Research Article

Measles Virus Induces Oncolysis of Mesothelioma Cells and Allows Dendritic Cells to Cross-Prime Tumor-Specific CD8 Response

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Abstract

Despite conventional medical and surgical treatments, malignant pleural mesothelioma (MPM) remains incurable. Oncovirotherapy (i.e., the use of replication-competent virus for cancer treatment) is currently explored in clinical trials. In this study, we investigated the antineoplastic potential of a new oncolytic viral agent, a live-attenuated measles virus (MV) strain derived from the Edmonston vaccine lineage (Schwarz strain). We evaluated both oncolytic activity and immunoadjuvant properties of the MV vaccine strain on mesothelioma tumor cells. Infectivity, syncytium formation, and cytolytic activity of MV were studied on a panel of mesothelioma cells derived from pleural effusions of MPM patients. We observed that MV infected preferentially MPM cell lines in comparison with nontransformed mesothelial cells, leading to an efficient killing of a significant fraction of tumor cells. A cytoreductive activity was also evidenced through formation of multinuclear cellular aggregates (syncytia). The susceptibility of MPM cell lines to measles infection was assessed by the analysis of cell surface expression of the MV vaccine receptor (CD46). We also evaluated whether MV infection of mesothelioma cells could elicit an autologous antitumor immune response. We showed that MV Schwarz strain induced apoptotic cell death of infected mesothelioma cells, which were efficiently phagocytosed by dendritic cells (DC). Loading of DCs with MV-infected MPM cells induced DC spontaneous maturation, as evidenced by the increased expression of MHC and costimulatory molecules along with the production of proinflammatory cytokines. Priming of autologous T cells by DCs loaded with MV-infected MPM cells led to a significant proliferation of tumor-specific CD8 T cells. Altogether, these data strongly support the potential of oncolytic MV as an efficient therapeutic agent for mesothelioma cancer. [Cancer Res 2008;68(12):4882–92]

Introduction

Malignant mesotheliomas are highly aggressive neoplasms that arise from the uncontrolled proliferation of mesothelial cells lining serosal cavities, most commonly the pleura [malignant pleural mesothelioma (MPM); ref. 1]. Exposure to asbestos is the main factor involved in MPM pathogenesis. In the absence of overt symptoms during the early period of tumor development, diagnosis is usually established late in disease evolution, which makes the prognosis for patients very poor (median survival of <1 year). MPM is also considered to be a cancer that is relatively refractory to all conventional treatment modalities, such as chemotherapy, radiotherapy, and/or surgery (2). Thus, in the absence of an efficient management strategy for this cancer, there is a pressing need for the development of new clinical approaches.

During the past decade, there has been an increasing interest in oncovirotherapy (i.e., the use of replicating viruses for cancer treatment). Numerous live-attenuated viruses, such as adenovirus (AdV), vesicular stomatitis virus (VSV), herpes simplex virus (HSV), Newcastle disease virus, vaccinia viruses, and measles virus (MV), are now considered as potential cancer therapeutics (3, 4). Among them, MV has already shown promising oncolytic properties (5). MV is an enveloped, nonsegmented, and negative single-strand RNA virus belonging to the Morbillivirus genus of the Paramyxoviridae family (6). Live-attenuated MV vaccines, such as the Schwarz strain, were derived from a clinical isolate by extensive passages through culture of chicken embryo fibroblasts (7). This vaccine has been administered to hundreds of millions of children since the 1970s and is considered as one of the most effective and safe human vaccines. Furthermore, the MV genome is very stable and the reversion of vaccine strains to pathogenic forms has never been observed.

Apart from its excellent safety profile, Edmonston-derived MV vaccine strains efficiently kill a clinically significant fraction of tumoral cells by direct cytolysis, leaving neighboring healthy tissue unharmed (8). Indeed, several *in vitro* studies have already shown that live-attenuated MV is selectively oncolytic, causing the formation of extensive multinucleated syncytia and killing a variety of human tumor cells, such as lymphoma (9), multiple myeloma (10), glioblastoma (11), and ovarian carcinoma (12) cell lines. Moreover, *in vivo* administration of the MV Edmonston strain resulted in either slower growth or even partial regression of tumors established in experimental models of lymphoma and myeloma cancers (9, 10). Another important component of the long-term therapeutic benefit of oncolytic viruses seems to rely on their capacity to trigger an autologous antitumoral immune response, as previously described for another paramyxovirus VSV (13, 14). Hence, both mechanisms could be implicated in spontaneous clinical remission previously observed in cancer patients naturally infected with MV (15–17). However, relatively little attention has been paid thus far to the role of MV-based virotherapy in the activation of antitumoral immunity (18).

In the present study, we investigated both the oncolytic activity and immunoadjuvant properties of Schwarz measles vaccine on a panel of MPM cells isolated from pleural effusions of mesothelioma patients. We used a cloned Schwarz MV produced from an infectious cDNA that we have previously described (19, 20). We analyzed infection susceptibility and cytolytic activity on both

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doi:10.1158/0008-5472.CAN-07-6265

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tumoral and nontransformed mesothelial cells. We observed that MPM tumor cells are more susceptible than nontransformed mesothelial cells to MV infection. The increased susceptibility of MPM cells to MV infection was assessed by the analysis of cell surface expression of the MV vaccine receptor (CD46). We also evaluated whether MV infection of mesothelioma cells could elicit an autologous immune response specific for a tumor-associated antigen (TAA), namely, mesothelin (MSLN). MSLN is a differentiation antigen that is abundantly expressed in normal mesothelial cells, from which malignant mesothelioma arises. Among MPM-associated antigens, MSLN seems as a promising target for immunotherapy (21). To determine whether MV could induce cellular immunity to MSLN, we cocultivated human primary monocyte-derived dendritic cells (DC) with MV-infected mesothelioma cells and analyzed the phagocytic properties of DCs, their activation surface phenotype and cytokine secretion profile, as well as their functional capacity to stimulate MSLN-specific CD8 T cells. We showed that phagocytosis of apoptotic MV-infected mesothelioma cells induced spontaneous DC maturation and activation, as evidenced by an increased expression of MHC and costimulatory molecules, the production of proinflammatory cytokines with Th1 polarizing capacities, and a significant amplification of MSLN-specific CD8 T cells.

Materials and Methods

Cell culture. Mesothelioma cell lines (M11, M13, M31, M47, M56, and M61) were established from pleural effusions collected by thoracocentesis of cancer patients after obtaining informed consent. The control mesothelial cell line (Met5A) was purchased from the American Type Culture Collection (LGC Promochem). Cell lines were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS (BioWest), 1% L-glutamine, and 1% penicillin-streptomycin antibiotics (all from Sigma). Cell cultures were routinely checked for *Mycoplasma* contamination using Hoechst 33258 staining (Sigma).

MV production. Attenuated Schwarz MV was produced from the pTM-MVSchw plasmid using the helper cell–based rescue system described by Radecke and colleagues (22) and modified by Parks and colleagues (20). Recombinant MV-enhanced green fluorescent protein (eGFP) virus was similarly rescued from the pTM-MV-eGFP plasmid, as previously described (19). Syncytia that appeared after coculture of helper cells with Vero cells were expanded on Vero cells in DMEM-5% FCS. When syncytia reached 80% to 90% confluence, the cells were scraped into a small volume of Opti-MEM (Invitrogen) and freeze thawed once. After low-speed centrifugation to pellet cellular debris, the virus-containing supernatant was stored at −80°C. Viral titer was determined by an end point limit dilution assay on Vero cells. The $TCID_{50}$ was calculated by the use of the Kärber method.

Apoptosis detection. Apoptosis was triggered by UVB exposure (312 nm-25 kJ/m^2) using an UV Stratalinker 2400 (Stratagene Europe) or by virus infection [multiplicity of infection (MOI), 1.0] with MV Schwarz strain. Cell death was evidenced by 15-min staining at room temperature using FITC-conjugated Annexin V labeling kit (5 μL per 10^5 cells) according to the manufacturer's instructions (BD Biosciences).

Reverse transcription-PCR. Confluent M13 cells were infected with MV Schwarz strain (MOI, 1.0), and cell pellets were collected at different times after infection. Total cell RNA was extracted using RNeasy kits (Qiagen) and reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen). Resulting cDNA were used as template for PCR amplification with primers specific for Toll-like receptor (TLR)-3, Mda-5, RIG-I, and PKR (Sigma). Gene-specific primer sets for β-actin were used as internal control of reverse transcription-PCR (RT-PCR) experiments.

