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1 **Oncolytic attenuated measles virus encoding NY-ESO-1 induces HLA I and II**  
2 **presentation of this tumor antigen by melanoma and dendritic cells.**

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19 **Short Title:** Measles virus encoding NY-ESO-1

20

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22 **Abstract**

23 Antitumor virotherapy stimulates the antitumor immune response during tumor cell lysis  
24 induced by oncolytic viruses (OV). OV can be modified to express additional transgenes that enhance  
25 their therapeutic potential. In this study, we armed the spontaneously oncolytic Schwarz strain of  
26 measles viruses (MV) with the gene encoding the cancer/testis antigen NY-ESO-1 to obtain MVny. We  
27 compared MV and MVny oncolytic activity and ability to induce NY-ESO-1 expression in six human  
28 melanoma cell lines. After MVny infection, we measured the capacity of melanoma cells to present NY-  
29 ESO-1 peptides to CD4<sup>+</sup> and CD8<sup>+</sup> T cell clones specific for this antigen. We assessed the ability of  
30 MVny to induce NY-ESO-1 expression and presentation in monocyte-derived dendritic cells (DC). Our  
31 results show that MVny and MV oncolytic activity are similar with a faster cell lysis induced by MVny.  
32 We also observed that melanoma cell lines and DC expressed the NY-ESO-1 protein after MVny  
33 infection. In addition, MVny-infected melanoma cells and DC were able to stimulate NY-ESO-1  
34 specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Finally, MVny was able to induce DC maturation. Altogether, these  
35 results show that MVny could be an interesting candidate to stimulate NY-ESO-1 specific T cells in  
36 melanoma patients with NY-ESO-1-expressing tumor cells.

37

38 **Keywords**

39 antitumor immunotherapy, measles virus, melanoma, tumor antigen, NY-ESO-1, T cells.

## 40 **Introduction**

41 Antitumor virotherapy consists in using oncolytic viruses (OV) to treat cancer. OV are non-  
42 pathogenic replicative viruses that infect, replicate in and kill tumor cells without harming healthy cells  
43 [1]. Furthermore, infection of tumor cells by OV should result in immunogenic cell death that potentially  
44 stimulate antitumor immune responses [2]. The combination of danger signals, tumor-associated  
45 antigens (TAA) and pro-inflammatory cytokines released by infected and dying tumor cells activates  
46 antigen-presenting cells such as dendritic cells (DC) that become able to initiate anti-tumor T cell  
47 responses [3, 4].

48 Numerous TAA that are recognized by T cells via HLA molecules on the surface of tumor cells  
49 have been identified [5]. They are classified as mutated, tumor-specific, differentiation or overexpressed  
50 antigens. Tumor-specific TAA, also known as cancer/testis or cancer-germline antigens share interesting  
51 properties for cancer immunotherapy with mutated neo-antigens. Their expression is restricted to tumor  
52 cells and they are recognized by a specific T cell repertoire of high avidity [6]. In addition, their  
53 expression is shared by a variety of tumors. New York esophageal squamous cell carcinoma 1 antigen  
54 (NY-ESO-1) that is encoded by the gene *CTAG1B* is a tumor-specific TAA that is expressed in several  
55 types of cancers such as melanoma or lung adenocarcinoma. This TAA is a good candidate for  
56 immunotherapy since it can be spontaneously recognized in patients not only by cytotoxic CD8+ T  
57 lymphocytes, but also by CD4+ T cells and B lymphocytes [7–9]. Several immunotherapy clinical trials  
58 using NY-ESO-1 as a target are undergoing [10].

59 Measles virus (MV) is an enveloped, negative sense ssRNA paramyxovirus responsible for  
60 Measles. The attenuated Schwarz strain of MV is commonly used for children vaccination. This strain  
61 displays spontaneous oncolytic activity against numerous types of cancers, notably melanoma [11], lung  
62 and colon adenocarcinoma [12] and malignant pleural mesothelioma [13, 14]. Other attenuated MV  
63 strains such as the Edmonston strain are evaluated in clinical trials for the treatment of ovarian cancer,  
64 glioblastoma multiforme, multiple myeloma, mesothelioma, head and neck, breast cancers and  
65 malignant peripheral nerve sheath tumors [15]. Despite a relatively small genome of around 16,000  
66 nucleotides, MV can accept additional transgenes for use in antitumor virotherapy [16–18] or

67 prophylactic vaccination [19, 20]. Concept of TAA encoding OV for tumor vaccination was introduced  
68 using Maraba virus encoding a melanoma antigen [21], and has been applied also to MV [22].

69 In this study, we generated MVny by insertion of the gene encoding NY-ESO-1 into the Schwarz  
70 MV genome. We then evaluated MVny oncolytic activity against human melanoma cell lines and  
71 observed that it was preserved compared to standard Schwarz MV. MVny induced a strong NY-ESO-1  
72 expression in infected melanoma cell lines and a moderate expression in human monocyte-derived DC.  
73 Finally, we demonstrated in vitro that melanoma cell lines or DC exposed to MVny present efficiently  
74 NY-ESO-1 peptides to NY-ESO-1-specific CD8<sup>+</sup> and CD4<sup>+</sup> T cell clones. Altogether, our results  
75 suggest that MVny might be an interesting OV to induce NY-ESO-1 presentation to T cells.

76

77 **Material and methods**

78 Tumor cells lines

79 The M18, M45, M88, M117 and M199 human melanoma cell lines were provided by Dr  
80 Nathalie Labarrière (INCIT, Nantes) that are part of the PC-U892-NL Biocollection (CHU Nantes,  
81 France). Samples were collected in accordance with the standards established by the Declaration of  
82 Helsinki. All recruited patients gave signed informed consent. Study was approved by local ethical  
83 committee (CPP Ouest-IV -Nantes). The IPC277/5 was provided by Dr C. Auben (INSERM, Marseille).  
84 The cell lines were maintained at 37°C, under 100% humidity and 5% CO<sub>2</sub> in Roswell Park Memorial  
85 Institute Medium (RPMI) 1640 (Gibco, France) supplemented with 100U/mL Penicillin (Gibco,  
86 France), 100µg/mL Streptomycin (Gibco, France), 2mM L-Glutamine (Gibco, France), and 10% Fetal  
87 Calf Serum (FCS, Eurobio) previously decomplexed. Cells were routinely checked for Mycoplasma  
88 contamination using the Plasmotest from InvivoGen.

89

90 Human NY-ESO-1-specific T cell clones

91 NY67, a CD4+ T cell clone specific for the HLA-DPb\*0401/NY-ESO-1(157-170) complex,  
92 and N5.14, a CD8+ T cell clone specific for the HLA-A\*0201/Mucin-1(950-958) complex were  
93 obtained from blood donors as previously described [23, 24]. M.117.167, a CD8+ T cell clone specific  
94 for the HLA A\*0201/NY-ESO-1(157-165) complex was obtained from tumor-infiltrating lymphocytes  
95 (TILs) from a melanoma patient biopsy [25]. The clones were seeded for amplification at 5,000  
96 cells/well in 150µL of RPMI 1640 (Gibco) supplemented with 100U/mL Penicillin (Gibco), 100µg/mL  
97 Streptomycin (Gibco), 2mM L-Glutamine (Gibco), 8% pooled human serum and containing 1µg/mL of  
98 Leucoagglutinin PHA-L (Sigma, cat #L2769450), 150 UI/mL IL-2 (Proleukin, CHIRON) and irradiated  
99 feeder cells in 96-well round bottom plates. The feeder cells consisted of a B lymphocyte cell line  
100 immortalized by the Epstein Barr virus and peripheral blood mononuclear cells (PBMC) irradiated at 70  
101 and 35 Gy and seeded at 1.10<sup>4</sup> and 1.10<sup>5</sup> cells per well, respectively. NY-ESO-1(157-165),  
102 SLLMWITQC, and NY-ESO-1(157-170), SLLMWITQCFLPVF, peptides were purchased from  
103 Eurogentech.

104

105 Human monocyte-derived DC

106 Blood monocytes were obtained by negative magnetic sorting (EasySep Human monocyte  
107 Enrichment Kit, StemCell) from PBMC of healthy donors provided by EFS (Etablissement Français du  
108 Sang, Nantes) as previously described [26]. Immature DCs were differentiated for 5 days from  
109 monocytes, seeded at  $2 \cdot 10^6$  cells/mL in a 6-well plate with culture medium composed of RPMI 1640  
110 (Gibco, France) supplemented with 100U/mL Penicillin (Gibco), 100 $\mu$ g/mL Streptomycin (Gibco,  
111 France), 2mM L-Glutamine (Gibco), 2% human albumin (Human Albumin 200 mg/mL, VIALEBEX),  
112 1000IU/mL of GM-CSF (CellGenix) and 200IU/mL of Interleukin-4 (CellGenix). After 5 days  
113 immature DCs were collected for experiments.

114

115 Attenuated Schwarz MV, MVeGFP and MVny

116 Live-attenuated Schwarz-strain measles virus vaccine (MV), MV recombinant for the enhanced  
117 green fluorescent protein (MVeGFP), and MV recombinant for NY-ESO-1 (MVny) were produced and  
118 purified as previously described [27]. To generate MVny recombinant virus, the cDNA sequence of  
119 human NY-ESO-1 ORF (*CTAG1B*, clone NM 001327) was codon optimized for MV expression,  
120 modified at the stop codon to ensure that the total number of nucleotides is a multiple of six [19], and  
121 subsequently cloned into an additional transcription unit localized between the P and M genes in the  
122 pTM-MV Schw plasmid encoding infectious MV Schw cDNA corresponding to the antigenome of the  
123 MV Schwarz vaccine strain [27]. Rescue of recombinant virus was performed as previously described  
124 using a helper-cell-based system [27]. Briefly, helper HEK293T cells expressing both the T7-RNA  
125 polymerase and the Schwarz MV N and P proteins (HEK293-T7-MV) were co-transfected with pTM-  
126 MV Schw-NY-ESO-1 vector plasmid and a plasmid expressing the Schwarz MV polymerase L.  
127 Transfected HEK293-T7-MV helper cells were gently harvested and co-cultured with Vero cells for  
128 amplification of MV Schw-NY-ESO-1 virus. Virus titers were determined by endpoint titration on Vero  
129 cells and expressed as TCID<sub>50</sub>/ml. Replication of MV Schw-NY-ESO-1 recombinant virus was  
130 analyzed by single-step growth curves on Vero cells using an MOI of 0.1. The growth rate of  
131 recombinant MV Schw-NY-ESO-1 virus was similar to empty MV Schw.

