Measles virus vaccine-infected tumor cells induce tumor antigen cross-presentation by human plasmacytoid dendritic cells

Jean-Baptiste Guillerme¹,²,³, Nicolas Boisgerault¹,²,³, David Roulois¹,²,³, Jérémie Ménager¹,²,³, Chantal Combredet⁴, Frédéric Tangy⁴, Jean-François Fonteneau¹,²,³,*, and Marc Gregoire¹,²,³*

¹INSERM, UMR892, Nantes, F-44000, France.
²CNRS, UMR6299, Nantes, F-44000, France
³Université de Nantes, Nantes, F-44000, France.
⁴Unité de Génomique Virale et Vaccination, CNRS-URA 3015, Institut Pasteur, Paris, France.

*authors participate equally in the last authorship position

Running title: cross-presentation of human tumor antigen by pDC

Keywords: antitumor virotherapy, measles virus vaccine, plasmacytoid dendritic cells, tumor antigen, cross-presentation.

Financial support: This study was financed by INSERM, La ligue régionale grand ousst contre le Cancer (CSIRGO), the Association pour la recherche contre le cancer (ARC), the Nantes Hospital and the ARSMESO44 association.

Corresponding author: Dr. Jean-François Fonteneau,

E-mail: jean-francois.fonteneau@inserm.fr

INSERM, UMR892, Institut de Recherche Thérapeutique de l’Université de Nantes, 8 quai Moncousu, BP70721, 44007 Nantes Cedex1, France

Phone: (+33)228 080 239 Fax: (+33)228 080 204

Conflicts of Interest: None

Total word count: 4892 before revision, 5850 after revision

Total number of figures: 5 figures and 6 supplemental figures (including 2 videos).
Statement of translational relevance

Measles virus vaccine (MV)-based antitumor virotherapy is a new therapeutic approach to treat cancers. It is based on the spontaneous capacity of MV to infect and kill preferentially tumor cells. Phase-I clinical trials against different malignancies are in progress with encouraging results.

This promising efficacy of MV antitumor virotherapy is mainly due to the lysis of tumor cells following MV infection. However, it is likely that a part of this efficacy is due to the effect of MV-infected tumor cells on the antitumor immune response.

In this study, we show that MV-infected tumor cells activate human plasmacytoid dendritic cells (pDC), a particular subset of DC specialized in the antiviral immune response. Human pDC are then able to cross-present a tumor antigen from MV-infected tumor cells to specific cytotoxic T cells. Our work, thus, suggests that MV-based antitumor virotherapy triggers an antitumor immune response, notably by the recruitment of pDC.
Abstract

Purpose: Plasmacytoid dendritic cells (pDC) are antigen-presenting cells specialized in antiviral response. The measles virus vaccine (MV) is proposed as an antitumor agent to target and specifically kill tumor cells without infecting healthy cells.

Experimental design: Here, we investigated, in vitro, the effects of MV-infected tumor cells on the phenotype and functions of human pDC. We studied maturation and tumor antigen cross-presentation by pDC, exposed either to the virus alone, or to MV-infected or UV-irradiated tumor cells.

Results: We found that only MV-infected cells induced pDC maturation with a strong production of IFN-α, whereas UV-irradiated tumor cells were unable to activate pDC. This IFN-α production was triggered by the interaction of MV ssRNA with TLR7. We observed that MV-infected tumor cells were phagocytosed by pDC. Interestingly we showed cross-presentation of the tumor antigen NYESO-1 to a specific CD8+ T cell clone when pDC were cocultured with MV-infected tumor cells, whereas pDC were unable to cross-present NYESO-1 after coculture with UV-irradiated tumor cells.

Conclusions: Altogether, our results suggest that the use of MV in antitumor virotherapy induces immunogenic tumor cell death, allowing pDC to mature, produce high amounts of IFN-α, and cross-present tumor antigen, thus representing a mode of recruiting these antigen presenting cells in the immune response.
Introduction

Measles virus vaccine (MV)-based antitumor virotherapy is a new therapeutic approach to treat cancers (1, 2). It is based on the ability of a vaccine-attenuated live strain of measles virus, such as Edmonston or Schwarz strains, to infect and kill preferentially tumor cells. MV uses mainly CD46 molecules to infect cells, whereas wild-type measles virus uses preferentially SLAM (CD150) (3, 4). During cancer development, tumor cells are often selected to express high levels of CD46 molecules, which inhibits the complement system (5, 6). This CD46 overexpression makes the tumor cells less sensitive to lysis by the complement but renders them sensitive to MV infection. We and others have reported that several types of cancer are sensitive to this approach in vitro, such as mesothelioma (7), ovarian cancer (8), multiple myeloma (9), breast cancer (10), hepatocellular carcinoma (11) and melanoma (12). In vivo efficacy of this approach has also been reported in models of human tumor xenografts in immunodeficient mice (8-11). Phase-I clinical trials against different malignancies are now in progress. In a first phase-I clinical trial, intratumoral injection of low doses of MV to five cutaneous-T-cell-lymphoma patients allowed stabilization of the disease in two patients and the observation of a partial response in one other (13). Results from a further phase I trial were published recently, in which patients with chemotherapy-resistant ovarian cancer were treated with intraperitoneal MV (14). Twenty-one patients were treated and improvements in serum tumor markers were noted in five.