Intracellular HSP immunostaining. M13 tumoral cells were stressed by heat shock (1 h at 42°C) or virus infection (2 h at 37°C; MOI, 1.0) and stained as follows. Briefly, cells were first suspended in 100 μL of 4% paraformaldehyde fixation solution for 15 min at room temperature and then incubated with 100 μL of 1:100 diluted anti-HSP70 (clone SPA810) or anti-Gp96 (clone SPA850) antibodies (Stressgen) in PBS-0.1% bovine serum albumin (BSA)-0.1% saponin for 30 min at 4°C and finally with 100 μL of 1:250 diluted Alexa Fluor 488–conjugated F(ab′)$_2$ fragment goat anti-mouse IgG (H+L) antibodies (Molecular Probes) for an additional 30 min at 4°C.

Monocyte-derived DC production. Monocytes were generated from leukapheresis harvests of healthy donors (Etablissement Français du Sang) after obtaining informed consent. The monocyte-enriched fraction (>85% purity) was isolated by incubation with RosetteSep human monocyte enrichment cocktail (StemCell Technologies), following the manufacturer's instructions, and then separated by Ficoll density gradient centrifugation (PAA Laboratories). For DC preparation, 2×10^6 monocytes per mL were cultured in RPMI 1640 supplemented with 70 ng/mL of granulocyte macrophage colony-stimulating factor and 50 ng/mL of interleukin (IL)-4 (AbCysSA).

DC loading and phagocytosis analysis. On day 5, immature DCs were collected from the monocyte culture supernatant and seeded into poly-HEMA–coated 12-well plates at 2×10^6 cells per mL. Because the harvest of both tumor cells and peripheral blood mononuclear cells (PBMC) from the same MPM patient proved to be difficult, an HLA-A2$^-$ allogeneic mesothelioma cell line (M13) was used for loading monocyte-derived DCs obtained from HLA-A2$^+$ healthy donors. For subsequent loading, DCs were incubated with 1×10^6 cells per mL of apoptotic material obtained from UV-irradiated or MV-infected M13 tumoral cells (ratio, 2:1). DC phagocytosis was assessed both by flow cytometry and confocal laser microscopy, as previously described (23). Briefly, UV- or MV-treated M13 cells were labeled with PKH-26 membrane dye colorant according to the manufacturer's protocol (Sigma). After 24-h coculture, DCs were stained with FITC-conjugated anti–HLA-DR antibodies (Immunotech). After PBS washes, cells were harvested and analyzed either on a FACSCalibur (BD Biosciences) or with a TCS NT microscope (Leica Instruments). DCs that had ingested apoptotic cells were identified as HLA-DR$^+$/PKH-26$^+$ double-stained cells.

DC phenotype. DCs were cocultured with apoptotic UV-treated or MV-infected M13 cells for 18 h and the surface expression of DC maturation markers was next analyzed by three-color flow cytometry. Immunostaining was performed with a panel of monoclonal antibodies (all purchased from Immunotech) specific for HLA-ABC (clone B9.12.1), HLA-DR (clone B8.12.2), CD80 (clone MAB104), CD83 (clone HB15a), CD86 (clone HA5.2B7), and CD40 (clone MAB89). Briefly, DCs were incubated with each of the above antibodies (1 μg/mL) at 4°C for 30 min. The cytokine secretion pattern was assayed in supernatants collected 24 h after coculture. IL-10, IL-12p70, IL-6, IL-1β, and tumor necrosis factor α (TNFα) concentrations were measured using commercially available BD Cytometric Beads Array (CBA) kits according to the manufacturer's protocol. The quantification of IFNα was performed with an ELISA test (BioSource).

***In vitro* T-cell sensitization.** CD8 T lymphocytes were prepared from HLA-A0201 healthy donor PBMCs by positive selection with the MACS column system using the CD8 multisort kit (Miltenyi Biotec). Purified CD8 T cells (>90% purity) were stimulated with autologous DCs loaded with different apoptotic preparations or unloaded DCs as a control. Coculture was performed in round-bottomed 96-well plates (BD Falcon) by mixing 2×10^4 mature DCs with 2×10^5 responder T cells (ratio, 1:10) in 200 μL of 8% horse serum RPMI 1640 supplemented with 10 ng/mL IL-12 for the first 3 d (AbCysSA) and with 10 units/mL IL-2 (Proleukin, Chiron Therapeutics) for the following days. IL-2 was added every 2 to 3 d, allowing regular culture medium renewal. After 7 to 8 d of culture, T cells were harvested and stained with MSLN-specific tetramers.

Tetramer immunostaining. Target peptides MSLN531-539 (24) and MSLN541-550 (25) were initially identified by scanning the MSLN amino acid sequence (NP 005814) for matches to consensus motifs for HLA-A0201 binding using two computer algorithms, BIMAS and SYFPEITHI (Fig. 5*A*). MSLN-derived peptides (synthesis performed by Eurogentec) were used for monomer production (recombinant protein production platform, U601-IFR26, Nantes, France), as previously described (26). HLA-A2 VLP9 and KLL10 monomers were then oligomerized with phycoerythrin (PE)-labeled streptavidin (BD Biosciences). Staining and washing were performed in 0.1% BSA-PBS. T cells were stained successively with 10 μg/mL of PE-labeled

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pMHC multimers at 4°C for 30 min and with 1 μg/mL diluted PE-Cy5–conjugated anti-CD8 antibodies (clone RPA-T8; BD Biosciences) for an additional 30 min at 4°C. Cells were washed and immediately analyzed on a FACSCalibur.

Results

Schwarz MV preferentially infects and kills mesothelioma cells. Although numerous studies have already established the potential efficiency of MV-based virotherapy for several cancers (5), no information has yet been provided about MV oncolytic activity on mesothelioma cancer. To evaluate the susceptibility of mesothelioma to the oncolytic activity of MV, we infected a panel of epithelioid (M11, M13, M47, M56, and M61) and one sarcomatoid (M31) MPM cell lines with the MV Schwarz strain (Fig. 1*A*). An immortalized mesothelial cell line (Met5A) was used as control. Three days after infection, we observed an important cytopathic effect (CPE) that was markedly increased on most epithelioid MPM-infected cells (four of five lines) compared with nontransformed Met5A cells (Fig. 1*A*). CPE was evidenced through the development of large syncytia, the appearance of inclusion bodies in dead tumoral cells, and finally by the complete dissociation of the cell layer 72 to 96 h after infection. We also observed that M31 is readily infected by Schwarz vaccine but displays a significant lower sensibility to MV oncolytic activity than epithelioid MPM cells (data not shown).

To compare MV infection sensitivity, we infected a representative epithelioid MPM cell line (M13), which belongs to the major mesothelioma phenotype, and an established mesothelial cell line (Met5A) with a recombinant Schwarz MV expressing eGFP (MOI, 1.0). The yield of infection was quantified by flow cytometry (Fig. 1*B* and *C*). The infection yield was dose dependent for both cell lines (MOI ranging from 0.01 to 5.0; data not shown). Tumor cells were significantly more susceptible to measles infection than normal cells were, both in individual culture (Fig. 1*B*) and in coculture (Fig. 1*C*) systems. Indeed, 2 days after infection, the number of infected tumoral M13 cells was repeatedly 2-fold higher than that of nontransformed Met5A cells (Fig. 1*B* and *C*). The development of multinuclear cell aggregates (syncytia) is characteristic of MV infection and indicates an efficient cell-to-cell spread of MV. Syncytia formation results from fusion of infected cells with noninfected neighboring cells through interactions between the glycoproteins (hemagglutinin and fusion) from viral envelope and the cellular receptor of MV vaccine strains (CD46). We thus compared the level of CD46 expression among the different cell lines tested. As it has already been reported for several cancers (27), we found that most mesothelioma tumor cells expressed a significant higher level of CD46 than nontransformed Met5A cells (Fig. 1*D*). Interestingly, we also observed that the susceptibility of MPM cells to MV could be related to their CD46 expression level. Indeed, M56 and M31 cell lines that expressed the lowest CD46 level were less sensitive to both MV infection (Fig. 1*D*) and cytolytic activity (Fig. 1*A*). Altogether, these results show that MPM tumor cells are more susceptible than nontransformed mesothelial cells to MV, suggesting that MV-based virotherapy could be a reliable therapeutic approach for mesothelioma cancer.