132

133 Western blot assay

134 Vero Cells, Tumor cell lines or monocyte-derived DC were seeded at  $5 \cdot 10^5$  cells/mL and  
135 infected with MV or MVny at a multiplicity of infection (MOI) of 1 in a 24-well plate in their respective  
136 media. After 48 hours of infection, cells were lysed in  $100 \mu\text{L} / 1 \cdot 10^6$  cells of RIPA lysis buffer (Radio  
137 immunoprecipitation assay buffer, Sigma-Aldrich) with 1% protease inhibitor (Sigma-Aldrich). Twenty  
138  $\mu\text{g}$  of proteins were denatured at  $95^\circ\text{C}$  for 5 minutes in Laemmli buffer (BioRad) with 10% beta-  
139 mercaptoethanol (Merck) before plating into a pre-cast SDS-polyacrylamide 4-15% electrophoresis gel  
140 (BioRad). Migration was performed at 200V for 30min in 10x Tris/Glycine/SDS migration buffer  
141 (BioRad, cat #1610772). After migration, proteins were transferred to a Polyvinylidene difluoride  
142 (PVDF) membrane, which was then blocked with 0.1% Tween tris-buffered saline (T-TBS) containing  
143 5% milk. The membrane was incubated with a rabbit anti-NY-ESO-1 primary antibody (Cell Signaling  
144 Technology, Cat #45437) at  $4^\circ\text{C}$  overnight at 1:1000 or with a rabbit anti-Actin primary antibody (Cell  
145 Signaling Technology, cat # 3700) for one hour at room temperature at 1:1000. After 3 washes with  
146 0.1% T-TBS, the membrane was incubated with horseradish peroxidase (HRP)-coupled anti-rabbit  
147 secondary antibody (Jackson Immuno Research) for one hour at 1:5000. Proteins were revealed with the  
148 Clarity TM Western ECL chemiluminescence kit (BioRad) by the Chemi DOC TM Imaging system  
149 (BioRad). The analysis of the images was performed with the ImageLab software.

150

151 Propidium iodide / annexin V Staining

152 CD8+ T cell clone N5.14, tumor cell lines or immature monocyte-derived DCs were seeded at  
153  $5 \cdot 10^5$  cells/mL and infected with MV or MVny (MOI=1) in a 48-well plate in their respective media.  
154 After 48h, 72h, 96h or 120h of infection, the cells were labeled with propidium iodide (PI) / FITC  
155 Annexin V Apoptosis Detection Kit I (BD Pharmingen Cat #556547) and analyzed by flow cytometry.

156

157 Intracytoplasmic staining of IFN- $\gamma$

158 HLA-A\*0201+ or HLA-DPb1\*0401+ immature DCs and tumor cells were seeded in a 96-well  
159 flat-bottom plate at  $1.2 \cdot 10^6$  cells/mL in  $100 \mu\text{L}$  of their culture medium and were infected or not with  
160 MV or MVny (MOI=1). After 48 hours, some uninfected DC and tumor cells were incubated 1 hour at



161 room temperature with 10 $\mu$ M of NY-ESO-1 (157-165) or (157-170) peptides and then washed.  
162 Uninfected, peptide-pulsed and unwashed infected DCs or tumor cells were co-cultured with the CD4+  
163 T cell clone NY67 or with the CD8+ T cell clone M.117.167 in presence of 10  $\mu$ g/mL of brefeldin A  
164 (BioLegend, cat #420601). After 5 h of co-culture, cells were fixed with PBS 4% paraformaldehyde  
165 (PFA) (Electron Microscopy Sciences) for 10 minutes and labeled with a FITC-conjugated anti-CD4  
166 (BD Pharmingen) or a FITC-conjugated anti-CD8 (BioLegend), and a PE-conjugated anti-IFN- $\gamma$  (BD  
167 /Pharmingen, Cat # 554701) mAb, diluted in PBS with 0.1% bovine serum albumin (BSA) and 0.1%  
168 saponin, for 30 minutes. Cells were then analyzed by flow cytometry with a BD FACS *Canto II*  
169 cytometer. The software *Diva* was used for the analysis.

170

#### 171 *MVgfp replication in monocyte-derived DC, M88 melanoma cell line and N5.14 CD8+ T cell clone*

172 N5.14 CD8+ T cell clone, M88 melanoma cell line and monocyte-derived DCs were seeded in  
173 a 48-well plate at 5.10<sup>5</sup> in 800 $\mu$ L of their respective culture media. They were exposed to MVeGFP  
174 (MOI=1). The fluorescence was recorded by time-lapse microscopy using a *Widefield Leica DMI8*,  
175 *driven by Metamorph v7.8 software* (Molecular Devices), with environmental control (Temperature,  
176 humidity and CO<sub>2</sub>) for 120h using a x10 objective. Fluorescence was calculated from two different fields  
177 per wells with the *ImageJ* software [28].

178

#### 179 *RT-qPCR*

180 The M88 melanoma cell line and iDCs were seeded in a 48-well plate at 5.10<sup>5</sup> cells/mL and  
181 2.10<sup>6</sup> cells/mL, respectively, in 500 $\mu$ L of their respective culture media. They were exposed to MV or  
182 MVny (MOI=1). After 48h, cellular RNAs were extracted using the Nucleospin® RNA plus kit  
183 (Macherey-Nagel, Cat #740984.10) and 250ng were reverse-transcribed (Invitrogen, Cat #28025013).  
184 The qPCR reaction is then performed using Master mix Maxima SYBR Green/ROX qPCR (Thermo  
185 Scientific, Cat #K0253). The expression of NY-ESO-1 was measured using a pair of QuantiTect primers  
186 for NY-ESO-1. NY-ESO-1 gene expression was normalized against the expression of the housekeeping  
187 gene encoding the Ribosomal Protein Lateral Stalk Subunit P0 (RPLP0). Each reaction sample was run  
188 in duplicate. To circumvent any issue of non-specific amplification melting curve analysis was

189 performed with a temperature gradient of 70-95°C. The  $2^{-\Delta\Delta C_t}$  method was used to calculate relative  
190 changes in expression [29].

191

#### 192 Surface staining of human monocyte-derived DCs

193 Monocyte-derived DCs were seeded in a 48-well plate at  $5.10^5$  cells/mL in 1mL of their culture  
194 medium. They were infected with MV or MVny (MOI=1), or treated with 1µg/mL lipopolysaccharide  
195 (LPS) (Sigma-Aldrich Cat #L2654-1 MG). After 48h of incubation, they were labeled 30 minutes with  
196 anti CD80 APC-H7-conjugated (IgG1k, BD Cat#561134), an anti-CD83 FITC-conjugated (IgG1k,  
197 Biosciences Cat #556910), an anti-CD86 APC-conjugated (IgG1k, BD Biosciences Cat#555660), an  
198 anti-HLA-ABC FITC-conjugated (IgG2a, Beckman Coulter, Cat#IM1838U), an anti-HLA-DP  
199 BUV395-conjugated (IgG1k, BD, 750866) or respective fluorescent isotype control mAb diluted in PBS  
200 with 0.1% BSA in presence of Fc block (BD Pharmingen, Cat#564220). Cells were washed and then  
201 fixed in PBS with 1% PFA for flow cytometric analysis with a BD FACS *Canto II* cytometer and the  
202 Diva software.

203

#### 204 Data formatting and statistical analysis

205 The data were formatted using *GraphPad Prism* v8. Statistical significance were determined by  
206 the Mann-Witney Wilcoxon signed ranks test and Kruskal Wallis test (\*  $p < 0.05$  ; \*\*  $p < 0.01$ )

207 **Results**

208 *MVny induces NY-ESO-1 expression in human melanoma cells and cell lysis*

209 MVny was generated by inserting the gene encoding the tumor associated antigen NY-ESO-1  
210 in the genome of the Schwarz MV between the genes encoding P/V/C and M proteins (Figure 1A). The  
211 recombinant MVny virus was obtained by reverse genetics using a helper-cell-based system and  
212 amplified on Vero cells. The modified virus replicated similarly to the parental Schwarz strain of MV  
213 and expressed high levels of NY-ESO-1 protein in infected Vero cells (Figures 1B).

214 We previously described that a majority of human melanoma cell lines are permissive *in vitro*  
215 to MV replication due to defects in their antiviral type I interferon pathway [11]. In this experiment, we  
216 evaluated by western blot if the tumor antigen NY-ESO-1 is expressed after *in vitro* exposure of five  
217 melanoma cell lines to MVny, using the human M18 melanoma cell line that expresses NY-ESO-1 as a  
218 control. First, we observed that among the melanoma cell lines studied, two cell lines, M18 and  
219 IPC277/5, expressed NY-ESO-1 (Figure 2A). After 48h of exposition to MVny, the five melanoma cell  
220 lines expressed high levels of NY-ESO-1 (Figure 2B). NY-ESO-1 expression induced by MVny was  
221 higher than its spontaneous expression in M18 and IPC277/5.

222 We then assessed by propidium iodide and annexinV staining if exposition of the 6 melanoma  
223 cell lines to MV or MVny led to cell lysis. After 48h, it was often too early to detect lysis induced by  
224 MV (Figures 3A and 3B). However, we detected about 15% more cell death when IPC277/5 cells were  
225 infected with both viruses. We observed around 40% and 30% additional cell death in M18 and M88  
226 induced by MVny compared to MV, respectively. After 120h, the same level of cell death was induced  
227 by MV and MVny in all cell lines, except for M199 that was better lysed by MV (Figure 3C and 3D).  
228 We also observed that M45 was quite resistant to the oncolytic activity of both viruses showing only  
229 around 10% lysis. Altogether, these results show that MVny has an equivalent oncolytic activity  
230 compared to MV that is faster for a few melanoma cell lines such as M18 and M88.

