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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. Thus, our work suggests that MV-based antitumor virotherapy trigger 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 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). A second phase-I clinical trial was published more recently (14). Clinical responses obtained after intraperitoneal injection of MV encoding the carcinoembryonic antigens (CEA) in 24 patients with a chemotherapy-resistant ovarian cancer were encouraging, with a doubling of the patient survival median.

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 ultra violet (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 (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 properties 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 to MV virus by producing IFN-α if they are cocultured with the virus and a survival factor such as IL-3. 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 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 to a CD8+ T cell clone, the human tumor antigen NYESO-1 expressed in tumor cells. Altogether, our results suggest that MV-based antitumor virotherapy, may trigger an antitumor immune response, in addition to a direct lysis effect on tumor cells, notably by the recruitment of 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% CO_2 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.

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 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. 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 donors PBMCs (Etablissement Français du Sang, Nantes, France) as previously described (27). 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 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, SanJose, CA, USA), CD83 (BioLegend, San Diego, CA-USA) and BDCA-4 (Myltenyi 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. 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 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 were measured by ELISA on pDC culture supernatants according to the manufacturer instructions.

Cross-presentation assay

NYESO-1^{+}/HLA-A*0201^{-} Melanoma (M18) and NYESO-1^{-}/HLA-A*0201^{-} pulmonary adenocarcinoma (A549) cell lines were MV-infected or UV-B irradiated and cultured for 72 hours. They were then cocultured with either HLA-A*0201^{+} 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 (28). 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 five 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 GFP+ cells for A549 to 90% of GFP+ cells for Meso13 (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).

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 apoptotic cell death was observed by irradiating the tumor cells with UV-B. Thus, MV infection induces tumor cell death for at least 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 2C).

We previously demonstrated that 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 observed when the pDC were exposed to R848. We also noted a slight induction of the expression of the costimulation molecules CD40 and CD86 on pDC exposed to MV-infected tumor cells. This induction was very 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 (29, 30). However the study from Duhen et Coll. reporting that MV activates pDC, was performed in the presence of IL-3, a pDC survival factor (29), 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 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 expression.

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 (31). Since MV alone 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 human. 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 (Figure 3A).

These results were confirmed by confocal microscopy (Figure 3B). 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. 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 as 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 to 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 very low amount (pg/ml range) after MV-infection (data not shown). UV-irradiated A549 or M18 tumor cells do not induce IFN-α production by pDC. These results show that MV-infected tumor cells were 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 previously showed that, three days after infection of the Meso13 tumor cell line, a large amount of virus is produced, reaching $1x10^8$ $TCID_{50}$/ml from a starting dose of virus of $1x10^6$ $TCID_{50}$/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 in these tumor cells. To test this hypothesis, we cultured pDC in the presence of increasing MV MOI ranging from 1 to 50, with or without IL-3 (Figure 4B). In 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. 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) (32). 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 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-depedent.

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, in 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*0201^{neg} M18 melanoma cell line expresses the cancer testis antigen, NYESO-1, whereas the A549 lung adenocarcinoma cell line is not (Figure 5A).

To determine whether HLA-A*0201^{pos} pDC are able to cross-present this TAA after exposure to MV-infected or UV-irradiated HLA-A*0201^{neg}/NYESO-1^{pos} 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). 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 of peptide was loaded onto pDC (16.3% IFN-γ+ 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 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). We also did not observe IFN-γ production in response to pDC cocultured with MV-infected NYESO-1^{neg} 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^+ 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^+ cells).

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 recruit 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 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, due to the presence of viral single-stranded DNA 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*0201^{neg} 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 is 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). The activation of pDC cultured with IL-3 and MV, notably their production of IFN-α, was increased when MV-MOI was increased. 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. 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 MV Schwarz failed to induce IFN-α by pDC cultured in the absence of IL-3 (30), 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 (29). However, our study does not support the claim that MV Schwarz inhibits IFN-α production by pDC (30), since pDC produce IFN-α in the presence of IL-3. Finally, both groups described the specific staining by a monoclonal antibody to MV hemagglutinin (H) of pDC cell surface exposed to the virus, but they interpreted this result differently. One group claimed that pDC were infected and amplified the virus (30), 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 CD40 and CD86 costimulatory molecules. Thus, this maturation phenotype resembles the maturation phenotype induced by HIV infection (33, 34), which, activates pDC by the TLR7 like MV (35). 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 M and colleagues, who showed that two TLR9 agonists, CpG-A and CpG-B, activate pDC production using two different pathways (36). More recently, the same dichotomy has been observed for TLR7 agonists (34). 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 (37). 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 (38). 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 (39). 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 apoptotic 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 determine 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 (40).