MV infection triggers danger signals in mesothelioma cells. Intracellular double-stranded RNA (dsRNA) production is a major sign of replication for many RNA viruses and a strong viral stimulus for pattern recognition receptors (PRR), such as TLRs or RIG-I–like receptors (28). MV infection has been previously shown to induce activation of specific PRR expression in lung epithelial cells (29). To assess the presence of viral nucleic acids in MV-infected MPM cells, we looked for the expression of *TLR-3, Mda-5, RIG-I*, and *PKR* genes by RT-PCR (the specific primers used are listed in Fig. 2*A*). Attesting to the presence of viral genome replication intermediates, the expression levels of Mda-5, and to a lower extent RIG-I, were increased during the progression of infection in M13 epithelial cells, peaking at 48 to 72 h after infection (Fig. 2*B*). This result confirms the implication of Mda-5 (29, 30) and RIG-I (31) in MV-specific innate response. In contrast, we observed a progressive decline of TLR-3 and no change in PKR gene expression (Fig. 2*B*). Overall, these data indicate, although indirectly, that dsRNA is readily produced in MV-infected M13 mesothelioma cells.

As viral infection may be related to a cellular stress, we thus explored the expression pattern of intracellular heat shock proteins (HSP) induced by MV infection. Heat shock of M13 cells was used as an internal positive control. Intracellular immunostaining was targeted to HSP70 and Gp96, two molecules previously related to a cross-presentation mechanism (32). Flow cytometry results evidenced constitutive cytoplasmic HSP70 [98.9% positive cells; mean fluorescence index (MFI), 51.05] and RE-associated Gp96 (85% positive cells; MFI, 18.8) expression by M13 tumoral cells (Fig. 2*C*). The kinetic study indicates that significant and maximum expression of inducible HSP70 is detected in MV-infected M13 cells at 72 h after infection (MFI, 194.2). These results are concordant with previous studies performed on HSP70 induction by MV infection (33). In comparison, heat shock–treated M13 cells expressed HSP70 as early as 8 h after treatment (MFI, 128.9; data not shown). The same experiment was conducted for Gp96, but in contrast to HSP70, no significant variation in Gp96 expression could be observed following MV infection (Fig. 2*C*).

MV infection induces mesothelioma cell death. As shown above, MV Schwarz strain infection caused severe alterations in MPM cell culture, which finally led to cell death (CPE). To discriminate between apoptosis- and necrosis-mediated cell death, we used Annexin V staining. Our results evidenced that apoptosis was the mode of MV-induced cell death (Fig. 3*A*). To follow the kinetic of mesothelioma cell killing, subconfluent monolayer M13 cell cultures were either MV infected (MOI, 1.0) or UV irradiated (25 kJ/m^2) and the percentage of Annexin V–positive cells was determined at different time points (Fig. 3*B*). A significant rate of tumoral cell death (70–80% of Annexin V–positive cells) was observed during 24 h following UVB irradiation or 72 h after infection with MV vaccine. These M13 cell death–induced conditions were thus used in the following experiments, performed to show that virotherapy could lead to the activation of an autologous antitumor immune response.

Apoptotic MV-infected mesothelioma cells are efficiently uptaken by immature DCs. We previously described that UV-irradiated apoptotic mesothelioma cells are efficiently phagocytosed by immature DCs (23). Here, we analyzed the uptake yield of MV-infected M13 cells in a similar phagocytosis assay. For that purpose, DCs were incubated with dead M13 cells stained with PKH-26 for 24 h. Interactions of HLA-DR–labeled DCs with PKH-26–positive M13 cells were then determined by flow cytometry analysis of double-positive ($PKH\text{-}26^+/FITC^+$) cells (Fig. 3*C*). More than 70% of the immature DCs efficiently engulfed apoptotic MV-treated M13 cells, similar to the phagocytic activity observed for UV-treated M13 cells (Fig. 3*D*). The phagocytosis process was confirmed by confocal laser scanning microscopy experiments that showed an efficient internalization of apoptotic M13 cells by

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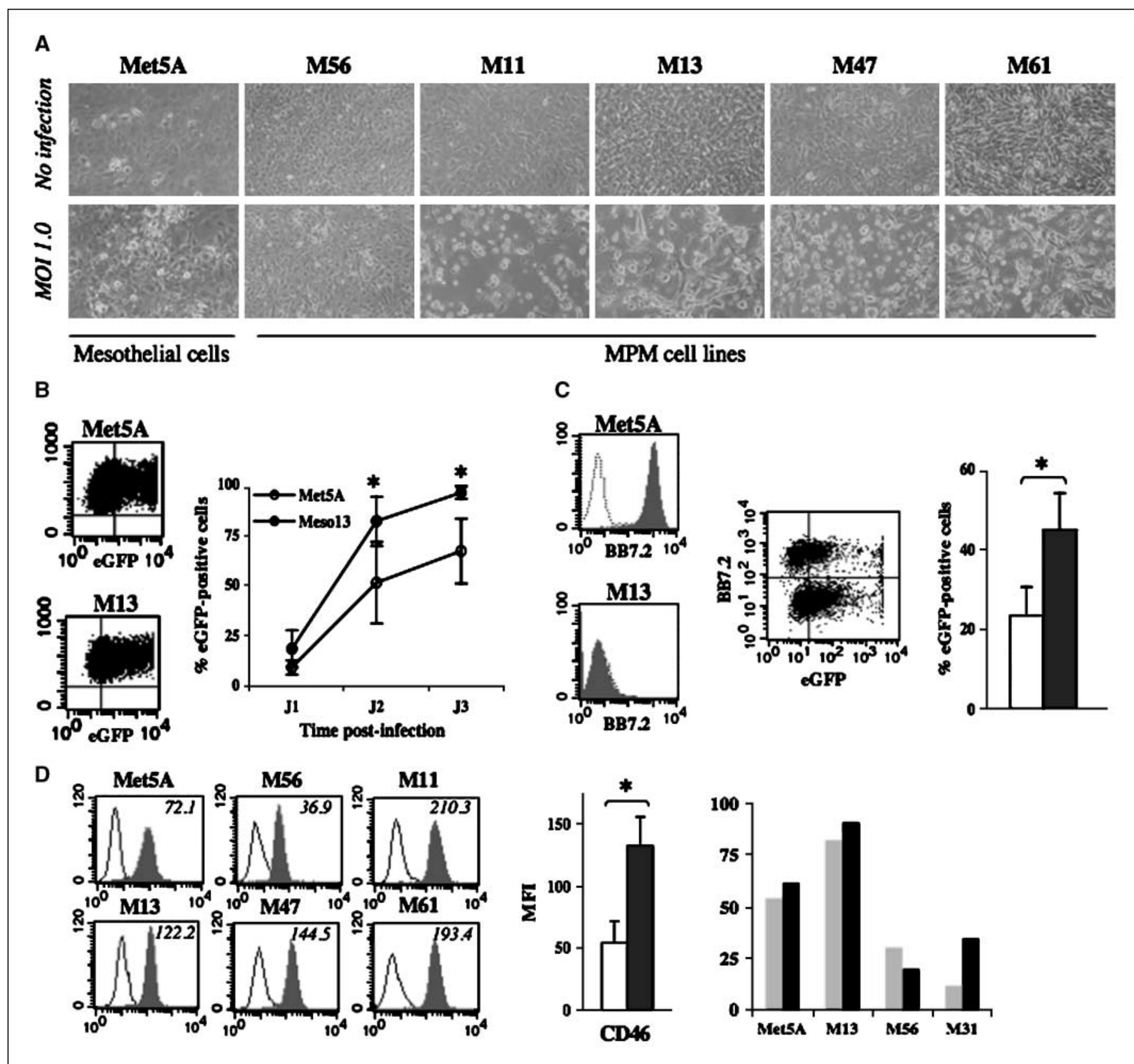