231

232 *MVny induces recognition of melanoma cell lines by NY-ESO-1-specific CD4+ and CD8+ T cell clones*

233 To determine whether HLA-A\*0201+ melanoma cell line M199 is able to present the NY-ESO-  
234 1(157-165) peptide after infection by MVny, we measured IFN- $\gamma$  production by M117.167, a human

235 CD8+ T cell clones specific for the complex HLA-A\*0201/NY-ESO-1(157-165) (Figure 4A-C). The  
236 M117.167 clone was unable to recognize M199 alone or exposed to MV, whereas it was able to produce  
237 IFN- $\gamma$  in response to M199 pulsed with NY-ESO-1(157-165) peptide or after infection with MVny. This  
238 production of IFN- $\gamma$  by M117.167 in response to MVny infected M199 cell lines was modest after 48h  
239 (Figures 4A and 4C), but reached a high level comparable to the production observed in response to  
240 10 $\mu$ M of peptide after 120h (Figures 4B and 4C). In the same experiments, we also assessed M117.167  
241 response to the autologous HLA-A\*0201+ tumor cell lines M117. This cell line and the M117.167 T  
242 cell clone were obtained from the same melanoma biopsy. Non-infected M117 was recognized by  
243 M117.167 and this recognition was comparable after peptide loading or infection by MV or MVny  
244 (Figure S1A and S1B). We failed to detect NY-ESO-1 protein by western blot in the non-infected M117  
245 cell line (Figure 2), but we found NY-ESO-1 mRNA by RT-PCR in this cell lines (Figure S1C). We  
246 performed an additional experiment that show that MVny does not induces the recognition of M199 by  
247 a MUC1(950-956) specific CD8+ T cell clones and that the NY-ESO-1 specific T cell clone M117.167  
248 does not recognize MV encoding cherry protein (MVch) infected M199 cells (Figure S2).

249 We also used NY67, a human CD4+ T cell clone specific for the HLA-DPB1\*0401/NY-ESO-  
250 1(157-170) complex, to determine if the HLA-DPB1\*0401+ melanoma cell line M88 was able to present  
251 the NY-ESO-1(157-170) peptide after infection by MVny (Figure 4D to 4F). The NY67 clone was  
252 unable to recognize M88 alone or exposed to MV, whereas IFN- $\gamma$  was produced in response to M88  
253 pulsed with NY-ESO-1(157-170) peptide or after infection with MVny. This production of IFN- $\gamma$  by  
254 NY67 in response to the MVny-infected M88 cell line was comparable to the production observed in  
255 response to 10 $\mu$ M of peptide after 48h of infection (Figure 4D and 4E) and then decreased after 120h  
256 (Figure 4D and 4F).

257 Finally, we measured HLA-DP and HLA-A\*0201 expression by M88 and M199 melanoma cell  
258 lines exposed to MV or MVny. After 48h, we observed no effects of both viruses on HLA-DP expression  
259 and slight decrease of HLA-A\*0201 expression induced by MVny (Figure S3).

260 Altogether, these experiments show that the expression of NY-ESO-1 by melanoma cell lines  
261 after infection by MVny allows the cells to present efficiently the tumor antigen to CD4+ and CD8+ T  
262 cells via HLA class II and I molecules, respectively.

263

#### 264 *MVny induces NY-ESO-1 expression in human monocyte-derived DC and cell lysis*

265 We first analyzed MV replication in monocyte-derived DC using MV encoding the enhanced  
266 green fluorescent protein (MVeGFP). Fluorescence was detected as soon as 20h after virus exposition  
267 in DC and M88 melanoma cells (Figure 5A). While in M88 cell line fluorescence reached a mean plateau  
268 around 250 FU after 96h of culture, in DC fluorescence peaked at a mean level of 8 FU after 30 hours  
269 and then slowly decreased. At fluorescence peak, DC that were permissive to MV replication appeared  
270 slightly green in the middle of non-fluorescent DC aggregates (Figure 5B). We failed to detect any  
271 fluorescence after exposition of the human CD8+ T cell clone N5.14 to MVegfp (Figure 5A). These  
272 results show that MV replicates moderately in monocyte-derived DC, confirming our previous  
273 observation [3].

274 We then measured by RT-PCR and western blot the NY-ESO-1 expression in monocyte-derived  
275 DC exposed for 48h to MV or MVny. As expected, NY-ESO-1 was not expressed by uninfected DC or  
276 DC infected with MV, whereas expression was detected in DC exposed to MVny (Figure 5C). However,  
277 the amount of NY-ESO-1 protein in MVny-infected DC was lower than in MVny-infected M88 cell line  
278 (Figure 5D).

279 We evaluated monocyte-derived DC cell death after exposition to MV or MVny using  
280 propidium iodide / annexin V staining (Figure 6). We observed that MVny induced cell death faster than  
281 MV in both monocyte-derived DC and M88 melanoma cells. We did not observe cell death of N5.14  
282 CD8+ T cells when exposed to MV or MVny.

283 We then assessed if MVny was able to induce DC maturation. DC maturation was observed 48h  
284 after infection by MV or MVny when more than a half of DC are still alive, using LPS exposed DC as  
285 a positive control. MVny induced DC maturation that is significant only for HLA-DR expression and  
286 this maturation was less intense than LPS (Figure 7).

287           Altogether these results show that MVny is able to induce maturation of monocyte-derived DC  
288 and expression of small amounts of NY-ESO-1 proteins, but also to induce significant DC cell death.

289

290 *MVny induces recognition of DC by NY-ESO-1-specific CD4+ and CD8+ T cell clones*

291           We finally determined if monocyte-derived DC exposed to MVny were able to present the NY-  
292 ESO-1 antigen to specific CD4+ and CD8+ T cells. The NY-ESO-1 specific CD8+ T cell clone  
293 M117.167 strongly responded by IFN- $\gamma$  production when exposed to MVny-infected HLA-A\*0201+  
294 DC or pulsed with 10 $\mu$ M specific peptide, while it produce low level of IFN- $\gamma$  in response to non-  
295 infected or MV-infected DC (Figure 8). Similarly, the NY-ESO-1 specific CD4+ T cell clone NY67  
296 produced high amounts of IFN- $\gamma$  in response to DC exposed to MVny-infected DC or pulsed with 10 $\mu$ M  
297 of peptide, while it produced low levels in response to non-infected or MV-infected HLA-DPB1\*0401+  
298 monocyte-derived DC (Figure 8). In this experiment, we also observed a low amount of IFN- $\gamma$  that is  
299 produced by both clones in response to MV-infected DC. This low IFN- $\gamma$  amount is due to type I IFN  
300 produced by DC in response to MV, since it can be inhibited by ruxolitinib that is an inhibitor of JAK1/2  
301 and consequently of the IFN- $\alpha$ / $\beta$  receptor signaling (Figure S4). In the meantime, the recognition of  
302 MVny-infected DC by the clones is not altered by ruxolitinib. Thus, MVny-infected DC are able to  
303 express the NY-ESO-1 and to present efficiently peptides from this tumor antigen to CD4+ and CD8+  
304 T lymphocytes through HLA molecules.

## 305 Discussion

306 In a previous study, we showed that a majority of melanoma cell lines are sensitive to the  
307 oncolytic activity of the Schwarz strain of MV due to defects of the antiviral type I interferon pathway  
308 in tumor cells [11]. In this study, we show that the attenuated Schwarz MV encoding human NY-ESO-  
309 1 is able to express this tumor antigen in infected melanoma tumor cells, while retaining its oncolytic  
310 activity. Upon infection of melanoma cells, NY-ESO-1 is strongly expressed and NY-ESO-1 peptides  
311 (157-165 and 157-170) are efficiently presented by infected tumor cells to specific CD8+ and CD4+ T  
312 cells by HLA class I and II molecules. We also show that MVny replicates modestly in monocyte-  
313 derived DCs compared to melanoma cells, allowing DCs to present NY-ESO-1 peptides to specific  
314 CD4+ and CD8+ T cells. Altogether, these results demonstrate that MVny displays an oncolytic activity  
315 against melanoma cell lines and induces NY-ESO-1 peptides presentation not only by tumor cells, but  
316 also by antigen presenting cells such as monocyte-derived DCs.

317 Recently, Elena Busch and collaborators showed that MV can be modified to encode TAAs  
318 [22]. They demonstrated using mouse cells that TAAs are expressed by tumor and dendritic cells after  
319 TAA-encoding MV infection and that they are presented to CD8+ T cells. In this study, we confirm  
320 their finding using human cells. We further show that MV encoding TAA can induce HLA class II  
321 presentation by tumor cells and DCs to CD4+ T cells.

322 We observed that MVny induced a faster cell death than MV in some melanoma cell lines and  
323 in DCs. Attenuated MV replication in human DCs has already been reported, albeit at a lower level than  
324 wild type MV [3, 30]. This weak replication in DC is believed to be part of its great effectiveness as a  
325 vaccine. The expression of NY-ESO-1 seems toxic for cells. It is difficult to formulate hypothesis  
326 regarding this increased toxicity of MVny since the role of NY-ESO-1 is not well defined [10]. It is also  
327 possible that this faster cell death may be due to the production of more non-encapsidated 5' copy-back  
328 defective interfering Genomes (DI-RNA) during MV replication due to the presence of a transgene [31,  
329 32]. DI-RNA are strong activator of the IFN I response via RIG-I that may lead to cell death induction.  
330 We and other previously showed that MV induces immunogenic cell death allowing the release of  
331 danger signals that mature DC [3, 33].