This promising efficacy of MV antitumor virotherapy is mainly a result of the lysis of tumor cells following MV infection, as shown by in vivo studies in immunodeficient mice (15, 16). In these immunodeficient mice, it has also been shown that MV infection triggers neutrophil infiltration of tumors, which probably participates in the efficiency of the antitumor virotherapy (17). Indeed, MV infection of tumor cells may also be able to trigger the immune system to induce an antitumor immune response. We previously showed, in vitro, that
mesothelioma cells killed by MV were able to produce danger signals, trigger the maturation of myeloid dendritic cells (DC) and cross-present tumor antigens to CD8+ T lymphocytes, whereas the same tumor cells killed by ultraviolet (UV) irradiation did not (7). Similar results were recently reported following lysis of melanoma tumor cells by MV (12).

Plasmacytoid DC (pDC) are a subset of DC involved in the antiviral immune response due to their expression of Toll-like receptors (TLR) specialized in the recognition of viral nucleic acids (TLR7, TLR9) (18). They respond to a wide range of viruses (inter alia influenza A virus, herpes simplex virus, HIV) in terms of activation and maturation by producing large amounts of type-I interferon (IFN-α, -β, -ω). They are also able to present viral antigens to CD8+ and CD4+ T cells when they are infected by a virus (19) and to cross-present viral antigens from virus-infected cells to CD8+ T lymphocytes (20-23). It has also been shown that these pDC could play a beneficial role in the immune response against tumors (24, 25). As an example, in a mouse melanoma model, pDC activation and antitumor immune response were observed inside tumors by topical treatment with the TLR7 ligand, imiquimod (24). As MV is single-stranded RNA (ssRNA), pDC should be able to detect the MV infection of tumor cells, because of their intravacuolar TLR7 expression which recognizes single-stranded RNA. In addition, although it has been well described that human pDC are able to cross-present viral antigens, there is as yet no evidence that these antigen-presenting cells are able to cross-present tumor antigens.

In our study we addressed, in vitro, the effect of tumor cell infection by MV Schwarz on the activation status of human pDC and their ability to cross-present a tumor antigen to a specific CD8+ T cell clone. We showed that, despite CD46 expression, pDC are not sensitive to MV infection. However, they are able to respond in vitro to MV by producing IFN-α with a greater sensitivity when IL-3 is added to the culture. We also demonstrated that MV-infected tumor cells triggered pDC activation, notably IFN-α
production, whereas UV-irradiated tumor cells did not. pDC activation was probably caused by the single-stranded RNA of MV, which triggers TLR7 in the pDC endocytic compartment following phagocytosis of MV-infected tumor cells. Interestingly, we showed for the first time that human pDC cocultured with MV-infected tumor cells were able to cross-present the NYESO-1 tumor antigen to a specific CD8+ T cell clone. Our results suggest that, in addition to a direct tumor lysis effect, MV-based antitumor virotherapy may trigger an antitumor immune response by activating pDC.
Materials and Methods

Cell culture

The mesothelioma Meso13 cell line was established and characterized in our laboratory (26), the melanoma M18 was a kind gift from Dr. Labarriere (INSERM U892, France), and the pulmonary adenocarcinoma A549 cell line was purchased from ATCC (Manassas, VA). All cell lines were cultured at 37°C, 5% CO₂ atmosphere in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100U/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamine (all reagents were purchased from Gibco-Invitrogen, Cergy-Pontoise, France). Cells were routinely checked for mycoplasma contamination, by PCR. A549 is HLA-A*2501+; -A*3001+ (27). M18 is HLA-A*2902 homozygous as determined by PCR (data not shown).

MV infection and UV irradiation

Live-attenuated Schwarz-strain measles virus (MV) and recombinant MV-enhanced green fluorescent protein (MV-eGFP) were produced as previously described (7). MV infection of tumor cells was performed for 2 hours at 37°C with a multiplicity of infection (MOI) of 1 unless otherwise indicated. Viral inoculum was then replaced by fresh cell medium for 72 hours. For pDC infection and maturation experiments, MV was not washed and stayed in the medium throughout the culture. Measurement of infection rate was performed by flow cytometry using MV-eGFP at 24, 48, and 72 hours post-infection. All other experiments were carried out using MV. Tumor cells were irradiated with UV-B (312nm – 100kj/m², Stratalinker, Stratagene). Medium was renewed every 72 hours.
DC isolation and culture

pDC were obtained from healthy donor PBMCs (Etablissement Français du Sang, Nantes, France) as previously described (28). Briefly, pDC were first enriched by counterflow centrifugation and then purified by magnetic bead negative selection as recommended in the manufacturer’s protocol (Stemcell Technologies, Grenoble, France). The purity of untouched pDC was always greater than 96%. pDC (3x10^5 per ml) were maintained in culture with 20ng/ml rhIL-3 (Sigma, Saint Quentin Fallavier, France) or activated in vitro with a TLR-7 agonist, R848 (InvivoGen, San Diego, USA) (5µg/ml). pDC were also cocultured with MV alone, MV and IL-3 (MOI=1), or MV-infected or UV-irradiated tumor cells (pDC:tumor cell/1:1) without rhIL-3 or maturation agent. After 18 hours, culture supernatants and pDC were harvested for use. For the TLR-7 inhibition assay we used immunoregulatory DNA sequences, which specifically inhibit signaling via TLR-7 [IRS 661], at concentrations ranging from 0.1µM to 1µM (Eurofins, Munich, Germany). As a control we used CpG-A at 5µg/ml to induce a TLR-9-dependent IFN-α secretion by pDC (InvivoGen, San Diego, USA).