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, 41). Liu and colleagues showed that murine pDC stimulated by TLR 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). pDC acquire a cytotoxic activity against tumor cells by TRAIL and granzyme B secretions by an IFNAR1-dependent mechanism. IFN-α secretions by pDC not only induce an antitumor cytotoxic activity on pDC by an autocrine loop, but can also act directly on tumor cells to induce apoptosis (42). Type-I IFN also plays a role in the NK activation and is required in a mouse model of NK-cell-dependent tumor rejection (43). 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 (44, 45). Thus, our study shows that MV-infected tumor cells induce a high quantity of IFN-α, which may be favorable for the development of multicell subsets involved in an antitumor immune response.

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.

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Figure Legends

Figure 1: MV receptor expression, MV infection sensitivity and survival of tumor cells and plasmacytoid dendritic cells determined by flow cytometry analysis. (A) Expression of CD46 and CD150/SLAM on the surface of tumor cell lines (M18, Meso13 and A549) and pDC (mAb staining : grey histogramme ; Isotype control : white histogramme) The values indicate on histogramme are the R-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. All results were obtained from three independent experiments.

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 $CD123^+$/$BDCA\text{-}4^+$ cells. (B) Histograms were obtained from three independent experiments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

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 either Mo-DC or 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. (B) 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=1), CpG-A or MV-infected M18, in the absence or presence of different concentration 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 M117.167 CD8+ T cell clone specific for HLA-A*0201/NYESO-1(157-165) 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) Mann Whitney statistical analysis was used to compare values with those obtained for lymphocytes in presence of pDC+IL-3 (*, $P < 0.05$). Results were obtained from three independent experiments.

Supplemental Figure Legends

Supplemental Figure 1: infection of the mesothelioma cell line Meso13 by MV-eGFP. Meso13 mesothelioma cell line was co-cultured with MV-eGFP (MOI=1). 16 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 co-cultured with MV-eGFP (MOI=1) in presence of IL-3. Fluorescence microscopy video was performed with a picture taken every 15 minutes with a time lapse confocal microscope (Nikon).

