-A1 peptide were used in parallel as stimulators also. Nontransduced DC or ALVAC-EGFP-transduced DC were used as negative controls for CTL activation and DC cocultured for 24 hours with the MEL-Z2 melanoma cell line as positive control.

capacity of those DC to further mature under poly I:C stimulation. Immature DC from different HLA-A1 melanoma patients were then transduced by an ALVAC vector carrying MAGE 1/3 minigenes. They appeared equal or even superior as APC than same DC pulsed with MAGE 3/A1 peptide to activate specifically a MAGE 3/A1 CTL clone, as demonstrated by increased IFN- γ secretion.

Since DC have a relatively unstable phenotype, nonperturbing methods of gene transfer should be employed so that their viability and their immunostimulatory capacity are not compromised. As we showed, these requirements were satisfied by ALVAC recombinant virus. On the contrary, vaccinia virus (VV) and especially herpes simplex virus (HSV) which has a strong cytopathogenic effect on immature DC³² inhibit DC maturation^{33,34} and efficient antigen presentation by selectively downregulating expression of MHC class I/II, CD 80 and CD 83 molecules^{32,35–37} or by interfering with the TAP-loading mechanism.¹⁹

We described in this report that ALVAC vectors were efficiently capable of infecting clinical-grade immature DC, but not with same efficacy mature DC (data not shown). These results are partly in contradiction with studies reporting important induction of apoptosis within ALVAC-infected immature DC.^{38–40} But it is likely that the protocol we set up to infect those DC (MOI 10, 1 hour of infection) was better for having good recombinant protein expression without inducing high mortality or apoptotic cell death. On the other hand, the fact that mature DC are more resistant to ALVAC infection was also reported by same authors.

Our data show no real maturation of DC induced by ALVAC infection since the “maturation marker” CD83 was not expressed at the DC surface although there was a slight upregulation of MHC-II and the CD86 molecules. Moreover, IL-12 production was as low as in noninfected DC. These results are in contradiction with the papers of Motta *et al*³⁹ and Marovich *et al*⁴⁰ which are suggesting or showing that infection of DC by canarypox viruses led to their maturation. They indeed observed that the expression of MHC-II, CD40, CD80 and CD86 antigens was upregulated and expression of CD83 induced. However, both studies used DC cultured in medium supplemented with serum (FCS or autologous plasma) whereas we generated and transduced DC in serum free X-VIVO 20 medium where it was quite more difficult to induce DC maturation (as shown in Fig 3). Besides, we have compared MHC-II, CD40, CD80, CD83 and CD86 expression between ALVAC-infected DC and control noninfected DC at same time-point, that is, after 24 hours or more of reculture, which is rarely done and could be the source of misinterpretation. Indeed, as shown in Figure 2, there is a notable increase in the DR or CD86 expression in control noninfected DC after 1 day reculture.

However, ALVAC does not interfere with the capacity of DC to mature following a poly I:C stimulus. We indeed noted a clear upregulation of the expression of CD80 and CD86 molecules as well as a slight expression of CD83.

The expression of this last marker is commonly used as the DC maturation marker and, although its function is not completely understood, its expression has been linked with the ability to induce protective immune responses to test antigens in human clinical trials.⁴¹ Also, the production of IL-12 by the ALVAC-infected DC which otherwise remains near control values, was induced after treatment with poly I:C.

Interestingly, our data show that immature DC from HLA-A1 melanoma patients transduced with an ALVAC vector carrying MAGE 1/3 minigenes were capable of activating a MAGE 3/A1 CTL clone more efficiently than same DC loaded with MAGE 3/A1 peptide, as shown by increased IFN- γ secretion. Moreover, we found that CTL activation was even obtained with very low efficiency of DC transduction. In agreement, direct immunization with several ALVAC vectors was shown to induce both humoral immunity and CTL responses, implying that these vectors can target both CD4+ and CD8+ T cells.²³ Also, strong CD8+ effector responses requiring CD4+ T-cell help were detected in HIV-1+ individuals vaccinated with even a small percentage of mature DC infected with canarypox virus encoding HIV antigens.³⁸ In accordance too, immature DC infected by recombinant ALVAC encoding MART-1 were recently shown to express and present this melanoma-associated antigen as evidenced by their capacity to stimulate *in vitro* an HLA-A2+, MART-specific CTL clone.³⁹ The recent discovery of the differential processing of class-I-restricted epitopes of (tumor) antigens by the standard proteasome and the immunoproteasome⁴² supports the use of an ALVAC vector carrying MAGE 1/3 minigenes to infect DC. Indeed immature DC carry equal amounts of both, whereas mature DC contain only immunoproteasome. Therefore transferring into DC minigenes encoding only the specific and relevant peptides should lead to better immune responses, not dependent on antigen processing.