332 We previously showed that melanoma cell lines expressing NY-ESO-1 and HLA class II  
333 molecules can present NY-ESO-1 peptides to NY-ESO-1-specific CD4+ T cells by an exogenous  
334 pathway [34]. Indeed, some NY-ESO-1 proteins are released by melanoma cells and re-captured to reach  
335 the endo-lysosomal HLA class II presentation pathway. We also reported that MV-induced cell death  
336 allows the release of high amounts of NY-ESO-1 proteins leading to an increase HLA class II  
337 presentation of this antigen by living tumor cells around [35]. Thus, MVny would reinforce NY-ESO-1  
338 expression in tumors and its release by tumor cells leading to a strong presentation to CD4+ T cells.  
339 Furthermore, NY-ESO-1 expression in melanoma tumors is often heterogeneous, exhibiting a mosaic-  
340 like pattern with only isolated parts of the tumor expressing this antigen [36]. MVny may be an  
341 interesting tools to spread NY-ESO-1 antigen presentation to regions of the tumor that do not express  
342 NY-ESO-1.

343 The frequency of patients with NY-ESO-1-expressing melanoma correlates with the stage of  
344 the disease. This expression is low in first stages and reaches around 45% in metastatic melanoma [36–  
345 38]. MVny may be interesting to induce or reinforce NY-ESO-1 specific T cell responses for patient  
346 with tumors that express this tumor antigen. Several immunotherapy clinical trials are on-going to  
347 induce CD4+ and CD8+ T cell response against NY-ESO-1 [10]. For exemple, the adoptive transfer of  
348 an autologous HLA-DPB\*0401/NY-ESO-1(157-170)-specific CD4+ T lymphocyte clone in a  
349 metastatic melanoma patient allowed the rejection of tumors and the emergence of other TAA-specific  
350 T cells, thereby suggesting the induction of antigen spreading [39]. In addition, the adoptive transfer of  
351 CD8+ T cells harboring a transgenic T cell receptor against NY-ESO-1 resulted in clinical benefits in  
352 HLA-A\*0201+ melanoma patients [40]. Thus, strategies aiming at increasing the presentation of this  
353 TAA by DC and tumor cells not only through the HLA class I pathway, but also through the class II  
354 pathway, should be envisaged to stimulate NY-ESO-1 antitumor immune responses. The use of  
355 oncolytic viruses expressing NY-ESO-1, such as MVny, may be a valuable therapeutic strategy to  
356 achieve this goal.



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361

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368

369 **Disclosure statement**

370 FT and JFF are authors of patents on MV. FT owns equity in Oncovita, an oncolytic virotherapy  
371 company.

372

373 **Author contributions**

374 Experiments conception: FT and JFF

375 Experiments: MG, MI, AA, CCh, LB, CCo, SD, VD, NB and JFF

376 Data analysis and manuscript writing: MG, NL, DF, NB, CB, FT and JFF.

377

378 **Data availability statement**

379 The data that support the findings of this study are available from the corresponding author (jean-  
380 francois.fonteneau@inserm.fr) upon reasonable request.

381

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- 487

488 **Figure legends**

489 Figure 1: Generation of MVny. (A) Schematic representation of pTM-MVny plasmid vector with the  
490 NY-ESO-1 ORF sequence inserted into an additional transcription unit between the PVC and M genes.  
491 The MV genome comprises the nucleoprotein (N), phosphoprotein (P), V and C accessory proteins,  
492 matrix (M), fusion (F), hemagglutinin (H) and polymerase (L) genes. Plasmid elements include the T7  
493 RNA polymerase promoter (T7), hammerhead ribozyme (hh), hepatitis delta virus ribozyme ( $\delta$ ), and T7  
494 RNA polymerase terminator (T7t). (B) Growth kinetics of MV (blue) and MVny (red) in Vero cells  
495 infected with an MOI of 0.1 (cell-associated virus titers are indicated in TCID<sub>50</sub>/ml).

496  
497 Figure 2: MVny induces NY-ESO-1 expression in human melanoma cell lines. (A) Western blot analysis  
498 of NY-ESO-1 antigen expression in six human melanoma cell lines (M18, M45, M88, M117, M199 and  
499 IPC 277/5). (B) Western blot analysis of NY-ESO-1 antigen expression in six melanoma lines (M18,  
500 M45, M88, M117, M199, and IPC277/5) non-infected (NI) or infected by MV or MVny for 48 hours.

501  
502 Figure 3: MVny induces the lysis of human melanoma cell lines. Melanoma cell lines were cultured  
503 alone (NI) or with MV or MVny (MOI=1). After 48h (A-B) or 120h (C-D), cell death was measured by  
504 annexin V and Propidium iodide (PI) staining and flow cytometry. (A and C) One representative  
505 experiment with M88, M117 and M199 cell lines is shown. (B and D) Results (means + SD) of three  
506 independent experiments performed with M18, M45, M88, M117, M199, and IPC277/5 cell lines are  
507 shown. Percentages of dead cells are the sums of Annexin V+/PI-, Annexin V+/PI+ and Annexin V-  
508 /PI+ cells.

509  
510 Figure 4: MVny induces the recognition of melanoma cell lines by NY-ESO-1-specific CD4+ and CD8+  
511 T cell clones. (A-C) The HLA-A\*0201+ melanoma cell line M199 was cultured alone (NI), with MV or  
512 MVny (MOI=1) during 48h (A) or 120h (B). Cells were collected and a part of M199 was pulsed with  
513 the NY-ESO-1 (157-165) for one hour and washed. Cells were co-cultured with the CD8+ T cell clone  
514 M117.167 for 5h in presence of brefeldin A and then fixed and stained for CD8 and intracellular IFN- $\gamma$ .  
515 Fluorescence was analyzed by flow cytometry. Results (means + SD) of three independent experiments

516 are shown (C). (D-F) The HLA-DPb1\*0401+ melanoma cell line M88 was cultured alone (NI), with  
517 MV or MVny (MOI=1) during 48h (D) or 120h (E). Cells were collected and a part of M88 was pulsed  
518 with the NY-ESO-1 (157-170) for one hour and washed. Cells were co-cultured with the CD4+ T cell  
519 clone NY67 for 5h in presence of brefeldin A, then fixed and stained for CD4 and intracellular IFN- $\gamma$ .  
520 Fluorescence was analyzed by flow cytometry. Results (means + SD) of three independent experiments  
521 are shown (F).

522

523 Figure 5: MVny replicates in human monocyte-derived DC and induces NY-ESO-1 expression. (A)

524 Human monocyte-derived DC, the M88 melanoma cell line and N5.14 CD8+ T cell clones were exposed  
525 to MVgfp (MOI=1) during 120h. The average fluorescence intensity is calculated from two different  
526 locations in the same culture well per condition. (B) Microscopy picture of monocyte-derived DC 30h  
527 after exposition to MVgfp. (C-D) Monocyte-derived DC and M88 melanoma cell line were exposed to  
528 MV or MVny (MOI=1). (C) After 48h, cells were collected and *CTAG1B* expression was measured by  
529 RT-qPCR and normalized to the expression of the housekeeping gene *RPLP0*. *CTAG1B* expression in  
530 infected cells were compared to that of non-infected cells and results are expressed as log2 of fold  
531 change. (D) After 48h, NY-ESO-1 and Actin were detected by western blot.

532

533 Figure 6: MVny induces the lysis of human monocyte-derived DC. Human monocyte-derived DC, the

534 M88 melanoma cell line and N5.14 CD8+ T cell clones were exposed to MV (MOI=1). (A-B) After  
535 48h, 72h, 96h and 120h, cell death was measured by annexin V and propidium iodide (PI) staining and  
536 flow cytometry. (B) Results (means + SD) of four independent experiments are shown. Percentages of  
537 dead cells are the sums of Annexin V+/PI-, Annexin V+/PI+ and Annexin V-/PI+ cells and was retracted  
538 from 100 to obtain % viability.

539

540 Figure 7: MVny induces the maturation of human monocyte-derived DC. Monocyte-derived DC were

541 cultured alone or with LPS, MV or MVny (MOI=1) for 48h. (A) Surface expressions of CD80, CD83,  
542 CD86, HLA class I and HLA-DP were measured by flow cytometry. (B) Results (means + SD) of the  
543 relative mean of fluorescence intensity (RMFI) from four independent experiments. RMFI were

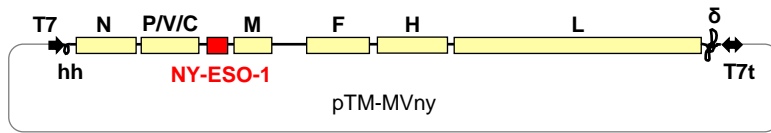
544 obtained by the ratio of the mean fluorescence of cells stained by the specific mAb by the mean  
545 fluorescence of cells stained with the isotype control. RMFI were normalized to non-infected cells  
546 RMFI.

547

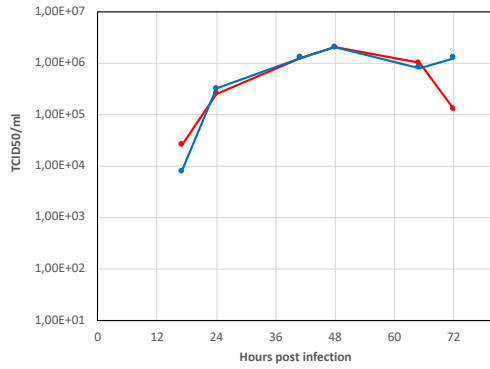
548 Figure 8: MVny induces the recognition of HLA-A\*0201+ and HLA-DPb1\*0401+ DC by NY-ESO-1-  
549 specific CD8+ and CD4+ T cell clones. (A) HLA-DPb1\*0401+ monocytes-derived DC were cultured  
550 alone (NI) or with MV or MVny (MOI=1) during 48h. Cells were collected and a part of DC were pulsed  
551 with the NY-ESO-1 (157-170) for one hour and washed. Cells were co-cultured with the CD4+ T cell  
552 clone NY67 for 5h in presence of brefeldin A, then fixed and stained for CD4 and intracellular IFN- $\gamma$ .  
553 Fluorescence was analyzed by flow cytometry. (B) HLA-A\*0201+ monocytes-derived DC were  
554 cultured alone (NI) or with MV or MVny (MOI=1) during 48h. Cells were collected and a part of DC  
555 were pulsed with the NY-ESO-1 (157-165) for one hour and washed. Cells were co-cultured with the  
556 CD8+ T cell clone M17.167 for 5h in presence of brefeldin A, then fixed and stained for CD8 and  
557 intracellular IFN- $\gamma$ . Fluorescence was analyzed by flow cytometry. (C) Results (means + SD) of the  
558 percentage of CD4+/IFN- $\gamma$ + or CD8+/IFN- $\gamma$ + cells of four independent experiments are shown.  
559 Significance was determined in comparison of the NI DC condition.