FIGURE 1

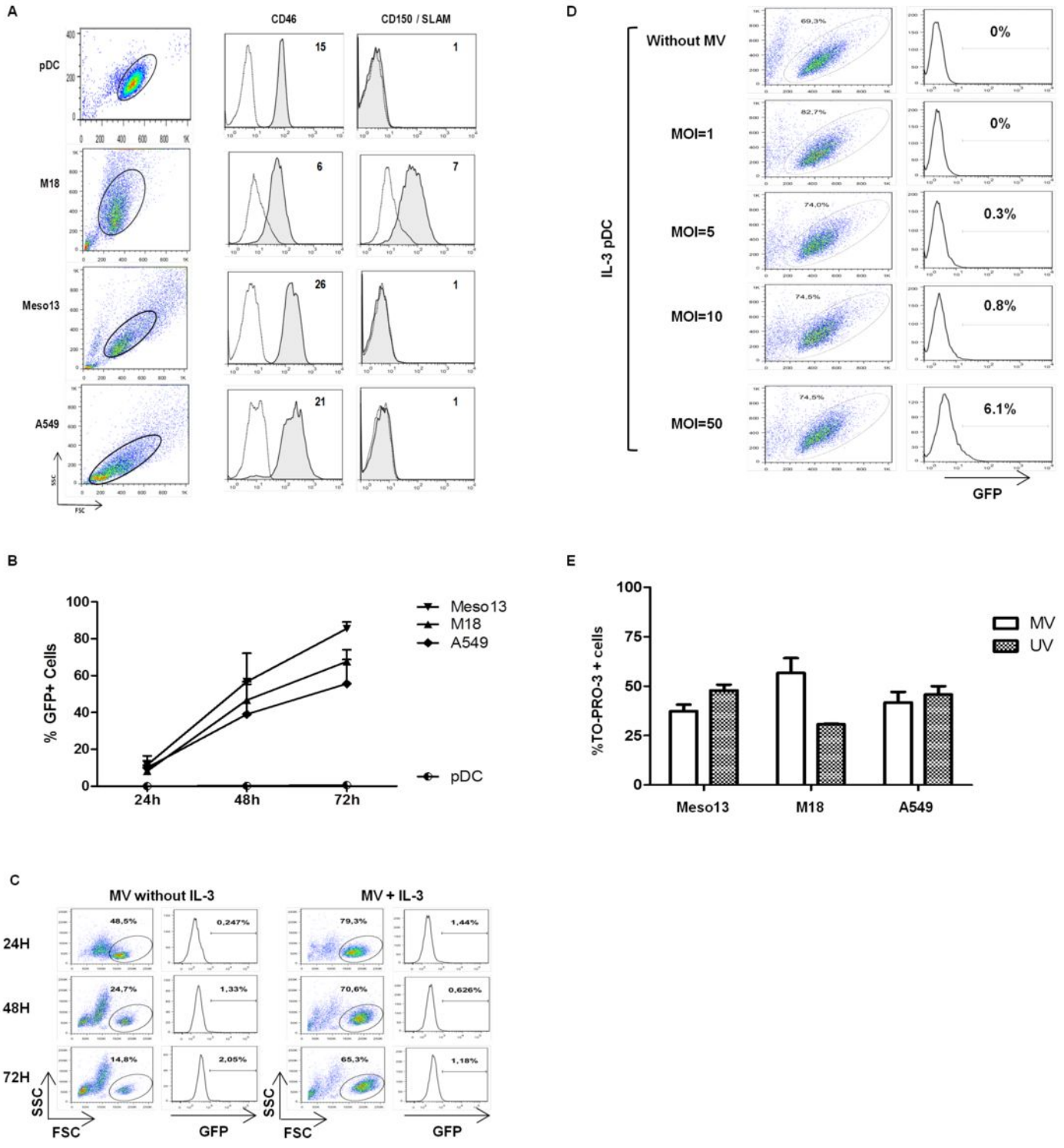

A
CD46
CD150 / SLAM
pDC
M18
Meso13
A549
15
1
6
7
26
1
21
1
SSC
FSC
B
% GFP+ Cells
Meso13
M18
A549
pDC
24h
48h
72h
C
MV without IL-3
MV + IL-3
24H
48H
72H
48,5%
0,247%
79,3%
1,44%
24,7%
1,33%
70,6%
0,626%
14,8%
2,05%
65,3%
1,18%
SSC
FSC
GFP
D
IL-3 pDC
Without MV
MOI=1
MOI=5
MOI=10
MOI=50
69,3%
0%
82,7%
0%
74,0%
0.3%
74,5%
0.8%
74,5%
6.1%
GFP
E
%TO-PRO-3 + cells
MV
UV
Meso13
M18
A549