Overall, our data may argue on using DC transduced by ALVAC vectors carrying MAGE sequences as a possible strategy in cancer immunotherapy. Canarypox vectors have an excellent profile in Phase I clinical trials and their effectiveness against infectious agents as well as cancer has been suggested both in animals and humans.⁴³ Combining the properties of ALVAC vectors with the unique properties of DC as APC could enhance antitumor responses, increasing simultaneously their durability and their magnitude. This strategy appears as an attractive approach in cancer immunotherapy, which merits to be clinically investigated in the future.

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References

1. Thurner B, Haendle I, Roder C, et al. Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. *J Exp Med*. 1999;190:1669–1678.
2. Kugler A, Stuhler G, Walden P, et al. Regression of human metastatic renal cell carcinoma after vaccination with tumor cell-dendritic cell hybrids. *Nat Med*. 2000;6:332–336.
3. Nestle FO, Aljagic S, Gilliet M, et al. Vaccination of melanoma patients with pep. *Nat Med*. 1998;4:328–332.
4. Tounouz M, Libin M, Bulte F, et al. Transient expansion of peptide-specific lymphocytes producing IFN- γ after vaccination with dendritic cells pulsed with MAGE peptides in patients with mage-A1/A3-positive tumors. *J Leukoc Biol*. 2001;69:937–943.
5. Ossendorp F, Mengede E, Camps M, et al. Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. *J Exp Med*. 1998;187:693–702.
6. Arthur JF, Butterfield LH, Roth MD, et al. A comparison of gene transfer methods in human dendritic cells. *Cancer Gene Ther*. 1997;4:17–25.
7. Zhong L, Granelli-Piperno A, Choi Y, et al. Recombinant adenovirus is an efficient and non-perturbing genetic vector for human dendritic cells. *Eur J Immunol*. 1999;29:964–972.
8. Van Tendeloo VF, Snoeck HW, Lardon F, et al. Nonviral transfection of distinct types of human dendritic cells: high-efficiency gene transfer by electroporation into hematopoietic progen. *Gene Therapy*. 1998;5:700–707.
9. Tuting T, DeLeo AB, Lotze MT, et al. Genetically modified bone marrow-derived dendritic cells expressing tumor-associated viral or “self” antigens induce antitumor immunity *in vivo*. *Eur J Immunol*. 1997;27:2702–2707.
10. Kim CJ, Prevetté T, Cormier J, et al. Dendritic cells infected with poxviruses encoding MART-1/Melan A sensitize T lymphocytes *in vitro*. *J Immunother*. 1997;20:276–286.
11. Brossart P, Goldrath AW, Butz EA, et al. Virus-mediated delivery of antigenic epitopes into dendritic cells as a means to induce CTL. *J Immunol*. 1997;158:3270–3276.
12. Kirk CJ, Mule JJ. Gene-modified dendritic cells for use in tumor vaccines. *Hum Gene Ther*. 2000;11:797–806.
13. Chen PW, Wang M, Bronte V, et al. Therapeutic antitumor response after immunization with a recombinant adenovirus encoding a model tumor-associated antigen. *J Immunol*. 1996;156:224–231.
14. Song W, Kong HL, Carpenter H, et al. Dendritic cells genetically modified with an adenovirus vector encoding the cDNA for a model antigen induce protective and therapeutic antitumor immunity. *J Exp Med*. 1997;186:1247–1256.
15. Kaplan JM, Yu Q, Piraino ST, et al. Induction of antitumor immunity with dendritic cells transduced with adenovirus vector-encoding endogenous tumor-associated antigens. *J Immunol*. 1999;163:699–707.
16. Specht JM, Wang G, Do MT, et al. Dendritic cells retrovirally transduced with a model antigen gene are therapeutically effective against established pulmonary metastases. *J Exp Med*. 1997;186:1213–1221.
17. Bronte V, Carroll MW, Goletz TJ, et al. Antigen expression by dendritic cells correlates with the therapeutic effectiveness of a model recombinant poxvirus tumor vaccine. *Proc Natl Acad Sci USA*. 1997;94:3183–3188.
18. Alcamí A, Koszinowski UH. Viral mechanisms of immune evasion. *Trends Microbiol*. 2000;8:410–418.
19. Tortorella D, Gewurz BE, Furman MH, et al. Viral subversion of the immune system. *Annu Rev Immunol*. 2000;18:861–926.
20. Bhardwaj N. Interactions of viruses with dendritic cells: a double-edged sword. *J Exp Med*. 1997;186:795–799.
21. Jenne L, Schuler G, Steinkasserer A. Viral vectors for dendritic cell-based immunotherapy. *Trends Immunol*. 2001;22:102–107.
22. Taylor J, Weinberg R, Tartaglia J, et al. Nonreplicating viral vectors as potential vaccines — recombinant canarypox virus expressing measles-virus fusion (F) and hemagglutinin (Ha) glycoproteins. *Virology*. 1992;187:321–328.
23. Paoletti E. Applications of pox virus vectors to vaccination: an update. *Proc Natl Acad Sci USA*. 1996;93:11349–11353.
24. Kawakita M, Rao GS, Ritchey JK, et al. Effect of canarypox virus (ALVAC)-mediated cytokine expression on murine prostate tumor growth. *J Natl Cancer Inst*. 1997;89:428–436.
25. Taylor J, Meignier B, Tartaglia J, et al. Biological and Immunogenic properties of a canarypox-rabies recombinant, Alvac Rg (Vcp65) in non-avian species. *Vaccine*. 1995;13:539–549.
26. Marshall JL, Hawkins MJ, Tsang KY, et al. Phase I study in cancer patients of a replication-defective avipox recombinant vaccine that expresses human carcinoembryonic antigen. *J Clin Oncol*. 1999;17:332–337.
27. Gorse GJ, Patel GB, Belshe RB. HIV type 1 vaccine-induced T cell memory and cytotoxic T lymphocyte responses in HIV type 1-uninfected volunteers. *AIDS Res Hum Retroviruses*. 2001;17:1175–1189.
28. Tartaglia J, Bonnet MC, Berinstein N, et al. Therapeutic vaccines against melanoma and colorectal cancer. *Vaccine*. 2001;19:2571–2575.
29. Boon T, Cerottini JC, Van den Enyde B, et al. Tumor antigens recognized by T lymphocytes. *Annu Rev Immunol*. 1994;12:337–365.
30. Renkvist N, Castelli C, Robbins P, et al. A listing of human tumor antigens recognized by T cells. *Cancer Immunol Immunother*. 2001;50:3–15.
31. Chaux P, Luiten R, Demotte N, et al. Identification of five MAGE-A1 epitopes recognized by cytolytic T lymphocytes obtained by *in vitro* stimulation with dendritic cells transduced with MAGE-A1. *J Immunol*. 1999;163:2928–2936.
32. Kruse M, Rosorius O, Kratzer, et al. Mature dendritic cells infected with herpes simplex virus type 1 exhibit inhibited T-cell stimulatory capacity. *J Virol*. 2000;74:7127–7136.
33. Engelmayer J, Larsson M, Subklewe M, et al. Vaccinia virus inhibits the maturation of human dendritic cells: a novel mechanism of immune evasion. *J Immunol*. 1999;163:6762–6768.
34. Salio M, Cella M, Suter M, et al. Inhibition of dendritic cell maturation by herpes simplex virus. *Eur J Immunol*. 1999;29:3245–3253.
35. Jenne L, Hauser C, Arrighi JF, et al. Poxvirus as a vector to transduce human dendritic cells for immunotherapy: abortive infection but reduced APC function. *Gene Therapy*. 2000;7:1575–1583.