Figure 1

A

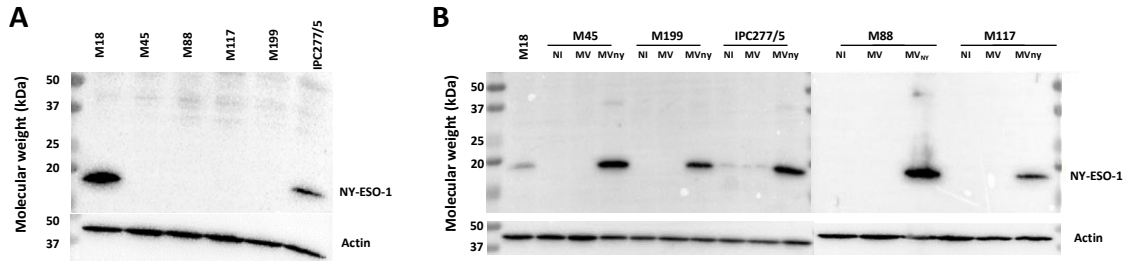


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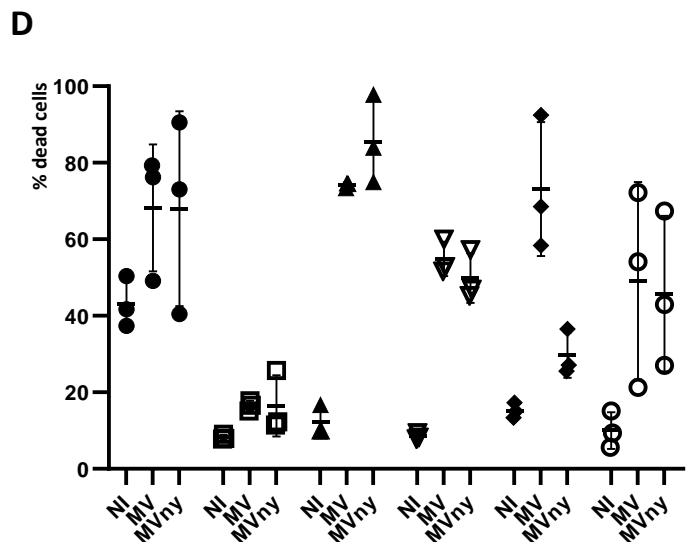
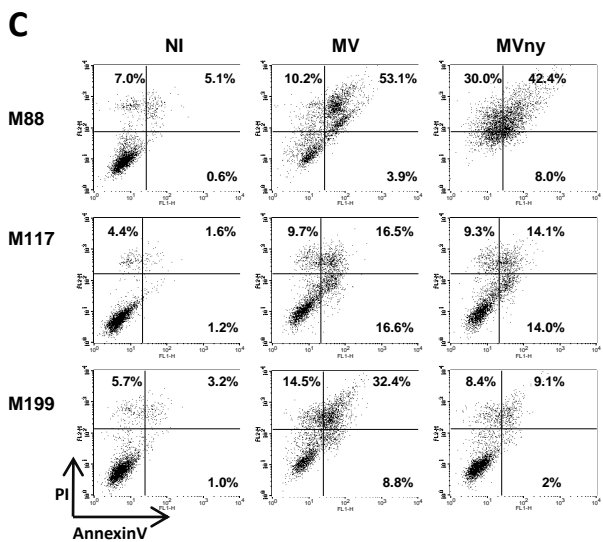
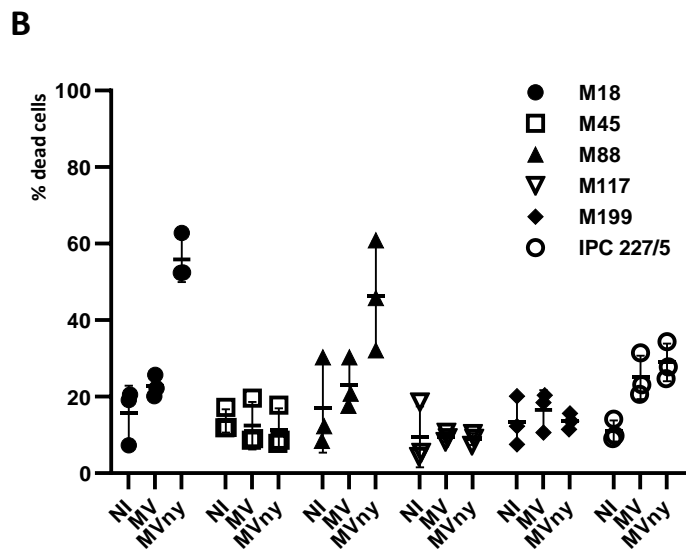
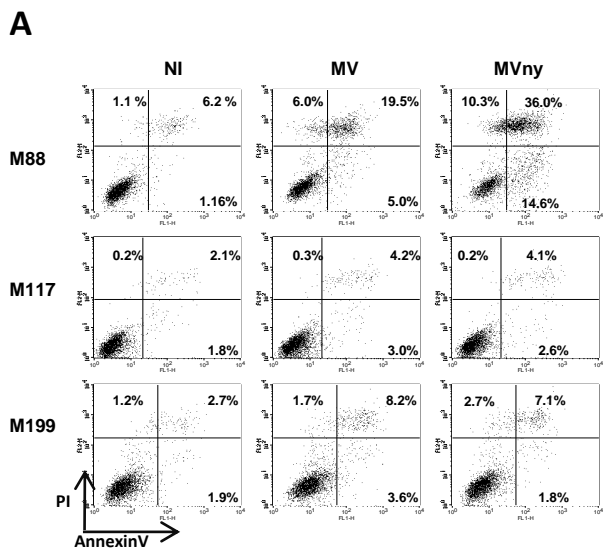