FIGURE 2

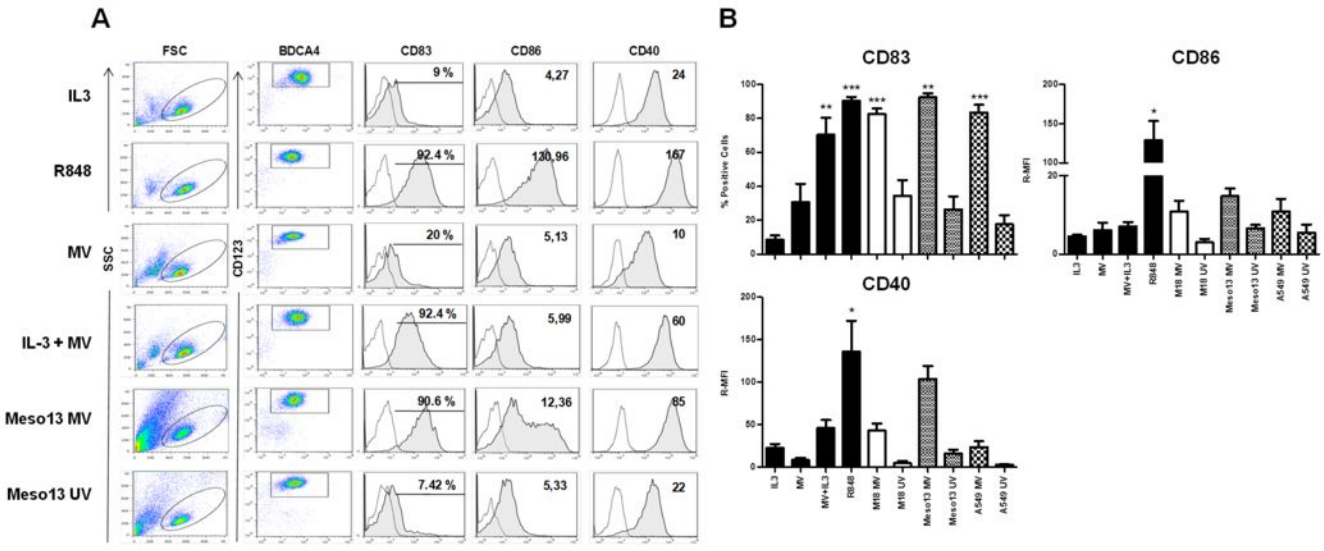
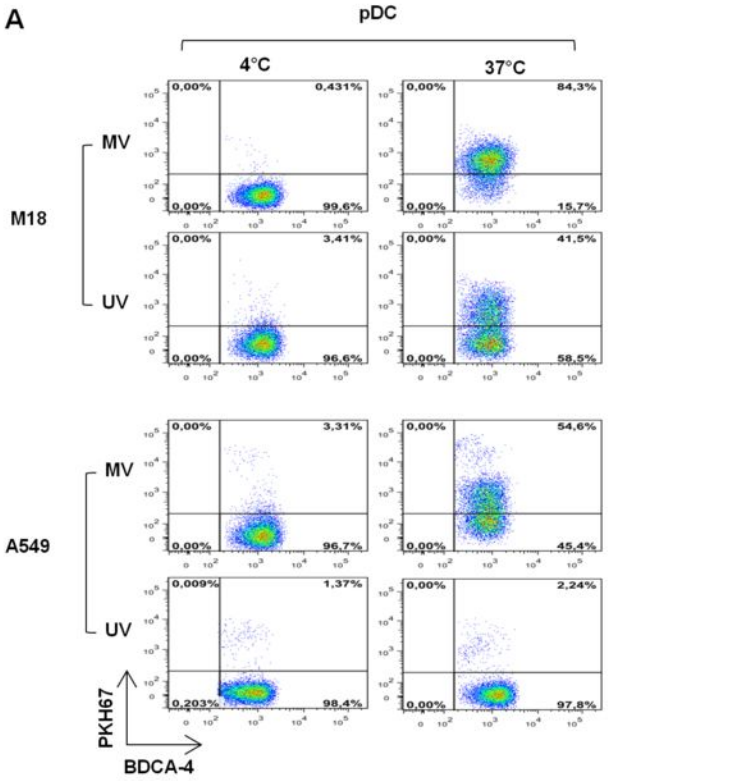

A
FSC
BDCA4
CD83
CD86
CD40
IL3
R848
MV
IL-3 + MV
Meso13 MV
Meso13 UV
SSC
CD123
9 %
4,27
24
92.4 %
130,96
167
20 %
5,13
10
92.4 %
5,99
60
90.6 %
12,36
85
7.42 %
5,33
22
B
CD83
% Positive Cells
CD86
R-MFI
CD40
R-MFI
IL3
MV
MV+IL3
R848
M18 MV
M18 UV
Meso13 MV
Meso13 UV
A549 MV
A549 UV

FIGURE 3


A
pDC
4°C
37°C
MV
UV
M18
A549
0,00%
0,431%
99,6%
3,41%
96,6%
84,3%
15,7%
41,5%
58,5%
3,31%
96,7%
54,6%
45,4%
0,009%
1,37%
98,4%
0,203%
2,24%
97,8%
PKH67
BDCA-4