Immunofluorescence and flow cytometry

The phenotypes of pDC were determined by immunofluorescence followed by flow cytometry. pDC were stained with monoclonal antibodies specific for CD40, CD86, HLA-DR (BD Biosciences, San Jose, CA, USA), CD83 (BioLegend, San Diego, CA-USA) and BDCA-4 (Miltenyi Biotec). pDC were gated as BDCA-4+/HLA-DR+ cells, to differentiate them from tumor cells. Tumor cell death was measured by TO-PRO®3 (Invitrogen, Saint Aubin, France) staining as recommended by the manufacturer. TO-PRO®3 is a carbocyanine monomer nucleic acid with far-red fluorescence that enters only in dead cells and stains the DNA. Fluorescence was analyzed on FACSCantoII (Becton Dickinson, New Jersey, USA) using FlowJo software.
Phagocytosis assay

MV-infected and UV-irradiated tumor cells were stained with PKH-67 according to the manufacturer’s protocol (Sigma, Saint Quentin Fallavier, France) and cocultured with pDC, for 18 hours at 4°C or 37°C (1 DC:1 tumor cell). Cocultures were washed with PBS-EDTA to dissociate the cell-conjugate. pDC were stained by an HorizonV450-conjugated, anti-HLA-DR-antibody (BD Biosciences, SanJose, CA, USA) and analyzed by flow cytometry (FACSCantoII, BD). pDC phagocytosis was observed by confocal microscopy (Nikon). MV-infected and UV-irradiated tumor cells were stained with PKH-67 and then cocultured with pDC in 24-well plates containing poly-lysine glass slides, for 18 hours (pDC:tumor cell, ratio 1:1). pDC were stained with uncoupled anti-HLA-DR (BD Bioscience). HLA-DR staining was revealed with a secondary anti-mouse IgG antibody coupled to AlexaFluor 568.

Cytokine detection

IFN-α (MabTech, Cincinnati, OH-USA) production was measured by ELISA on pDC culture supernatants according to the manufacturer’s instructions.

Cross-presentation assay

NYESO-1pos/HLA-A*0201neg melanoma (M18) and NYESO-1neg/HLA-A*0201neg pulmonary adenocarcinoma (A549) cell lines were MV-infected or UV-B irradiated and cultured for 72 hours. They were then cocultured with HLA-A*0201pos pDC (pDC:tumor cell ratio 1:1). After 18 hours, pDC were cocultured with the HLA-A*0201/NYESO-1(156-165)-specific CD8+ T cell clone, M117.167, for 6 hours in the presence of Brefeldin-A (Sigma, Saint Quentin Fallavier, France). The M117.167 clone was obtained by cloning in a limiting dilution of tumor-infiltrating lymphocytes from a melanoma patient. The clone was
cultured as described (29). As control, we used pDC pulsed for 1 hour with 0.1 or 1µM NYESO-1(156-165) peptide and washed. Cells were then fixed with PBS containing 4% paraformaldehyde, for 10min at room temperature, and permeabilized and stained with IFN-γ and CD8-specific antibodies (BD Biosciences, SanJose, CA, USA), as previously described (31). IFN-γ production was analyzed by flow cytometry with a gate on CD8+ T cells.

Real-time RT-PCR

One microgram of total RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (InVitroGen, Saint Aubin, France). PCR reactions were performed using QuantiTect primers (Qiagen, Foster City-USA) and RT² Real-Time SYBR-Green/ROX PCR mastermix (Tebu-bio, Le Perray-en-Yvelines, France), according to the manufacturers’ instructions.

Statistics

GraphPad Prism (Inc., San Diego, CA-USA) software using a nonparametric Mann Whitney comparison test was used. P values < 0.05 were considered to be statistically significant.
Results

Sensitivity of tumor cells and pDC to MV infection.

During infection, MV enters cells mainly via the CD46 and, to a lesser extent, CD150/SLAM (3, 4). In a first experiment, we studied the expression of these two major MV receptors, CD46 and CD150/SLAM, on pDC, melanoma (M18), mesothelioma (Meso13) and pulmonary adenocarcinoma (A549) cell lines (Figure 1A). We observed CD46 expression on all cell types, with higher expression on Meso13 and A549. Regarding CD150/SLAM expression, we found a positive expression on the melanoma cell line, M18. These results suggest that all these cell types may be sensitive to MV infection, as they all express CD46.

We then studied the sensitivity to MV infection of these four cell types using a recombinant MV encoding the green fluorescent protein (MV-GFP). Seventy-two hours after exposure to MV with a MOI=1, the three tumor cell lines were productively infected with MV, ranging from 50% of A549 cells positive for GFP to 90% of Meso13 cells (Figure 1B). Furthermore, we observed syncytia formation for the three tumor cell lines (Supplemental Figure video 1). pDC were not permissive at MOI=1 (Supplemental Figure video 2). Without a survival signal such as IL-3, the pDC died during the 72 hours of culture. Thus, we also performed experiments where we added IL-3 to the pDC exposed to MV (Figure 1C). In the presence of IL-3, they survived during the 72 hours, but were not productively infected by MV. To confirm this result, we increased the MOI up to 50 in the presence of IL-3, but we still failed to detect infected pDC (Figure 1D). However, we observed a small shift of fluorescence at MOI=50, which was probably due to uptake of soluble GFP during the 72-hour culture, which contaminates the MV-GFP preparation, as when we used UV-irradiated MV-GFP which is not able to replicate, we still observed this slight fluorescence shift (Supplemental Figure 3). Finally, when the MV-GFP was
incubated for 2 hours at MOI=50 with pDC and washed, we failed to detect the small shift of fluorescence 70 hours later (Supplemental Figure 3).