Figure 1. Mesothelioma susceptibility to MV infection and oncolytic activity. *A,* selective oncolytic activity of MV vaccine strain. A panel of human epithelioid mesothelioma cell lines (*M11, M13, M47, M56*, and *M61*) and a mesothelial (*Met5A*) cell line were infected with MV Schwarz strain (MOI, 1.0) and microscopic observations of morphologic modifications of MV-infected cultures were performed 72 h later. *B* and *C,* MV Schwarz strain preferentially infects transformed tumoral cells. Equal numbers of M13 and Met5A cells were cultured separately (*B*) or cocultured (*C*) overnight allowing cellular adherence, and infection was carried out at a MOI of 1.0 with eGFP recombinant MV strain. *B,* in separate cultures, analysis of eGFP expression was performed at different times after infection (24, 48, and 72 h) by flow cytometry. In the coculture model, the same experiment was conducted along with HLA-A2 staining (BB7.2 antibody was kindly provided by F. Jotereau, U601, Nantes, France), as HLA allele differential expression allowed a distinction between the two cell lines. *C,* percentage eGFP-positive cells for Met5A (*white column*) and M13 (*black column*) cells from coculture. *, $P < 0.05$ (*B* and *C*). *D,* up-regulated surface expression of CD46 receptors for tumoral cells in comparison with their normal cell counterparts. Cells were stained with FITC-conjugated CD46-specific antibodies (*gray histogram*) or related isotype Ig control (*white histogram*). Numbers indicate the MFI. *Left histogram,* mean values of CD46 expression level obtained for mesothelial (*white column*) and mesothelioma (*black column*) cell lines. *, $P < 0.05$. *Right histogram,* correlation between the values of CD46 expression level (*gray column*) and MV infection yield 48 h after infection (*black column*) obtained for mesothelial (*Met5A*) and mesothelioma (*M13, M56*, and *M31*) cell lines.

immature DCs within 24 h of coculture, whatever the death-induced strategy used (data not shown).

Apoptotic MV-infected mesothelioma cells induce DC maturation. Depending on the danger signal produced by dying cells and the PRR expression pattern of the phagocytic cells, the clearance of apoptotic cells can trigger an immune response from tolerance to immunity (34). We thus analyzed the DC maturation status following engulfment of killed MV-infected (MV-M13) or UV-irradiated (UV-M13) tumoral cells compared with direct infection by MV. Coculture was performed for 18 h at 37°C at a ratio of two DCs to one M13 cell (ratio, 2:1), and the phenotype of viable DCs was investigated by surface expression analysis of class I and II

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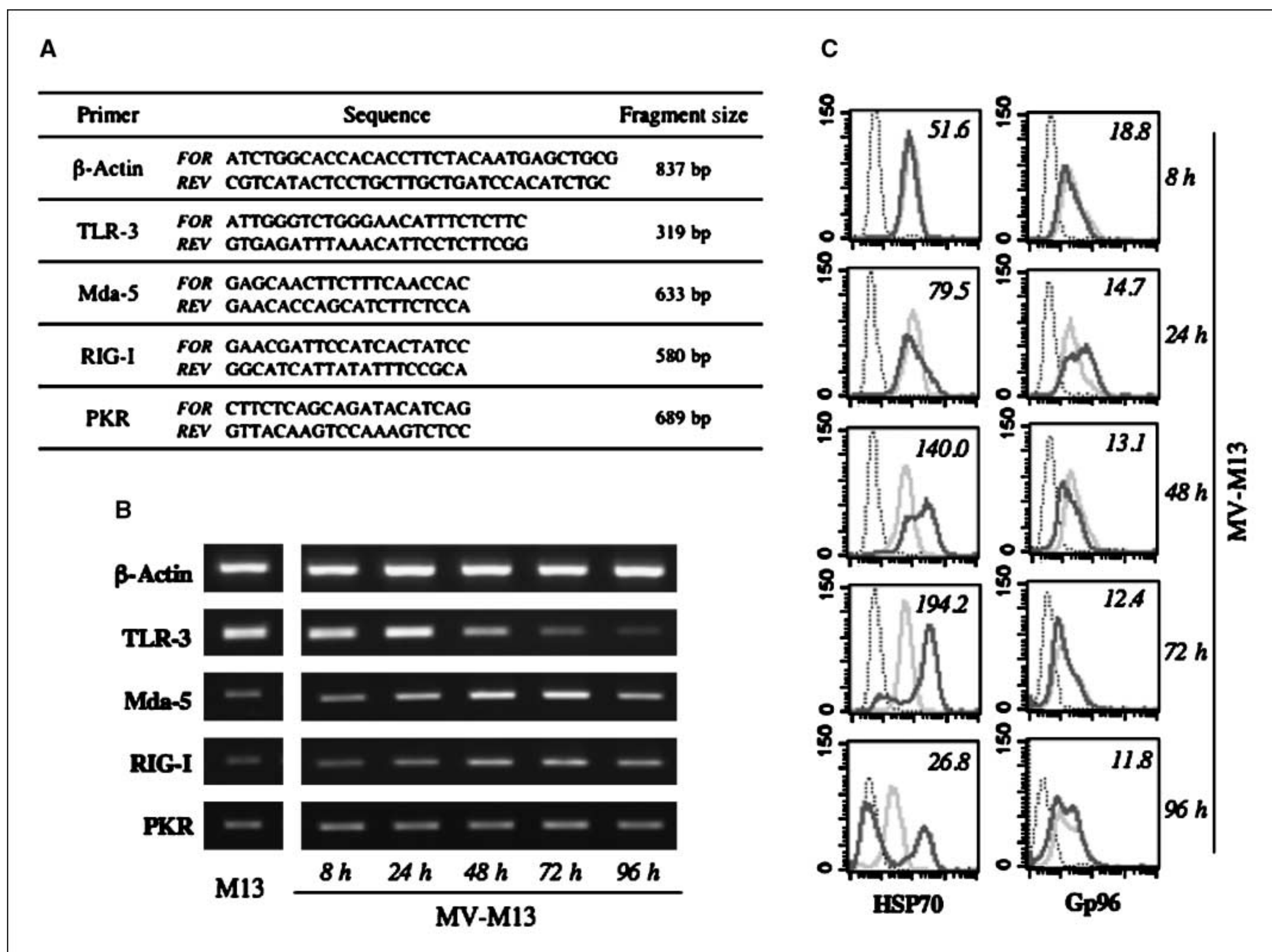
HLA molecules (Fig. 4*A*) and costimulation molecules CD80, CD86, CD83, and CD40 (Fig. 4*B*). We also analyzed the pattern of cytokine secretion in coculture supernatants (Fig. 4*C*). Unloaded DCs (mock), otherwise treated similarly, were used as a negative control. As a maturation positive control, DCs were incubated with a synthetic analogue of viral dsRNA, poly(inosinic-cytidylic) acid [poly(I:C)], in association with a major cytokine produced in response to viral infection IFNα (I:C/IFNα).

Only DCs loaded with MV-infected apoptotic M13 cells expressed a significant maturation profile at a level almost equivalent to that observed for DCs treated with the maturation cocktail I:C/IFNα (Fig. 4*A* and *B*). In contrast, DCs pulsed with UV-irradiated apoptotic M13 cells display a similar activation profile to immature DCs (Fig. 4*A* and *B*), as we previously described (23). Spontaneous maturation of DCs coincubated with MV-infected apoptotic mesothelioma cells was evidenced by a significant up-regulated surface expression of the major maturation markers ($P < 0.05$, for CD80, CD83, CD86, CD40, and HLA-DR; Fig. 4*A* and *B*), associated with a relevant production of numerous proinflammatory cytokines ($P < 0.05$, for IL-6, IFNα, TNFα, and IL-1β; Fig. 4*C*). However, we noticed a slightly, but not significant, IL-12p70 secretion combined with no IL-10 production (Fig. 4*C*). Therefore, these results are rather in favor of the production of an inflammatory environment with Th1 polarizing capacities. The same spontaneous maturation was shown for DCs cocultured with several other apoptotic MV-infected MPM cells (as M11 and M61; data not shown), thus strengthening the significant increased immunogenicity of measles-infected tumor cells.