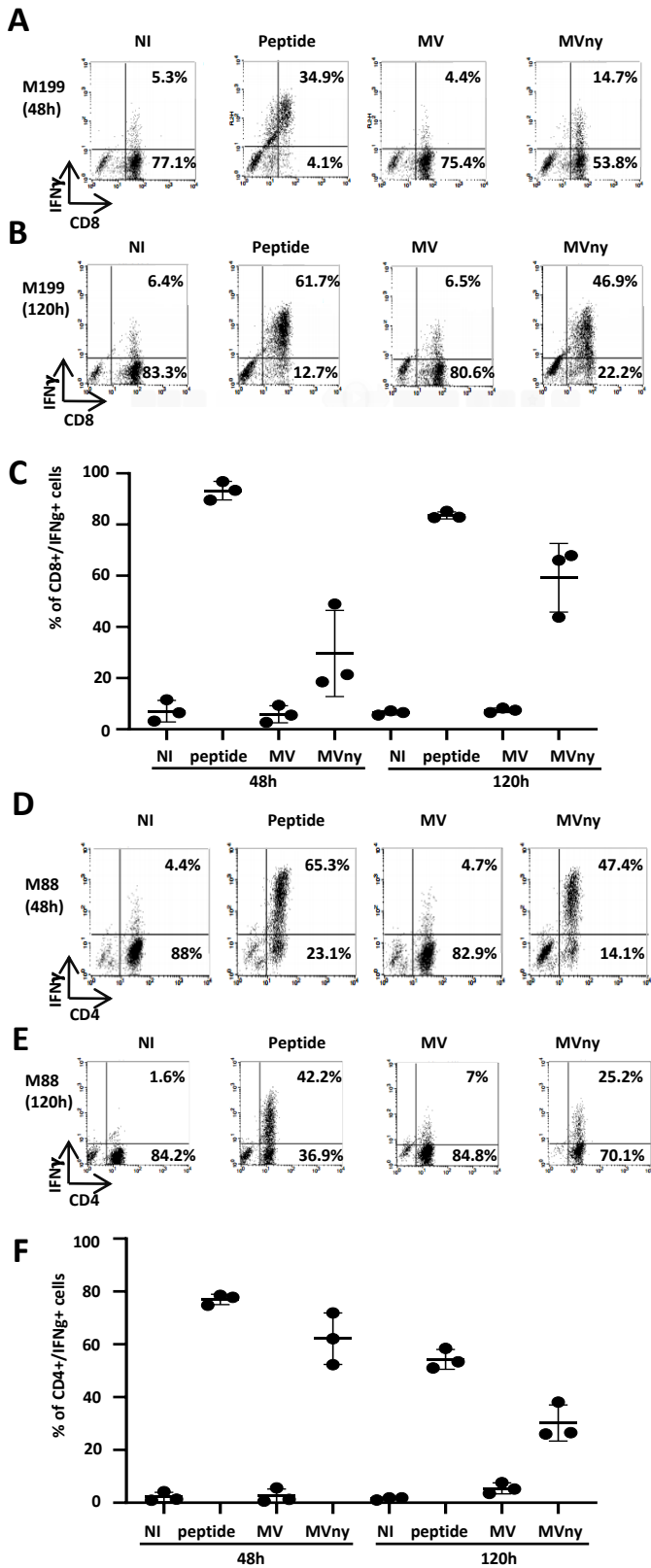
**Figure 2**



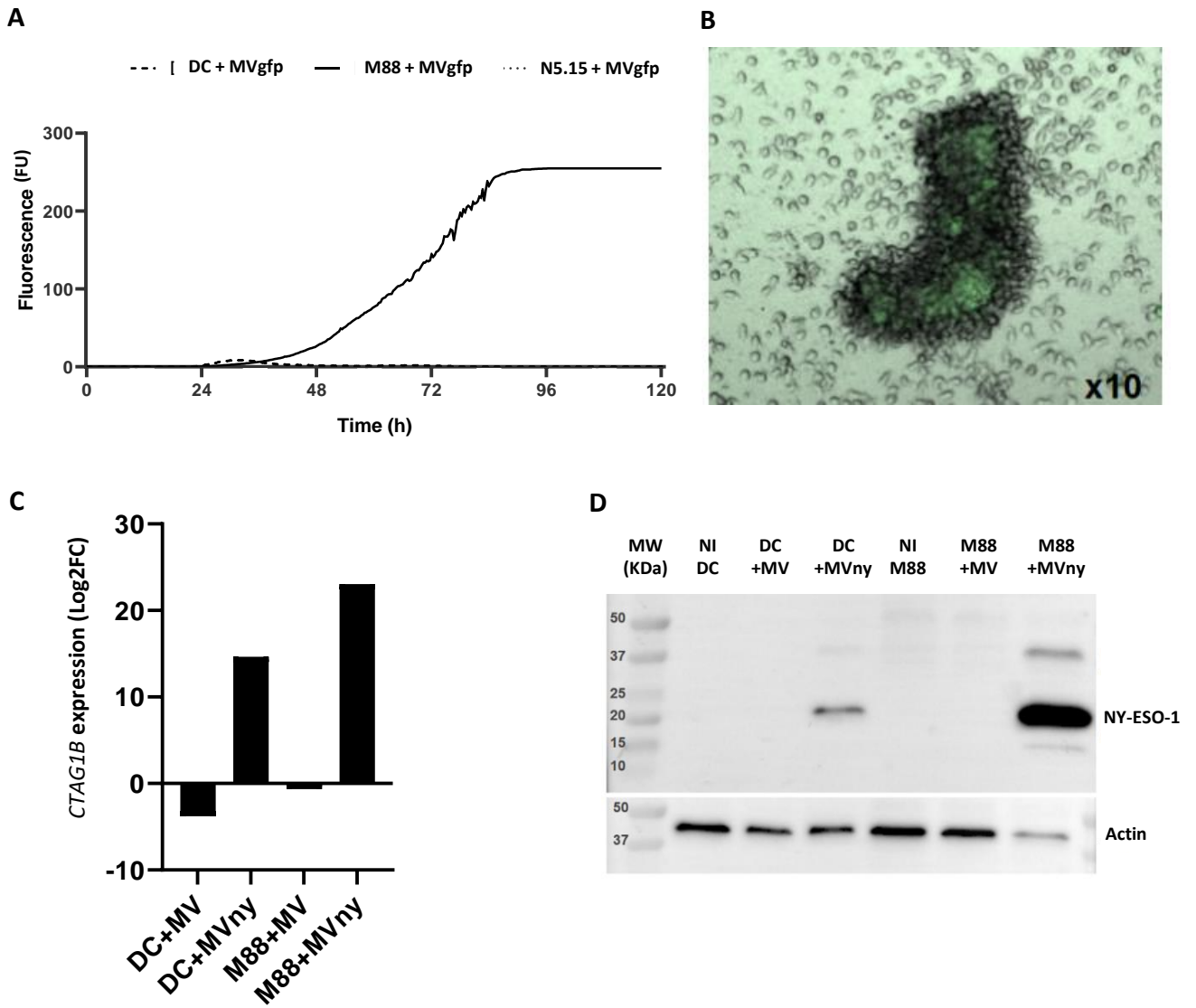
**Figure 3**



**Figure 4**



**Figure 5**



**Figure 6**

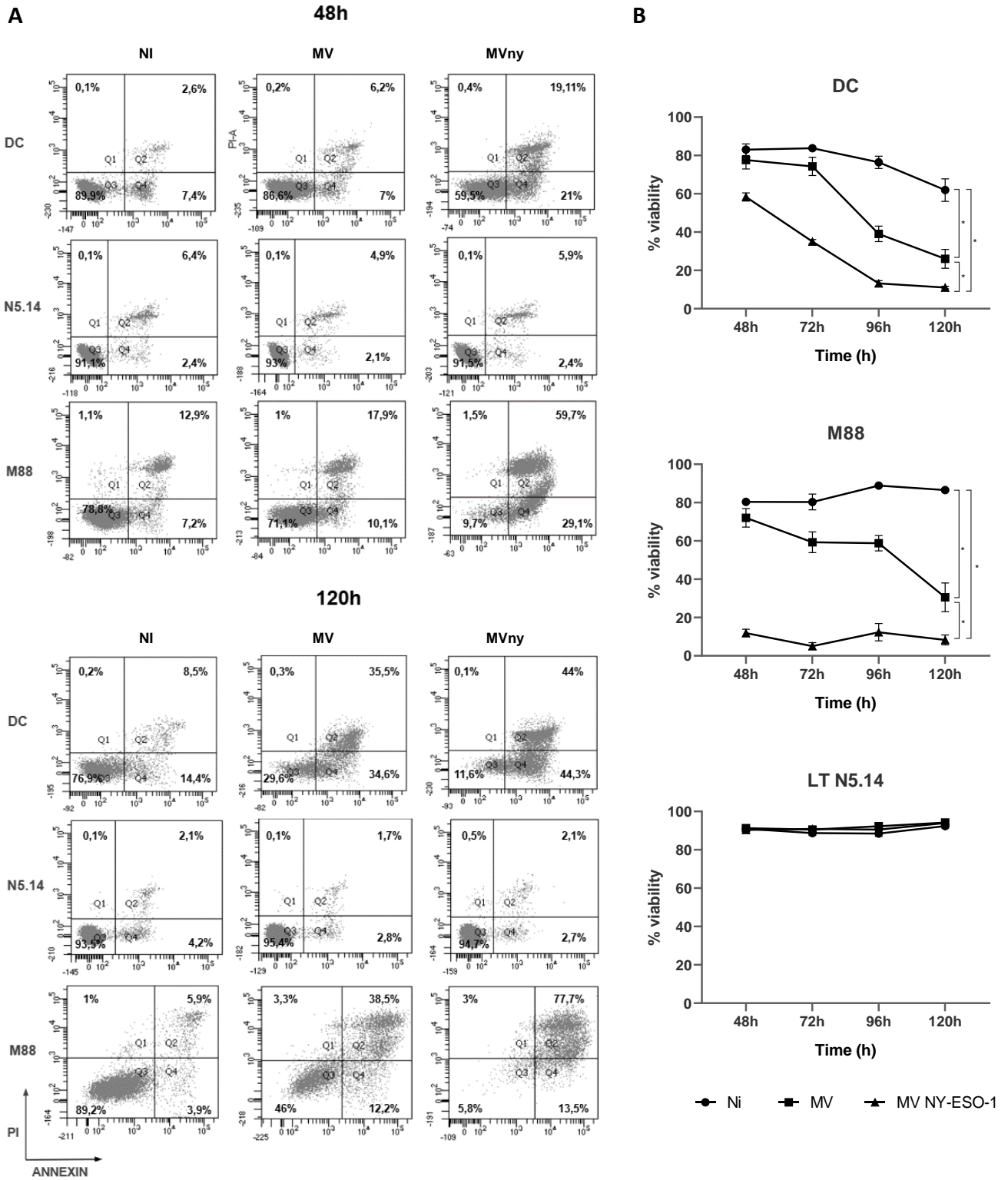
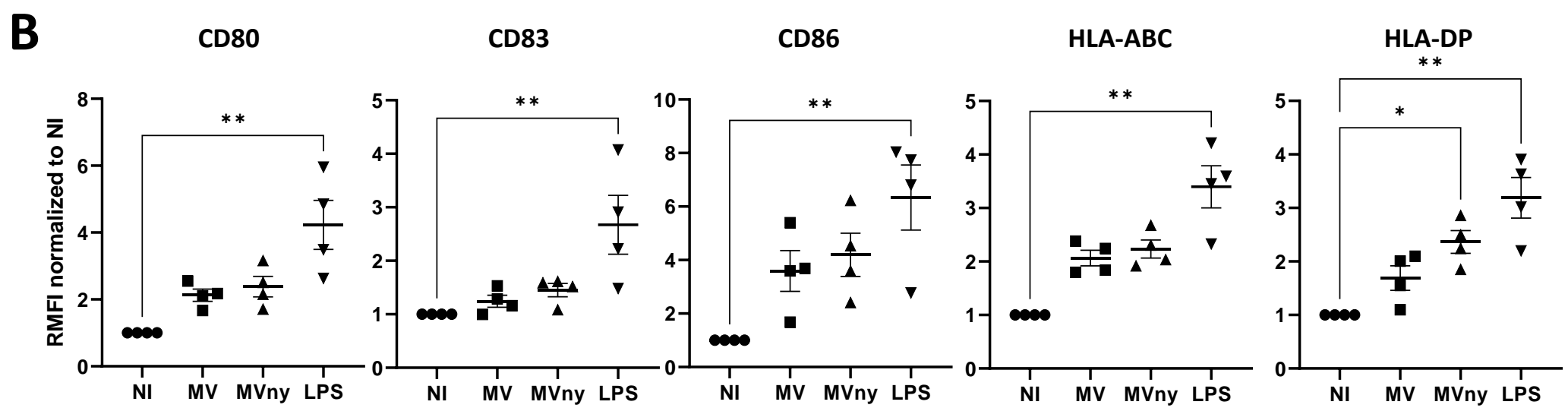