We then measured tumor cell death 72 hours after infection. We found that nearly half of MV-infected tumor cells were TO-PRO+ after 72 hours (Figure 1E). A similar level of cell death was observed by irradiating the tumor cells with UV-B. Thus, MV infection induces tumor cell death for approximately half of the tumor cells 72 hours after infection.

MV-infected tumor cells induce maturation of pDC.

We next investigated the effects of MV alone and MV-infected cells on pDC maturation (Figure 2). In these experiments, we evaluated how MV infection of tumor cells in comparison with UV irradiation, another inducer of tumor cell death, affects pDC maturation. As a control for maturation, we exposed pDC to the TLR7/8 agonist, R848 (Figures 2A and 2B).

We previously demonstrated that the MV-infected MPM tumor cell line, Meso13, induced maturation of monocyte-derived DC, without additional adjuvants, whereas the virus alone or UV-irradiated Meso13 did not (7). We presently performed a set of experiments on pDC to determine the effects of MV alone, MV-infected or UV-irradiated tumor cells on pDC maturation status. We compared the effect of MV-infected and UV-irradiated tumor cells on the maturation status of pDC (Figure 2). We observed maturation of pDC cocultured with MV-infected tumor cells, whereas UV-irradiated tumor cells failed to activate pDC. Indeed, CD83 maturation marker expression was induced by MV-infected cells to a similar level as that observed when the pDC were exposed to R848. We also noted an induction of the expression of the costimulation molecules, CD40 and CD86, on pDC exposed to MV-infected tumor cells, although this induction was low compared with the levels triggered by R848 alone.
Two studies have been reported which describe conflicting results on the ability of MV alone to trigger pDC maturation (30, 31). However the study from Duhen et Coll, reporting that MV activates pDC, was performed in the presence of IL-3, a pDC survival factor (30), whereas the other study, from Schlender and Coll, who observed that pDC cultured with MV does not induce pDC maturation, was carried out without IL-3. Thus, we performed and compared the two conditions and found similar results to those described by these authors. Indeed, MV at MOI=1 induced pDC maturation only in the presence of IL-3 (Figure 2). As observed for R848 alone, MV in the presence of IL3 induced pDC maturation, mainly characterized by a significant increase of CD83 and, to a lesser extent, CD40 and CD86 expression. **We also observed survival and maturation of pDC in the absence of IL-3 only when we exposed them to a high quantity of MV (MOI=50) (data not shown). At a lower viral concentration in the absence of IL-3, the pDC died.**

In the last set of experiments, we tested whether MV infection and replication in pDC were needed to induce their activation. We exposed pDC to UV-irradiated MV (MV*), which is unable to replicate, and we observed a similar level of maturation (CD83, CD80 and CD86 expressions) and IFN-α production as with non-irradiated MV (Supplemental Figure 4A and 4C). The presence of a blocking anti-CD46-specific antibody in the culture of pDC exposed to IL-3 and MV did not affect maturation of pDC (Supplemental Figure 4A). The same experiment was performed with pDC exposed to MV-infected tumor cells. We still observed maturation and IFN-α production when MV-infected tumor cells were UV-irradiated before exposure to pDC (Supplemental Figure 4B and 4C). Finally, we tested whether a CD46-specific monoclonal antibody was able to inhibit pDC maturation in response to MV-infected tumor cells (Supplemental Figure 4B and 4C). We did not observe inhibition, whereas the anti-CD46 antibody completely inhibited infection of Meso13 as a control (Supplemental Figure 4D).
Altogether, these results suggest that MV infection and replication in pDC are not necessary for pDC activation in response to MV.

*pDC capture cellular components from MV-infected tumor cells.*

Due to endo/lysosomal expression of TLR-7 and TLR-9, pDC are specialized in viral nucleic acid detection (18). These two receptors are the major innate receptors that activate pDC (32). Since MV, in the presence of IL-3 or MV-infected tumor cells, are able to induce pDC maturation, it is likely that the maturation stimulus is MV ssRNA which activates TLR7 in the endo/lysosomal compartment. This hypothesis is strengthened by the fact that MV alone does not induce DC maturation, as these cells do not express TLR7 in humans. This implies that some MV are endocytosed by pDC when they are cultured with MV and IL-3 or with MV-infected cancer cells. We then investigated whether pDC efficiently take up cellular material from MV-infected and UV-irradiated tumor cells (Figure 3). MV-infected and UV-irradiated M18 and A549 tumor cells were labeled with PKH67 and cocultured with pDC. We observed that pDCs efficiently take up MV-infected tumor cells at 37°C, whereas UV-irradiated tumor cells were less efficiently taken up (Figures 3A and 3B).

In two additional experiments, we observed that the presence of the CD46 monoclonal antibody in the culture did not inhibit phagocytosis of MV-infected tumor cells (data not shown).

These results were confirmed by confocal microscopy (Figure 3C). pDC were cocultured for 18 hours with PKH-67-labeled, MV-infected tumor cells. The optical sections showed fluorescent fragments of MV-infected tumor cells inside the pDC, confirming the internalization of MV-infected tumor cell pieces by pDC. Interestingly, we never observed syncitia formation between pDC and tumor cells. Altogether, these results suggest that
some MV contained in infected tumor cells could access compartments where TLR7 is located.