We observed that the infection (Fig. 5*A*) and replication (Fig. 5*B*) properties of the MV Schwarz strain in monocyte-derived DCs were greatly limited compared with those observed in mesothelioma M13 cells. This may be partly related to their respective CD46 expression level (Fig. 5*C*). We also observed a significant increase in virus amplification in DCs cocultured with MV-infected M13 tumor cells compared with DCs infected with MV (Fig. 5*B*). In addition, we did not evidence any modification of DC maturation status



Primer	Sequence	Fragment size
β-Actin	*FOR* ATCTGGCACCACACCTTCTACAATGAGCTGCG *REV* CGTCATACTCCTGCTTGCTGATCCACATCTGC	837 bp
TLR-3	*FOR* ATTGGGTCTGGGAACATTTCTCTTC *REV* GTGAGATTTAAACATTCCTCTTCGG	319 bp
Mda-5	*FOR* GAGCAACTTCTTTCAACCAC *REV* GAACACCAGCATCTTCTCCA	633 bp
RIG-I	*FOR* GAACGATTCCATCACTATCC *REV* GGCATCATTATATTTCCGCA	580 bp
PKR	*FOR* CTTCTCAGCAGATACATCAG *REV* GTTACAAGTCCAAAGTCTCC	689 bp

Figure 2. Immunogenicity of MV-infected M13 cells. *A* and *B,* dsRNA production in M13 cells. Mesothelioma M13 cells were left untreated or cocultured with Schwarz MV (MOI, 1.0). Total cellular RNA was extracted from cell pellets, and the expression of Mda-5, TLR-3, RIG-I, PKR, and IFNβ was assessed by RT-PCR. Gene-specific primer sets used in RT-PCR experiments are listed in *A*. *B,* PCR products were visualized by ethidium bromide staining after electrophoresis through a 2% agarose gel. *C,* HSP expression by M13 cells. Flow cytometric kinetic assays of HSP70 and Gp96 expression before (*gray histogram*) and after (*black histogram*) viral infection (MOI, 1.0). Cells were collected at the indicated time points by a brief trypsinization and washed in PBS. Intracellular staining was performed by indirect staining with anti-HSP70 and anti-Gp96 primary antibodies followed by incubation with Alexa Fluor 488–conjugated secondary antibodies. For *B* and *C*, data shown are representative of two independent experiments.

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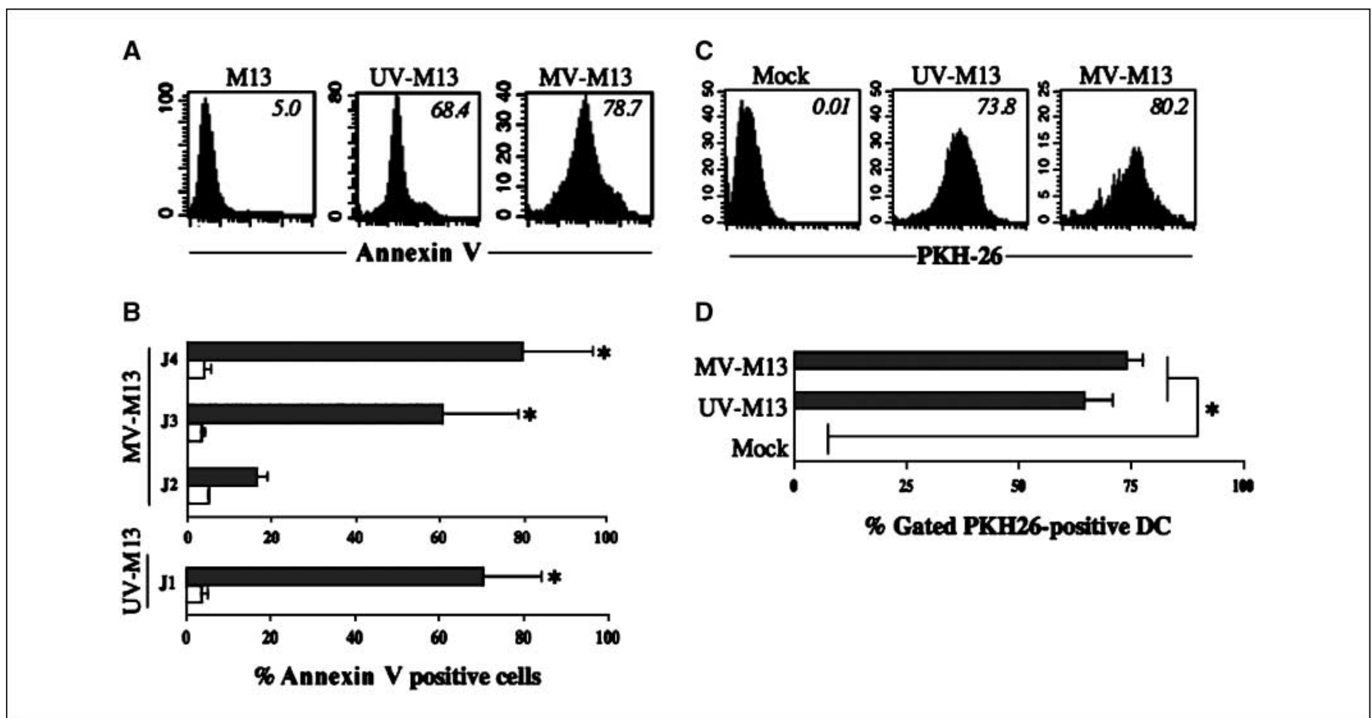

Figure 3. Phagocytosis yield of apoptotic MV-infected M13 cells by DCs. *A* and *B,* flow cytometry kinetic analysis of M13 tumoral cell apoptosis triggered by UV exposure (5 kJ/cm^2) or MV infection (MOI, 1.0). Mesothelioma M13 cells were grown on 24-well plates to obtain confluent cultures. From days 0 to 4 after MV or UV treatment, both adherent and nonadherent tumoral cells were collected for cell death quantification with a FITC-conjugated Annexin V BD reagent. *A,* one representative experiment of three with similar results. *B, columns,* mean values of percentage of Annexin V–positive cells for each apoptosis condition tested. *, $P < 0.05$. *C* and *D,* UV- or MV-treated M13 tumor cells were labeled with PKH-26 and cocultured with J5 immature DCs for 24 h (ratio, 2:1). Harvested DCs were subsequently stained with FITC-conjugated anti–HLA-DR antibodies and analyzed by flow cytometry. The number of double-positive DCs (PKH-26$^+$/FITC$^+$), that is, the percentage of PKH-26–positive DCs gated on the basis of HLA-DR expression (FITC-conjugated antibodies, clone B8.12.2; Immunotech), indicates phagocytosis efficiency. *C,* one representative experiment of three with similar results. *D, columns,* mean values of uptake yield for each loading condition tested. Statistical analysis compared values with those obtained for DC mock. *, $P < 0.05$.

within 24 h following direct infection with the Schwarz vaccine (Fig. 4*A* and *B*). Therefore, the evolution of the DC phenotype observed early after 24 h of coculture with MV-infected mesothelioma cells might have been rather induced by the phagocytosis of infected MPM cells than by a direct infection of the DCs. However, we cannot exclude the possibility of a direct infection of a fraction of the DCs present in the coculture depending on their activation status.

DCs loaded with MV-infected M13 cells cross-prime MSLN-specific CD8 T cells. Our data strongly support an increased immunogenicity of MV-infected M13 tumoral cells, allowing spontaneous maturation of DCs. Consequently, DCs produce a cocktail of proinflammatory cytokines with related activities on the CD8 T-cell response. Therefore, we tested whether these different DC preparations differ in their abilities to stimulate CD8 T cells specific for MPM-associated tumor antigen, such as MSLN. To assess this question, tetramer immunostaining specific for two previously described MSLN-derived CD8 epitopes [i.e., MSLN531-539 (for HLA-A2 VLP9) and MSLN541-550 (for HLA-A2 KLL10)] was performed (Fig. 6*A*). Purified CD8 T lymphocytes were sensitized for 1 week with autologous DCs unloaded or loaded with apoptotic material (UV-M13 or MV-M13 cells) but without any additional maturation agents.

Among the CD8-positive gated population, we observed a significant proliferation of MSLN-specific T cells (0.63% for HLA-A2 VLP9 and 0.76% for HLA-A2 KLL10) only in the coculture of DCs loaded with apoptotic MV-infected mesothelioma M13 cells (Fig. 6*B*). These tetramer-positive staining values are of real interest in comparison with a low frequency of precursors specific for a self-tumor antigen, such as MSLN (<0.1%; data not shown). Further, the possibility of a cross-reaction with epitopes processed from viral antigens may be ruled out, as T cells stimulated by DCs infected with MV Schwarz strain did not lead to a positive signal of tetramer immunostaining (data not shown). However, the functional activity of tumor-specific CD8 T lymphocytes still remains to be determined to confirm their effective implication in virotherapy-mediated cancer regression. Finally, our results also suggest that phagocytosed MV-infected apoptotic M13 cells are a possible source of TAAs for a potent cross-priming of specific CD8 T cells. We also confirm a cross-presentable status for MSLN antigen (24), giving additional arguments for its use in immunotherapy protocols (21). Altogether, our results show that measles virotherapy is able to elicit an autologous antitumor immune response, thus strengthening the immunoadjuvant properties of the oncolytic viruses.