36. Drillien R, Spehner D, Bohbot A, et al. Vaccinia virus-related events and phenotypic changes after infection of dendritic cells derived from human monocytes. *Virology*. 2000;268:471–481.
37. Harris NL, Ronchese F. The role of B7 costimulation in T-cell immunity. *Immunol Cell Biol*. 1999;77:304–311.
38. Engelmayer J, Larsson M, Lee A, et al. Mature dendritic cells infected with canarypox virus elicit strong anti-human immunodeficiency virus CD8⁺ and CD4⁺ T-cell responses from chronically infected individuals. *J Virol*. 2001;75:2142–2153.
39. Motta I, Andre F, Lim A, et al. Cross-presentation by dendritic cells of tumor antigen expressed in apoptotic recombinant canarypox virus-infected dendritic cells. *J Immunol*. 2001;167:1795–1802.
40. Marovich MA, Mascola JR, Eller MA, et al. Preparation of clinical-grade recombinant canarypox-human immunodeficiency virus vaccine-loaded human dendritic cells. *J Infect Dis*. 2002;186:1242–1252.
41. Jonuleit H, Giesecke-Tuettenberg A, Tutting T, et al. A comparison of two types of dendritic cell as adjuvants for the induction of melanoma-specific T-cell responses in humans following intranodal injection. *Int J Cancer*. 2001;93:243–251.
42. Van den Eynde BJ, Morel S. Differential processing of class-I-restricted epitopes by the standard proteasome and the immunoproteasome. *Curr Opin Immunol*. 2001;13:147–153.
43. Karanikas V, Lurquin C, Colau D, et al. Monoclonal anti-MAGE-3 CTL responses in melanoma patients displaying tumor regression after vaccination with a recombinant canarypox virus. *J Immunol*. 2003;171:4898–4904.