**MV-infected tumor cells induce strong type-I IFN secretion by triggering TLR7.**

pDCs are known to be the strongest producers of type-I IFN, notably against virus, upon TLR-7 or TLR-9 activation (18). Thus, we measured IFN-α production by pDC following exposure to MV, MV-infected or UV-irradiated tumor cells, by ELISA (Figure 4A). Direct exposure to MV induced IFN-α secretion by pDC only in the presence of IL-3, matching the cell maturation observed earlier in Figure 2. The amount of IFN-α produced in response to MV in the presence of IL-3 was comparable with the amount induced by R848 alone, a potent TLR7/8 agonist. Strikingly, we found high amounts of IFN-α in coculture supernatants after exposure of pDC to MV-infected tumor cells (20-40 times more than observed in response to MV in the presence of IL-3 or R848 alone). These high quantities of IFN-α were produced by the pDC, since tumor cells did not produce IFN-α or a very low amount (pg/ml range) after MV infection (data not shown). UV-irradiated A549 or M18 tumor cells did not induce IFN-α production by pDC. These results show that MV-infected tumor cells are able to trigger the production of high levels of IFN-α by pDC, considerably higher than the levels produced by pDC exposed to MV in the presence of IL-3 or to R848 alone.

We have previously shown that, three days after infection of the Meso13 tumor cell line, a large amount of virus is produced, reaching $1 \times 10^8$ TCID$_{50}$/ml corresponding to an MOI greater than 100 from a starting dose of virus of $1 \times 10^6$ TCID$_{50}$/ml, corresponding to an MOI=1 (7). It is thus likely that the huge quantity of IFN-α produced by pDC in response to MV-infected tumor cells is the result of the intense MV replication
these tumor cells. To test this hypothesis, we cultured pDC in the presence of increasing MOI ranging from 1 to 50, with or without IL-3 (Figure 4B). In the presence of IL-3, we observed that IFN-α production by pDC increased with the MOI. On the contrary, pDC did not produce IFN-α in the absence of IL-3, except for the highest MOI (MOI=50). These results suggest that the level of IFN-α production by pDC is dependent on the quantity of MV and the presence of either IL-3 or other survival signals, explaining the huge quantity of IFN-α produced in response to the high titer of virus after infection of tumor cells.

Since MV and MV-infected tumor cells contain viral ssRNA, it is likely that IFN-α production by pDC is mainly due to the triggering of TLR-7. Thus, we carried out an inhibition of TLR7. We used specific immunoregulatory DNA sequences (IRS) that inhibit IFN-α expression mediated by TLR-7 (IRS661) (33). We showed that IFN-α production by pDC cultured in the presence of MV and IL-3 was inhibited when we added the IRS661 (Figure 4C). We also observed a similar IFN-α inhibition when IRS661 was added to pDC exposed to MV-infected tumor cells. As a control, we showed that IRS661 did not inhibit the CpG-A-induced IFN-α production by pDC, which is TLR9 dependent. Altogether, these results demonstrate that IFN-α production induced by MV or MV-infected cells is TLR7 dependent.

*pDC are able to cross-present a tumor-associated antigen from MV-infected tumor cells.*

The capacity of human pDC to cross-present viral antigens has been reported (20-22), but cross-presentation of tumor-associated antigens (TAA) has not yet been described. We wondered whether human pDC exposed to MV-infected tumor cells would be able to cross-present a human TAA spontaneously expressed by tumor cells. We showed by RT-PCR that the HLA-A*0201neg M18 melanoma cell line expresses the cancer testis antigen, NYESO-1, whereas the A549 lung adenocarcinoma cell line does not (Figure 5A).

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To determine whether HLA-A*0201<sup>pos</sup> pDC are able to cross-present this TAA after exposure to an MV-infected or UV-irradiated HLA-A*0201<sup>neg</sup>/NYESO-1<sup>pos</sup> M18 tumor cell line, we used the CD8+ T cell clone, M117.167, which is specific for HLA-A*0201/NYESO-1(157-165) complexes (Figures 5B-D). A schematic of this experiment is shown in Supplemental Figure 5. The M117.167 T cell clone did not produce IFN-γ, alone or in the presence of IL-3 pDC, but was activated in the presence of pDC pulsed with NYESO-1 [157-161] peptides (Figure 5B). The clone was activated as soon as 0.1µM peptide was loaded onto pDC (16.3% IFN-γ<sup>+</sup> cells) and was more intensely activated by pDC pulsed with 1µM peptide (77.5%). In the presence of pDC cultured with MV-infected M18 tumor cells, 11.5% of the clone population was activated, whereas the clone did not produce IFN-γ in response to pDC cultured with UV-irradiated M18 tumor cells (Figure 5B). In response to pDC cocultured with MV-infected M18, the clone had an IFN-γ production profile comparable with that observed in response to pDC pulsed with 0.1µM NYESO-1(157-165) peptide.