Discussion

Pleural mesothelioma is an aggressive tumor rarely suitable for radical surgical resection and highly resistant to current therapeutic approaches, such as chemotherapy and radiotherapy (1, 2). An alternative therapeutic option could be the use of viruses as anticancer agents. Indeed, during the past decade, there has been an increasing interest in oncovirotherapy (i.e., the use of replicating

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viruses for cancer treatment; ref. 3). Some intrinsic features of pleural mesothelioma make it a suitable candidate for virotherapy, such as its accessibility and its localized nature, associated with a relative lack of distant metastasis. MPM tumors spread early and aggressively in the serosal cavity but rarely metastasize at distant sites through lymphatic or systemic circulation (1). Moreover, the pleural cavity is a confined compartment that could allow an efficient interaction between cancer cells and a viral therapeutic agent, and therefore constitutes an ideal target for the local administration of high virus concentrations (35, 36). This intrapleural administration pathway could also be a solution to limit virus inactivation by circulating MV neutralizing antibodies (4). Indeed, the feasibility of a virotherapy approach for this cancer has already been shown by the successful use of other oncolytic viruses, such as HSV (36) and AdV (35) strains. Thus, oncovirotherapy might be an interesting opportunity for an efficient improvement in mesothelioma cancer treatment.

Although numerous studies have already established the potential efficiency of MV-based virotherapy for several cancers (3, 4), there is still no available information on the antineoplastic activity of the MV vaccine on mesothelioma cancer. Our study showed that MV Schwarz strain efficiently kills a significant fraction of tumoral cells, as we observed an important CPE on most MPM cell lines at 72 to 96 h after infection. The susceptibility of MPM cells to measles infection could be related to the natural tropism of MV for epithelial cells of the upper respiratory tract (6). As 70% to 80% of MPM cases belong to the epithelioid subtype (1), measles virotherapy could be particularly relevant in the treatment of this cancer. Moreover, we observed that MV Schwarz vaccine exerts a potent lytic activity on tumoral MPM cells but only minimal cytopathic damage to nontransformed Met5A cells. As well, the large multinucleated syncytia and cytotoxic killing observed in the tumoral cell cultures infected with the live-attenuated Edmonston MV strain were never seen in normal

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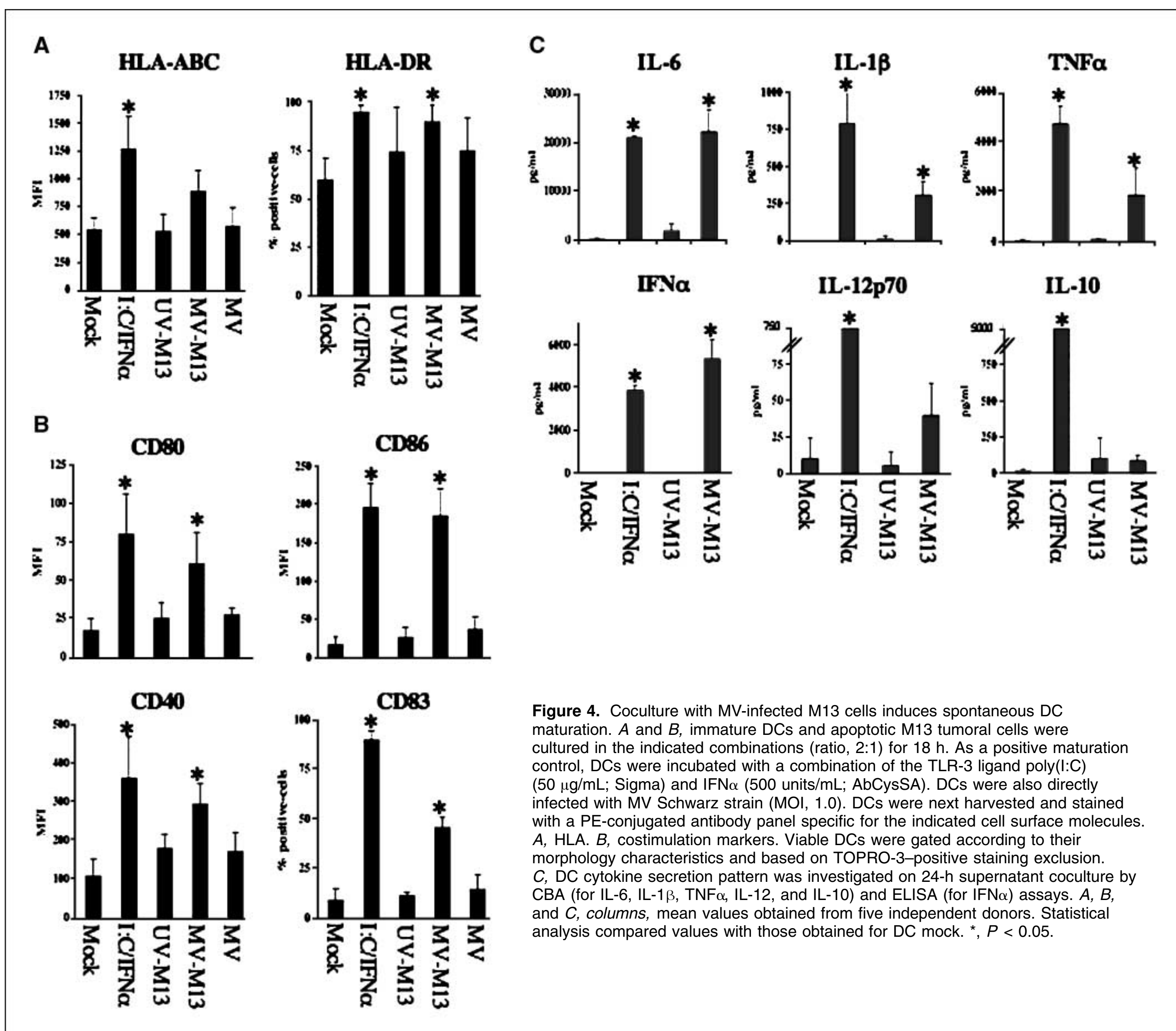

Figure 4. Coculture with MV-infected M13 cells induces spontaneous DC maturation. *A* and *B,* immature DCs and apoptotic M13 tumoral cells were cultured in the indicated combinations (ratio, 2:1) for 18 h. As a positive maturation control, DCs were incubated with a combination of the TLR-3 ligand poly(I:C) (50 μg/mL; Sigma) and IFNα (500 units/mL; AbCysSA). DCs were also directly infected with MV Schwarz strain (MOI, 1.0). DCs were next harvested and stained with a PE-conjugated antibody panel specific for the indicated cell surface molecules. *A,* HLA. *B,* costimulation markers. Viable DCs were gated according to their morphology characteristics and based on TOPRO-3–positive staining exclusion. *C,* DC cytokine secretion pattern was investigated on 24-h supernatant coculture by CBA (for IL-6, IL-1β, TNFα, IL-12, and IL-10) and ELISA (for IFNα) assays. *A, B,* and *C, columns,* mean values obtained from five independent donors. Statistical analysis compared values with those obtained for DC mock. *, $P < 0.05$.