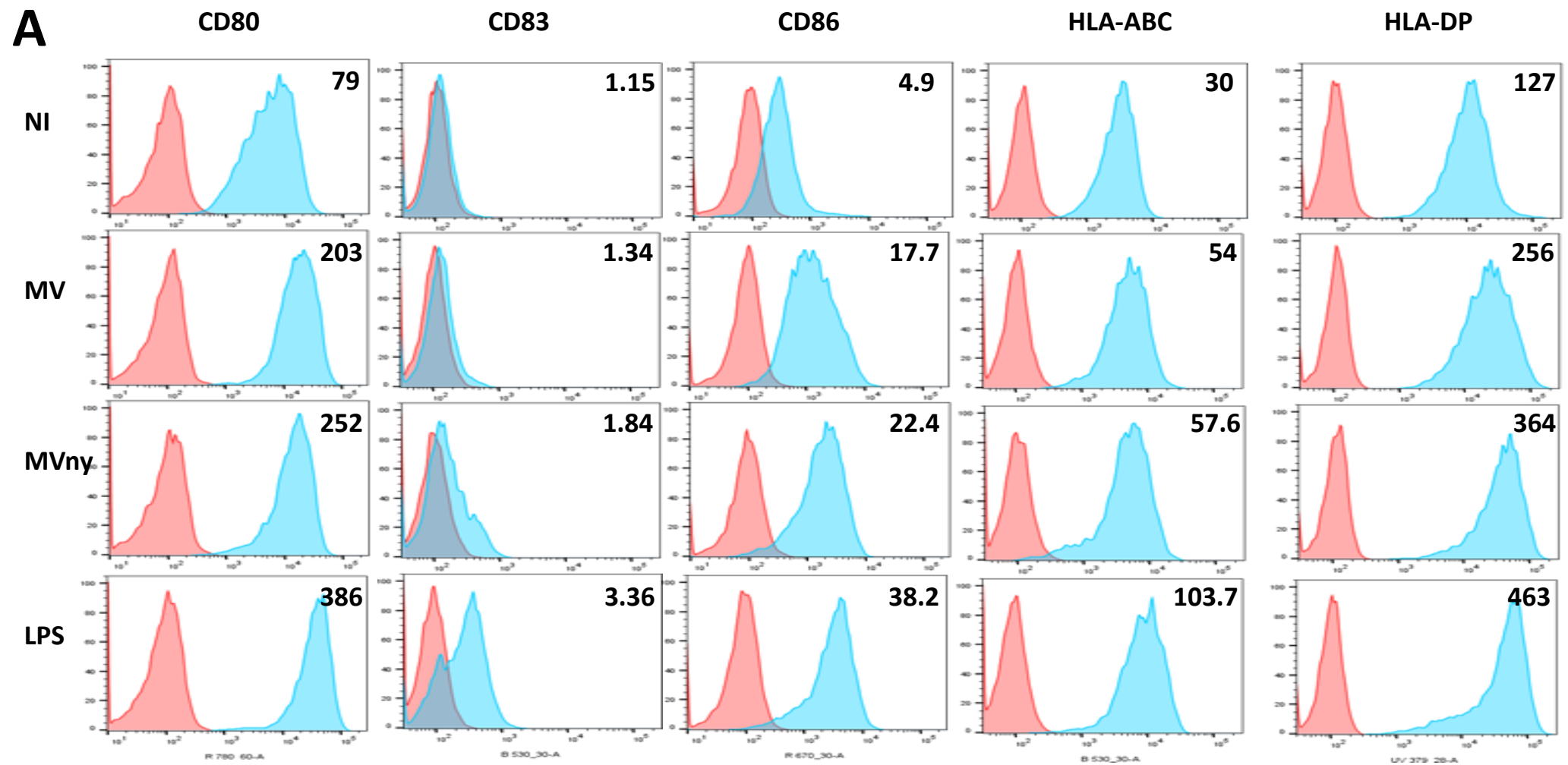
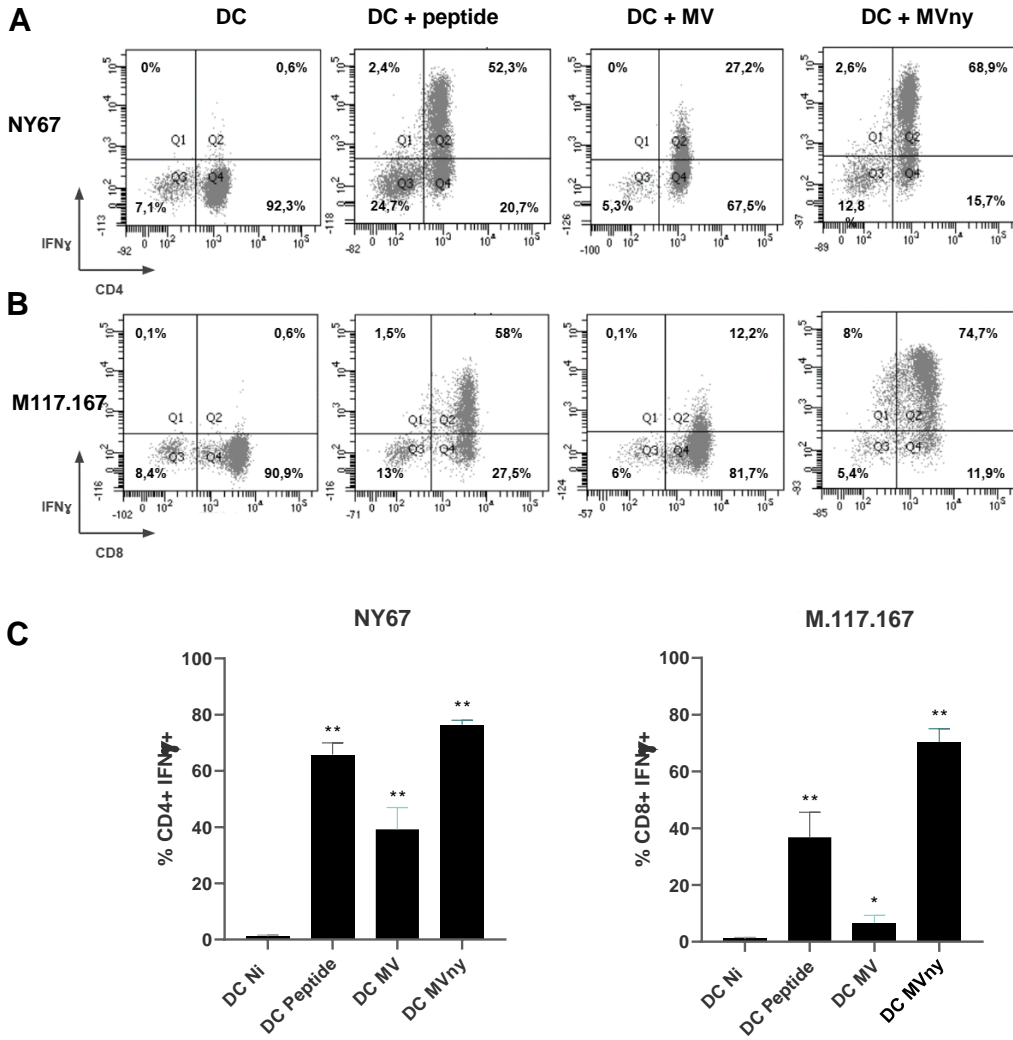
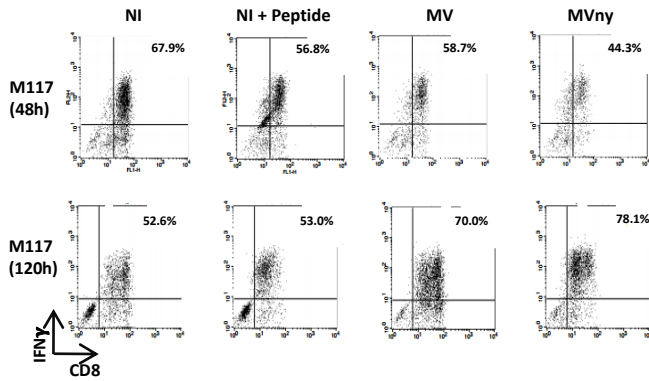
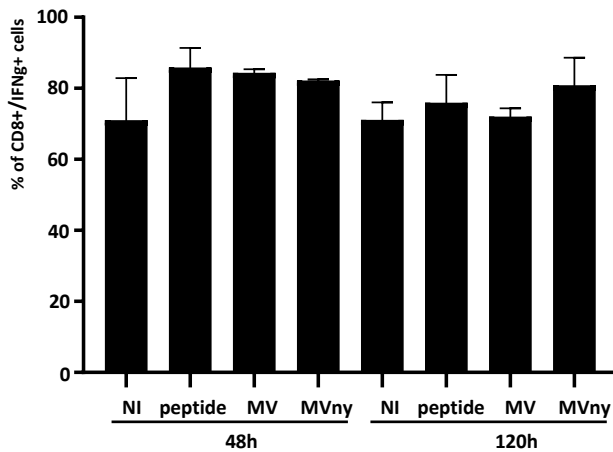
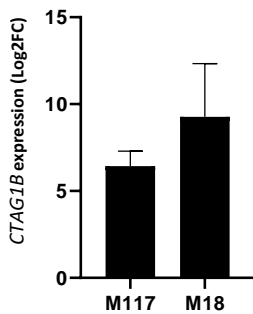