As a control, we failed to detect activation of the M117.167 T cell clone in response to MV-infected or UV-irradiated M18 tumor cells alone (Figure 5C). This result was expected, as the M18 tumor cell line is HLA-A*0201<sup>neg</sup>, thus unable to directly present NYESO-1(157-165) peptide to the clone. This demonstrates that IFN-γ production by the clone in response to HLA-A*0201<sup>pos</sup> pDC cocultured with MV-infected M18 tumor cells is due to cross-presentation. We also did not observe IFN-γ production in response to pDC cocultured with MV-infected NYESO-1<sup>neg</sup> A549 tumor cells. In this representative experiment, the clone produced IFN-γ in response to pDC cocultured with MV-infected M18 (6.5% IFN-γ<sup>+</sup> cells), a production rate close to the one observed in response to pDC pulsed with 0.1µM NYESO-1(157-165) peptide (10.8% IFN-γ<sup>+</sup> cells). In a final set of experiments, we compared NYESO-1 cross-presentation by pDC with cross-presentation by Mo-DC (Supplemental
Figure 6). We found a similar level of cross-presentation between the two types of DC when they were cocultured with MV-infected M18 tumor cells, whereas no cross-presentation was observed in response to pDC or Mo-DC cocultured with UV-irradiated M18.

Altogether, our results show that pDC are able to cross-present tumor antigen such as NYESO-1 from MV-infected tumor cells, but not from UV-irradiated ones. Thus, MV-based antitumor virotherapy should be able to hire pDC in the antitumor immune response by activating their ability to produce high quantities of IFN-α and to cross-present TAA from MV-infected tumor cells to tumor-specific CD8+ T lymphocytes.
Discussion

In this study, we characterized, *in vitro*, the consequences of MV-based antitumor virotherapy on human pDC functions. Firstly, we showed that pDC are not sensitive to MV infection despite expression of CD46. **However, they are able to detect the virus by producing IFN-α in response to high virus quantity in the absence of a survival signal, and to low virus quantity when a survival signal, such as IL-3, is added to the culture.** Secondly, when the pDC were cocultured with MV-infected tumor cells, they underwent a maturation characterized by the induction of CD83 expression and strong production of IFN-α, with a slightly increased expression of costimulatory molecules. Conversely, the pDC cocultured with UV-irradiated tumor cells retained an immature phenotype similar to that observed when they were cocultured with IL-3 alone. **We then identified TLR7 as the pDC receptor responsible for their activation, probably due to the presence of single-stranded viral RNA in the endocytic compartment of pDC following internalization of MV-infected tumor cell fragments.** Finally, using an HLA-A*0201/NYESO-1(157-165)-specific CD8+ T cell clone, we showed that HLA-A*0201+ pDC were able to cross-present this tumor-associated antigen (TAA) from NYESO-1+ HLA-A*0201neg MV-infected tumor cells, but not from UV-irradiated ones. This is the first time, to our knowledge, that the capacity of human pDC to cross-present a TAA from dead tumor cells to CD8+ T cells has been demonstrated. Altogether, these results suggest that MV-based antitumor virotherapy, in addition to its direct lysis of infected tumor cells, is able to recruit pDC in the antitumor immune response, to activate their ability to produce high levels of type-I IFN and to cross-present TAA.

In the first part of our work, we showed that human pDC exposed *in vitro* to MV at an MOI=1 did not undergo maturation without IL-3. In this condition, with no survival signal, pDC undergo apoptosis and fail to acquire MV in the endosomal compartment to engage in a
maturation process by the ligation of viral ssRNA to TLR7. When pDC were exposed to MV in the presence of IL-3, they survived and maturation was observed (low IFN-α production and induction of CD83 expression). **We observed the activation of pDC by MV in the absence of IL-3, only when we used a high quantity of MV (MOI=50).** At this high MV concentration, we think that enough MV reached the endocytic compartment of pDC to provide a survival/maturation signal, before their apoptosis program was engaged. Thus, when pDC are exposed to MV in the presence of IL-3, the pDC survive and MV is internalized and allows triggering of TLR7 by the viral ssRNA. When pDC are exposed to MV in the absence of IL-3, they undergo apoptosis unless enough MV reaches the endocytic compartment to activate and mature them. Our results explain the contradictory reports in the literature, due to differences in experimental settings. Indeed, we obtained similar results to Schlender and collaborators who reported that a **low quantity of MV Schwarz** failed to induce IFN-α by pDC cultured in the absence of IL-3 (31), and to Duhen and colleagues who claimed that MV Schwarz induces high quantities of IFN-α production by pDC in the presence of IL-3 (30). However, our study does not support the claim that MV Schwarz inhibits IFN-α production by pDC (31), as pDC produce IFN-α in the presence of IL-3. **Finally, both groups described staining of pDC by a monoclonal antibody to MV hemagglutinin (H), but interpreted the result differently.** One group claimed that pDC were infected and amplified the virus (31), while the other group concluded that, despite the H protein staining on pDC, MV replication was low. Our results support this latter conclusion, as we did not observe productive infection using MV-eGFP, even at high MOI, in the absence nor presence of IL-3.