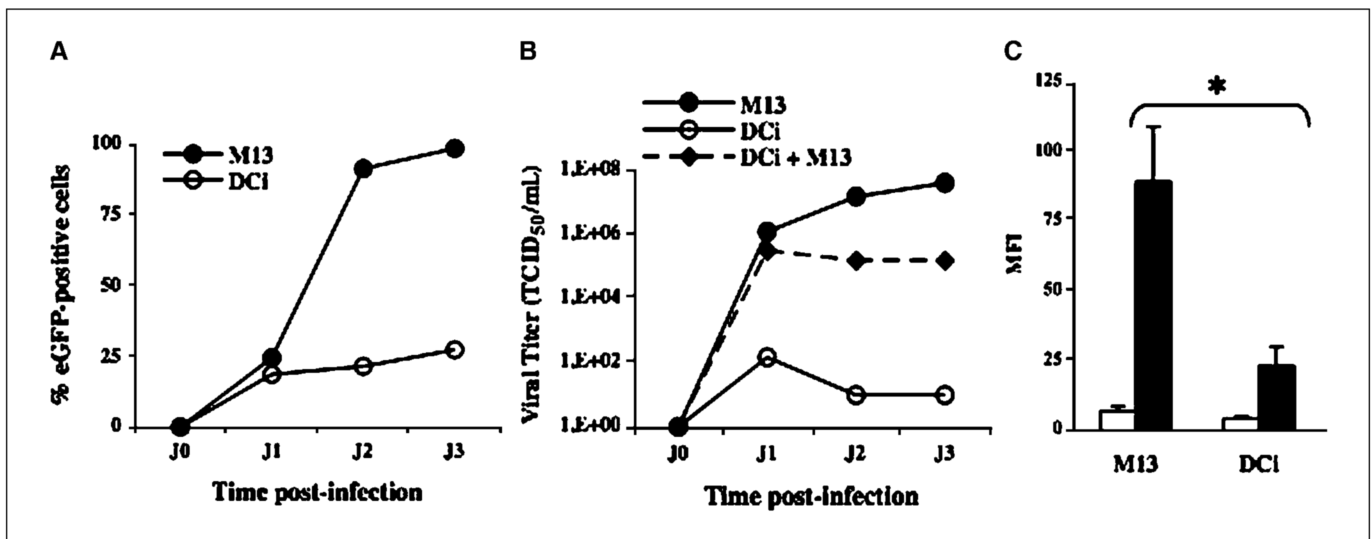

Figure 5. Infection and replication properties of MV Schwarz vaccine. *A* and *B,* infection was carried out at a MOI of 1.0 with eGFP recombinant MV strain in mesothelioma cells (*M13*) and in immature monocyte-derived DC (*DCi*). *A,* MV infection yield was quantified by a flow cytometry analysis of eGFP expression at different times after infection. *B,* replication rate was evaluated by the production of infectious viral particles in the supernatant and pellet of MV-infected cells. MV titers were determined on Vero cells, as previously described (20, 22). *C, columns,* mean values of CD46 expression level obtained for mesothelioma cells (*M13*) and DCi. Cells were stained with FITC-conjugated CD46-specific antibodies (*black columns*) or related isotype Ig control (*white columns*). Statistical analyses compared CD46 expression level between mesothelioma cells (*M13*) and DCi. *, $P < 0.05$.

mesothelial cell primary cultures established from peritoneal dialysis of cancer-free patients (12). As tissue culture–adapted strains of MV display attenuated pathogenicity for healthy tissue and are simultaneously more lytic against tumoral tissue, some question arises about the potential mechanism(s) involved in this selective activity (8). Although MV wild-type strains preferentially use the signaling lymphocyte activation molecule (CDw150) as their cellular receptor (37), Edmonston-derived MV vaccine strains have evolved to also infect target cells via the membrane complement cofactor protein (CD46; ref. 38). Because of its role as a membrane regulatory protein of the complement activity, CD46 is frequently overexpressed by tumoral cell lines compared with their normal tissue counterparts (27). This may represent an immune escape mechanism for cancer cells to overcome complement lytic activity (39). Hence, this cell mutation acquired during neoplastic transformation is in favor of tumor development while paradoxically rendering the tumor more susceptible to infection and lysis by MV vaccine strains. As previously described for other cancers (8), we observed a significant up-regulation of CD46 expression by mesothelioma cancer cells in comparison with nontransformed mesothelial cells. We also noted a correlation between the expression level of CD46 receptor and MV infection yield, thus providing a mechanistic basis for the increased susceptibility of MPM to MV oncolytic activity.

Although *in vitro* models have clearly shown that replication-competent oncolytic viruses efficiently spread and kill tumoral cell cultures, *in vivo* studies indicated that many physical barriers (circulating complement and neutralizing antibodies, and architecture of the tumoral environment) could severely hamper the virus from reaching to the tumor site (4) and restrict viral replication to delimited peripheral areas within the tumoral mass (9, 10, 40). These observations strongly suggest that antitumoral properties of oncolytic viruses probably involve more than just tumor cell killing. Currently, there is increasing evidence that additional immune mechanisms may be implicated in virotherapy-mediated therapeutic effect. Indeed, several experimental studies have shown that neutrophils and CD8 T cells contribute to HSV-related (41), VSV-related (13, 14), and MV-related (18) virotherapy efficiency. This new approach of cancer immunotherapy, based on *in vivo* infection of tumors, raises the possibility to eliminate tumoral cells because they are recognized as infected rather than malignant (42). This could lead to the lysis of at least a limited set of infected tumor cells, thus providing an inflammatory state within the tumoral environment in favor of the induction of an efficient immune response (13). Indeed, this infectious site might release numerous proinflammatory factors that could lead to the recruitment of innate immune effectors (such as neutrophils and natural killer cells) and to the loading and activation of antigen-presenting cells (such as macrophages and DCs). This would provide an immunogenic presentation context of released tumor-associated cargo for the activation of an efficient tumor-specific CTL response (42). As previously described in the melanoma model, tumoral cell death promoted by oncolytic virus infection, with modified virus Ankara poxvirus (43) or H-1 parvovirus (44), was previously related to an increased capacity for tumor-specific CD8 T-cell priming. We also showed that DCs loaded with apoptotic MV-infected human mesothelioma cells stimulate a significant proliferation of MSLN-specific CD8 effector T cells. This important result strongly supports the hypothesis on the participation of virus-induced antitumor immunity in therapeutic efficiency of MV-based oncolytic treatment (18).

Although previous studies showed a potential adjuvant activity of oncolytic virus in CTL response induction (43, 44), they did not describe spontaneous DC maturation such as we readily observed in this study. To our knowledge, these experimental data represent the first case wherein virus-induced death of human tumor cells induces spontaneous DC maturation without any additional adjuvant maturation stimuli (such as cytokine cocktails). Thus, our experimental results raise an interesting point about the mechanism(s) potentially implicated in a significantly increased immunogenicity of apoptotic measles-infected tumoral cells. Until now, apoptotic dying cells were thought to be weakly immunogenic, thereby modulating the immune response toward tolerance

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rather than immunity (34). However, several reports have already shown the possibility of overriding the inhibitory effects of apoptotic cell ingestion on DC maturation by triggering apoptosis in the presence of inflammatory "danger signals," such as those produced by viral infection. The increased immunogenicity of infected tumoral cells may thus rely on innate immune stimuli, such as HSP70 (32, 33, 45) or dsRNA (28, 29, 31), produced by virally infected cells. Indeed, we readily observed that measles infection of M13 mesothelioma cells induces both HSP70 and dsRNA production peaking at 72 h after infection, a time delay necessary to detect a relevant DC spontaneous maturation (data not shown). As previously described by our group, DCs loaded with apoptotic HSP70-expressing M13 cells and activated by exogenous maturation agents are potent *in vitro* inducers of mesothelioma-specific CTLs (23). Interestingly, recent studies indicated a relevant adjuvant effect of cellular-associated dsRNA for tumor cells loaded with poly(I:C) (46, 47) and for virally Semliki Forest virus–infected Vero cells (48). So, although not directly correlated by our results, we hypothesize that apoptotic mesothelioma cells loaded with viral dsRNA produced by MV infection might be a strong immunogenic signal, leading to the spontaneous maturation of monocyte-derived DCs and the subsequent activation of a relevant MSLN-specific CD8 T-cell response. Hence, our results suggest that MV-based virotherapy therapeutic efficiency may rely not only on viral oncolytic activity but also on virus immunoadjuvant properties, which may lead to priming of an efficient antitumor immune response.