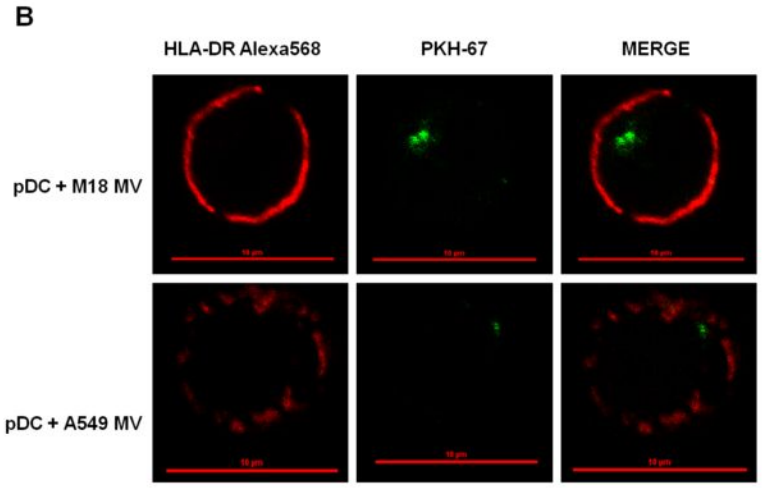

B
HLA-DR Alexa568
PKH-67
MERGE
pDC + M18 MV
pDC + A549 MV

FIGURE 4

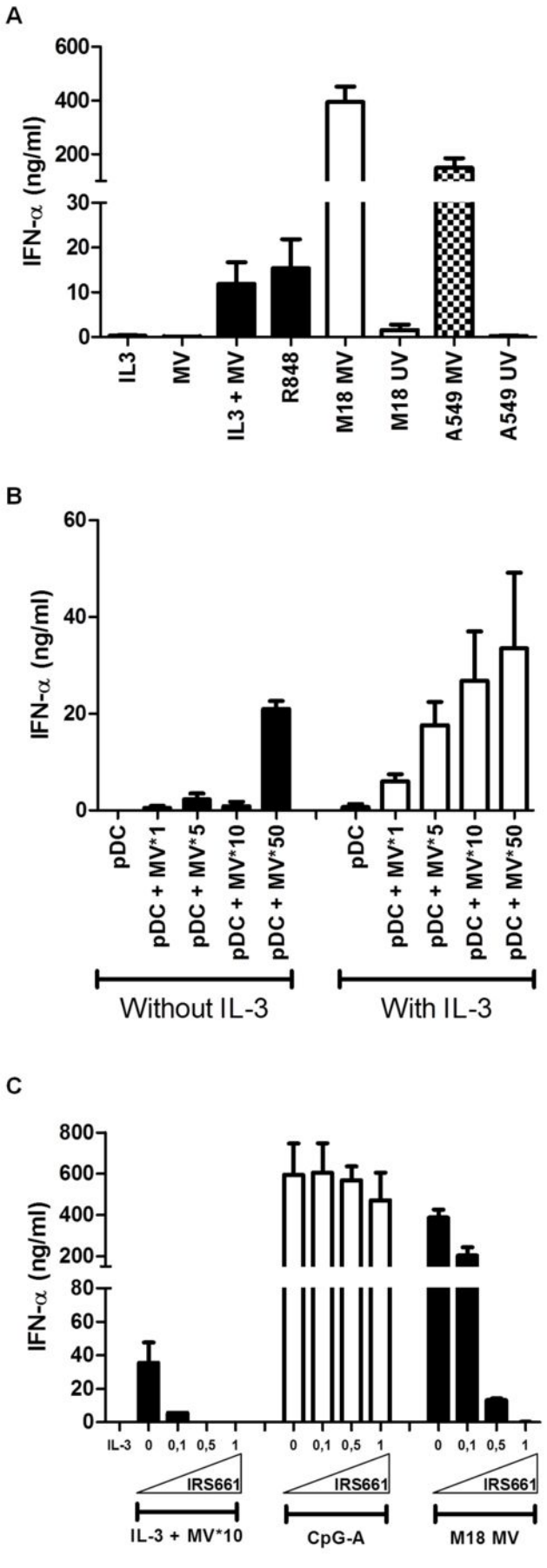
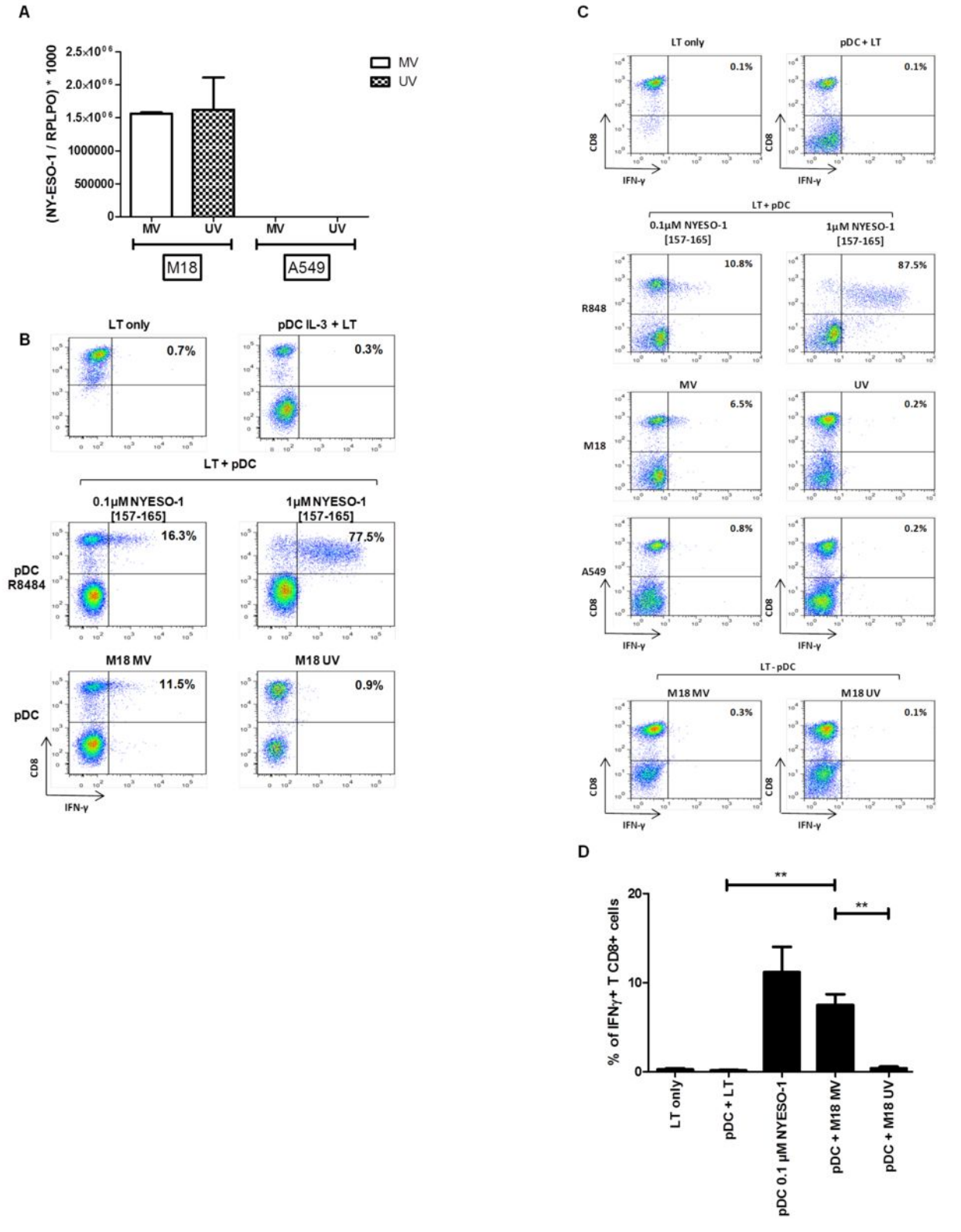