We also showed that, in the presence of MV or MV-infected tumor cells, pDC undergo maturation characterized by the upregulation of CD83 molecule expression at the cell surface. In the presence of MV or MV-infected tumor cells, the pDC produce higher
quantities of IFN-α in response to high viral load than pDC stimulated with R848 alone. However, these cells do not express as much of the CD40 and CD86 costimulatory molecules. Thus, this maturation phenotype resembles the maturation phenotype induced by HIV infection (34, 35), which activates pDC by the TLR7, as does MV (36). Indeed, it is now clear that, depending on the nature of the TLR agonist used, two main pathways of activation can be triggered in human pDC. This dichotomy was first reported by Kerkmann and colleagues, who showed that two TLR9 agonists, CpG-A and CpG-B, activate pDC maturation using two different pathways (37). More recently, the same dichotomy has been observed for TLR7 agonists (35). Indeed, HIV behaves like CpG-A by triggering TLR7 and the IRF7 signaling pathway in the early endosome of pDC, and by inducing strong production of IFN-α. Our results show that the maturation induced by MV+IL-3 or MV-infected cells is similar to the activation induced by HIV, suggesting an early endosomal triggering of TLR7 by MV ssRNA. This early endosome activation pathway is compatible with antigen cross-presentation expressed by virus-infected cells, as cross-presentation of viral antigens from infected cells has been demonstrated (21) and cross-presentation of the TAA from MV-infected cells, in our present study. Conversely, Schnurr and colleagues reported that, in vitro, pDC, contrary to myeloid DC, were not able to cross-present a TAA from a full-length protein alone or as an immune complex form (38). However, these authors used a soluble protein and did not use NYESO-1-expressing tumor cells as the antigen source. In vivo, antigen cross-presentation by pDC is also controversial. Salio and colleagues reported that murine pDC stimulated by CpG are not able to cross-present antigens, whereas they can mount a T cell response against endogenous antigens (39). Mouries and colleagues showed, in vivo and in vitro, also in a murine model, that soluble OVA protein and TLR agonists (CpG or R848) activate pDC to cross-prime OVA to specific CD8+ T cells (23). Similarly, presentation and cross-presentation of soluble OVA peptide or whole protein, following TLR9 stimulation by
CpG or by infection with influenza virus containing OVA epitopes, was confirmed recently, *in vitro*, by Kool and colleagues (40). Finally, Liu and colleagues reported that intratumoral injection of CpG-A-stimulated pDC to mice bearing B16 melanoma induced a tumor antigen cross-priming, but this cross-priming was performed by CD11c+ DC, not by pDC (25). We have shown here that, *in vitro*, human pDC exposed to MV-infected tumor cells are able to cross-present NYESO-1 to a CD8+ T cell clone specific for this TAA. **We demonstrated that** MV-infected tumor cells undergo cell death and are then phagocytosed by pDC. These MV-infected cells are capable of activating pDC without the addition of adjuvants or TLR agonists. **It remains to be determined** whether this cross-presentation would result in cross-priming *in vivo* since MV activates pDC by the early endosomal pathway, which is thought to be more implicated in IFN-α production than in T cell stimulatory capacity (41).

The efficiency of MV-based antitumor virotherapy has been demonstrated *in vivo* in different models of human tumor xenografts in immunodeficient mice (8-11). The first clinical trials of MV-based virotherapy have shown encouraging results (13, 14). The efficiency of MV-based virotherapy is likely due to the lysis of tumor cells by the virus. However, a part of its efficiency may also be due to the capacity of MV-infected tumor cells to activate cells of the immune system, notably pDC. Indeed, activation of pDC by TLR agonist in tumor-bearing mice has been shown to induce an antitumor immune response and tumor regression (24, 25, 42). **Liu and colleagues showed that murine pDC stimulated by a TLR9 agonist induced NK cell activation and recruitment to the tumor, triggering tumor antigen cross-presentation by CD11c+ DC** (25). Drobits and colleagues showed that topical treatment of melanoma tumors in mice with the TLR7 agonist, imiquimod, induced activation and recruitment of pDC into the tumor and caused tumor regression (24). **They demonstrated that** pDC acquire a cytotoxic activity against tumor cells by secreting TRAIL and granzyme B, in an IFNAR1-dependent mechanism. IFN-α secretion by pDC...
not only induces an antitumor cytotoxic activity on pDC by an autocrine loop, but can also act
directly on tumor cells to induce apoptosis (43). Type-I IFN also plays a role in the NK
activation and is required in a mouse model of NK-cell-dependent tumor rejection (44).
Finally, these NK cells probably also participate in the initiation of the antitumor response by
stimulating myeloid DC, since in IFNAR1- and STAT1-deficient mice the antitumor T cell
response failed to develop (45, 46). Thus, our study shows that MV-infected tumor cells
induce a high quantity of IFN-α by pDC, which may be favorable for the development of
multicell subsets involved in an antitumor immune response. Furthermore, other oncolytic
viruses known to activate pDC are being used in clinical trials of antitumor virotherapy,
such as vaccinia (47), Herpes Simplex Virus (48) and adenovirus (49). Tumor cells
infected by these viruses may also be able to induce IFN-α production and tumor
antigen cross-presentation by pDC.

MV-based antitumor virotherapy is a promising approach for treating cancer through
the oncolytic activity of the virus. Furthermore, we show here that MV-infected tumor cells
activate the maturation and tumor antigen cross-presentation capacities of human pDC. Thus,
MV-based antitumor virotherapy may represent an interesting approach to the recruitment of
pDC in the antitumor immune response.
Acknowledgements

We thank Delphine Coulais and Clarisse Panterne for their technical assistance and the Platform of Clinical Transfer and Development for the PBMC and pDC facilities. We thank Philippe Hulin and the cellular and tissular core facility of Nantes University (MicroPiCell) for their expertise in confocal microscopy. We also thank Juliette Desfrançois and the core facilities of flow cytometry. We thank Dr Yves Delnette and Pascale Jeannin for their critical discussion of the manuscript.
References