This work raises a controversial question about the relative contribution of cross-priming and direct priming in the activation of the host immune response. One of the major human immunosuppressive viral diseases is related to measles infection (6). Indeed, MV induces a transient but profound systemic immunosuppression that is associated with an increased susceptibility to opportunistic secondary infections. However, at the same time, MV infection also stimulates a specific antiviral immune response that provides an efficient life-long protective immunity in infected individuals. Although not clearly understood, this immunologic paradox is believed to be determined by early interactions of the virus with DCs (49). Indeed, all DC sources tested were shown to be productively infected with MV (50, 51, 52). Both wild-type MV strains and Edmonston-derived vaccines induce the maturation of DCs, albeit with different kinetics (50). Activation of DCs was observed within 24 h following wild-type infection, but only 48 to 72 h after Edmonston infection. In our study, we

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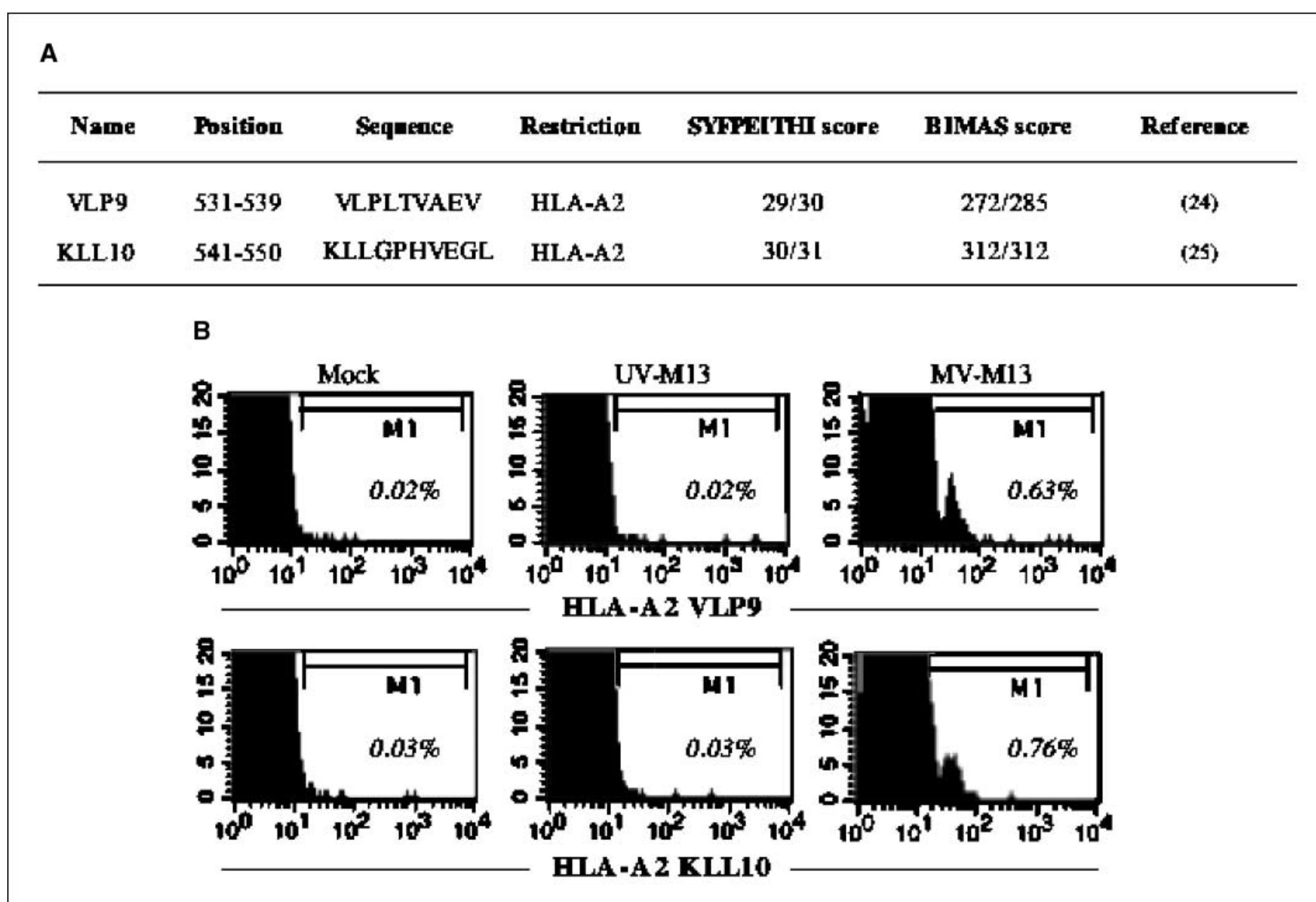

A

Name	Position	Sequence	Restriction	SYFPEITHI score	BIMAS score	Reference
VLP9	531-539	VLPLTVAEV	HLA-A2	29/30	272/285	(24)
KLL10	541-550	KLLGPHVEGL	HLA-A2	30/31	312/312	(25)

Figure 6. DCs loaded with MV-infected M13 cells stimulate MSLN-specific CD8 T-cell proliferation. *A,* table shows candidate HLA-A0201–restricted human MSLN-derived CTL epitopes. Target peptides MSLN531-539 and MSLN541-550 were initially identified by scanning the MSLN amino acid sequence (NP 005814) for matches to consensus motifs for HLA-A0201 binding using two computer algorithms, BIMAS and SYFPEITHI. The positions, sequences, and MHC binding scores of selected peptides were determined. *B,* number of MSLN-specific CD8 T cells, derived from 1-wk sensitization coculture with unpulsed, UV-M13–pulsed, or MV-M13–pulsed DCs, were analyzed by flow cytometry. The histogram indicates the percentage of PE tetramer-positive cells among T cells gated on the basis of human CD8α expression (PE-Cy5–conjugated antibodies, clone RPA-T8). One example of two independent donors with similar results is shown.

observed that monocyte-derived DCs are infected by the MV Schwarz strain with a lower yield than mesothelioma tumor cells. The direct contact of DCs with the MV Schwarz vaccine leads to the up-regulation of the major maturation markers 2 to 3 days after infection (data not shown). So, we thought that the evolution of the DC phenotype observed only after 24 h of coculture might rather be induced by the phagocytosis of MV-infected mesothelioma cells than by a direct infection of DCs.

In measles pathogenesis, DCs are believed to be a reservoir for MV spread into the draining lymph nodes during DC-T-cell cross-talk (49). Several *in vitro* studies already showed that the interaction of MV-infected DCs with T cells not only induces syncytia formation, where MV undergoes massive replication, but also leads to an impairment of DC and T-cell functions (51, 52). Indeed, MV-infected DCs are not able to stimulate the proliferation of naive T cells (52), and they also exhibit a cytotoxic activity on activated T cells (51). Because direct infection of DCs strongly altered their ability to stimulate effector T lymphocytes, the proliferation of MSLN-specific CD8 T-cell precursors detected in the coculture might rather be induced by DCs loaded with MV-infected tumor cells. So, the immunologic mechanisms underlying measles-induced immune suppression seem to be at least partially mediated through a direct targeting of DCs. In these conditions, it remains to be understood how DCs could stimulate MV-specific CD8 T cells to elicit an efficient life-long protection to MV (49). Several pathways for the presentation of viral antigens to CD8 T cells by DCs have been identified (direct priming versus cross-priming). Cross-presentation of viral antigens by DC, by the uptake of virus-infected cells (48) or viral immune complexes (53), can serve as a means by which the host immune system can circumvent viral interference. Cross-priming may be dedicated to viruses that do not have natural tropism for DCs or viruses such as MV that may exert immunosuppressive activities on infected DCs (48). Because a viral-specific immune response might be rather induced through the cross-presentation pathway (53), we hypothesize that the reactivation of an antitumoral immune response by measles virotherapy could also use this indirect pathway.

Today, it seems likely that no conventional treatment modality will be effective enough by itself for an efficient MPM management (2). So, immunotherapy could be used as an adjuvant protocol to optimize the efficiency of current mesothelioma treatments, such as chemotherapy with pemetrexed-cisplatin drugs (54), or in the future pathogen-based therapy with bacterial immunotoxin (SS1P; ref. 55) or oncolytic viruses (such as AdV, HSV, or MV; ref. 4). We believe that the results obtained from our study can help to design a new kind of therapeutic pathway based on a synergistic association between virotherapy and immunotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 11/15/2007; revised 3/17/2008; accepted 4/8/2008.

Grant support: Institut National de la Sante et de la Recherche Medicale, Pasteur Institut, Fondation Weisbrem-Beneson, and La Ligue Régionale Contre le Cancer (Vendée, Morbihan, Pays de Loire). A. Gauvrit is funded by a postdoctoral fellowship from La Ligue Nationale Contre le Cancer. S. Brandler is a fellow of Departamento de Desarrollo Sostenible de Total Oil and Gas Venezuela and of Fondation Charles de Gaulle.

We thank Dr. P. Despres for his helpful comment on the manuscript, C. Combredet for her technical assistance in MV titrations, and K. Kean for the English editing.

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