A
IFN-α (ng/ml)
600
400
200
30
20
10
0
IL3
MV
IL3 + MV
R848
M18 MV
M18 UV
A549 MV
A549 UV
B
IFN-α (ng/ml)
60
40
20
0
pDC
pDC + MV*1
pDC + MV*5
pDC + MV*10
pDC + MV*50
pDC
pDC + MV*1
pDC + MV*5
pDC + MV*10
pDC + MV*50
Without IL-3
With IL-3
C
IFN-α (ng/ml)
800
600
400
200
80
60
40
20
0
IL-3
0
0,1
0,5
1
IRS661
IL-3 + MV*10
CpG-A
M18 MV

FIGURE 5


A
(NY-ESO-1 / RPLPO) * 1000
2.5x10^06
2.0x10^06
1.5x10^06
1000000
500000
0
MV
UV
MV
UV
MV
UV
M18
A549
B
LT only
0.7%
pDC IL-3 + LT
0.3%
LT + pDC
0.1µM NYESO-1 [157-165]
16.3%
1µM NYESO-1 [157-165]
77.5%
pDC R8484
M18 MV
11.5%
M18 UV
0.9%
pDC
CD8
IFN-γ
C
LT only
0.1%
pDC + LT
0.1%
LT + pDC
0.1µM NYESO-1 [157-165]
1µM NYESO-1 [157-165]
R848
10.8%
87.5%
MV
UV
M18
6.5%
0.2%
A549
0.8%
0.2%
LT - pDC
M18 MV
0.3%
M18 UV
0.1%
D
% of IFNγ+ T CD8+ cells
20
10
0
**
**
LT only
pDC + LT
pDC 0.1 µM NYESO-1
pDC + M18 MV
pDC + M18 UV