Figure Legends

**Figure 1: MV receptor expression, MV infection sensitivity and survival of tumor cells and pDC.** (A) Expression of CD46 and CD150/SLAM on the surface of tumor cell lines (M18, Meso13 and A549) and pDC (mAb staining: grey histogram; Isotype control: white histogram; the values on histograms are the R-MFI, relative mean fluorescence intensity, defined as the mAb staining MFI divided by Isotype control MFI). (B) Infection of tumor cell lines (M18, Meso13 and A549) and pDC by MV-eGFP (MOI=1). (C) Infection of pDC by MV-eGFP (MOI=1), in the presence or absence of IL-3. (D) Infection of pDC by MV-eGFP with increasing MOI, in the presence or absence of IL-3. (E) Survival of tumor cell lines following MV infection or UV irradiation. Three days after infection or UV irradiation, cells were incubated with TO-PRO®3 which stains dead cells. Fluorescence was analyzed by flow cytometry. *Results in Figures 1A, 1C and 1E are representative of three independent experiments. Results in Figures 1B and 1E reflect the mean of three independent experiments. Error bars represent the standard deviation.*

**Figure 2: MV-infected tumor cells induce pDC maturation.** pDC were cultured for 18 hours with either IL-3, MV (MOI=1), MV and IL-3, R848, UV-irradiated- or MV-infected tumor cells. (A) Expression of CD83, CD86 and CD40 by pDC was measured by flow cytometry with a gate on CD123+/BDCA-4+ cells. (B) Histograms were obtained from three independent experiments. *A nonparametric Mann Whitney comparison test was used to determine the P value, which was obtained by comparison of the sample result with the IL-3 pDC result (*p <0.05, **p < 0.01, ***p < 0.001).*

**Figure 3: Phagocytosis of MV-infected or UV-irradiated tumor cells by pDC.** (A) MV-infected and UV-irradiated tumor cells were stained with PKH-67 and cocultured with
pDC for 18 hours at 4°C or 37°C (1 DC:1 tumor cell). Cells were stained with HLA-DR-specific mAb. Fluorescence was analyzed by flow cytometry. This experiment is representative of four experiments. (B) Scatter plot representation of the four phagocytosis experiments. Error bars represent the standard deviation. (C) MV-infected tumor cells were stained with PKH-67 (green) and cocultured with pDC for 18 hours. Cells were stained with HLA-DR-specific mAb (red). Fluorescence was analyzed by confocal microscopy.

Figure 4: Production of IFN-α by pDC in response to MV is TLR7 dependent. (A) pDC were cultured for 18 hours with IL-3, MV (MOI=1), MV and IL-3, R848, UV-irradiated- or MV-infected M18 or A549 tumor cells. IFN-α production was measured by ELISA in the culture supernatants. (B) pDC were cultured for 18 hours with or without IL-3 and increasing quantities of MV. IFN-α production was measured by ELISA in the culture supernatants. (C) pDC were cultured for 18 hours with IL-3 and MV (MOI=10), CpG-A or MV-infected M18, in the absence or presence of different concentrations of IRS661 (TLR7 inhibitor). IFN-α production was measured by ELISA in the culture supernatants. Results were obtained from three independent experiments.

Figure 5: Cross-presentation of NYESO-1 by HLA-A*0201+ pDC after coculture with NYESO-1+/HLA-A*0201- M18 tumor cells infected with MV. (A) Expression of NYESO-1 by M18 and A549 tumor cell lines determined by real-time PCR (n=3). (B) pDC were cultured for 18 hours with IL-3, R848, or UV-irradiated- or MV-infected M18 tumor cells. Some pDC cultured with R848 were pulsed with NYESO-1(157-165) peptide for 1 hour and washed. pDC were then cocultured for 6 hours with the M117.167 CD8+ T cell clone specific for HLA-A*0201/NYESO-1(157-165) (defined as LT) in the presence of brefeldin A.
Production of IFN-γ by the M117.167 T cell clone was analyzed by flow cytometry after staining with CD8 and IFN-γ-specific mAb. (C) pDC were cultured for 18 hours with R848, or UV-irradiated- or MV-infected M18 (NYESO-1+/HLA-A*0201) or A549 (NYESO-1- /HLA-A*0201) tumor cells. Some pDC cultured with R848 were pulsed with NYESO-1(157-165) peptide for 1 hour and washed. pDC were then cocultured for 6 hours with the M117.167 CD8+ T cell clone specific for HLA-A*0201/NYESO-1(157-165) in the presence of brefeldin A. The production of IFN-γ by the M117.167 T cell clone was analyzed by flow cytometry after staining with CD8- and IFN-γ-specific mAb. (D) Scatter plot representation of cross-presentation experiments. “n” represent the number of experiments performed. “n” is different from one condition to another, since we were not able to perform all controls in each experiment due to the limited quantity of available pDC.
Supplemental Figure Legends

*Supplemental Figure 1: infection of the mesothelioma cell line Meso13 by MV-eGFP.* Meso13 mesothelioma cell line was cocultured 2 hours with MV-eGFP and then washed (MOI=1). Sixteen hours later, fluorescence microscopy video was performed with a picture taken every 15 minutes with a time-lapse confocal microscope (Nikon).

*Supplemental Figure 2: absence of infection of pDC by MV-eGFP.* pDC were cocultured with MV-eGFP (MOI=1) in the presence of IL-3. Fluorescence microscopy video-recording was performed with a picture taken every 15 minutes with a time lapse confocal microscope (Nikon). One cell becomes infected after 12 hours.
Clinical Cancer Research

Measles virus-vaccine infected tumor cells induce tumor antigen cross-presentation by human plasmacytoid dendritic cells

Jean-Baptiste Guillerme, Nicolas Boisgerault, David Roulois, et al.

Clin Cancer Res  Published OnlineFirst January 21, 2013.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-12-2733

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