Figure 7



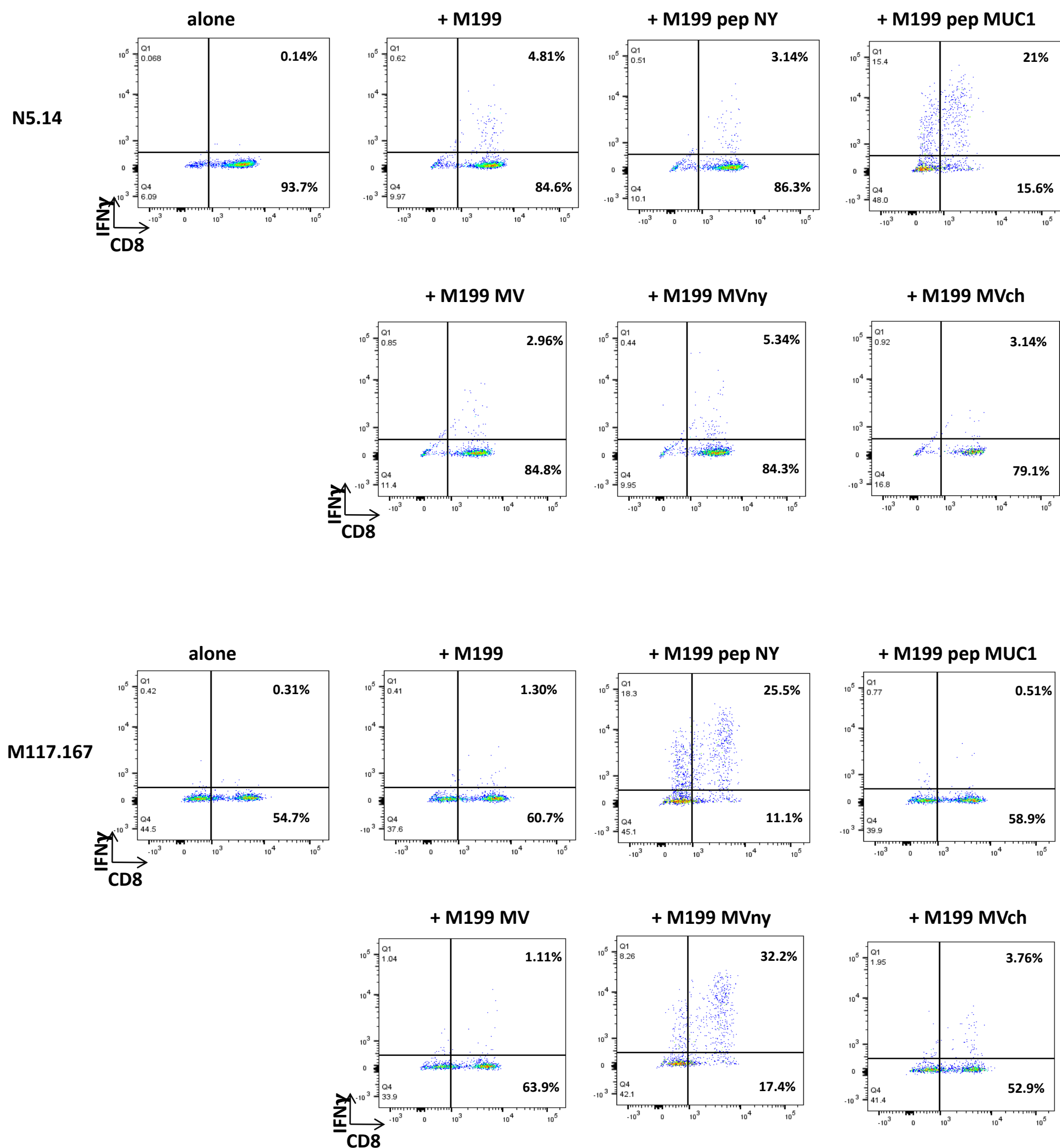
**Figure 8**



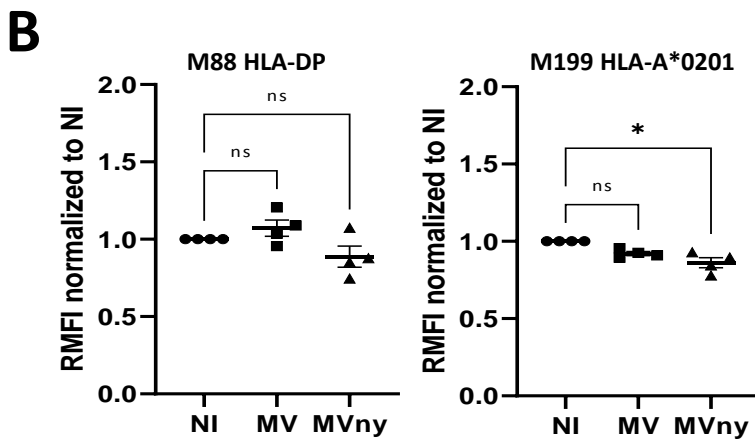
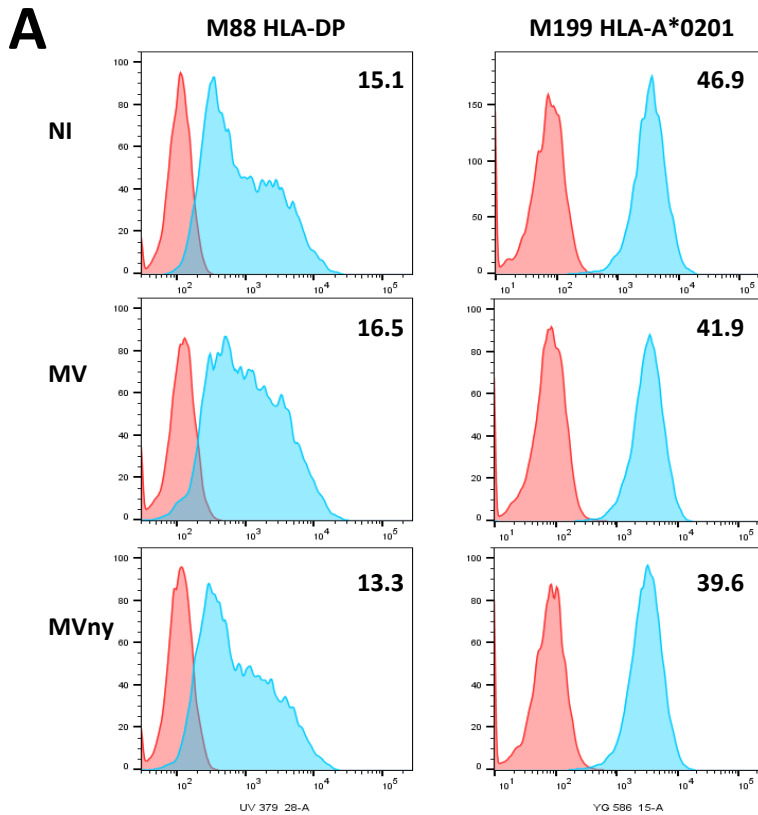
**A****B****C**

**Figure S1: MVny does not modify recognition of the autologous HLA-A\*0201+melanoma cell lines M117 by a NY-ESO-1 specific CD8+ T cell clone.** The HLA-A\*0201+melanoma cell lines M117 was cultured alone (NI) or with MV or MVny (MOI=1) during 48h or 120h. (A) Cells were collected and a part of M117 was pulsed with the peptide NY-ESO-1 (157-165) for one hour and washed. Cells were co-cultured with the CD8+ T cell clone M117.167 for 5h in presence of brefeldin A and then fixed stained for CD8 and IFN-g. (B) Results represents the mean with SD of three independent experiments. (C) NY-ESO-1 expression was measured in M18, M88 and M117 cell lines by RT-qPCR using RPLPO as a reference gene. M18 and M117 CTAG1B expression are expressed as log2 of fold change compared to M88 CTAG1B expression.

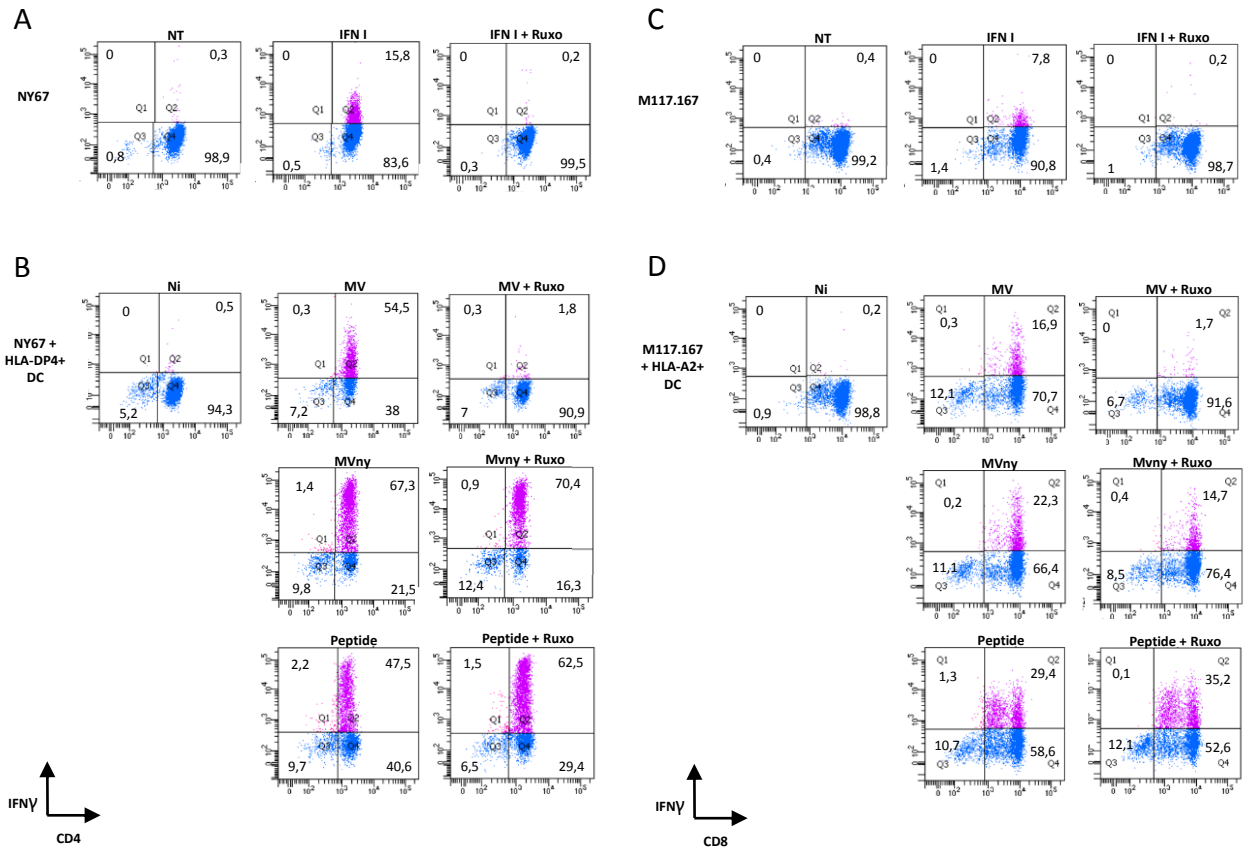




**Figure S2: *MVny does not induces the recognition of melanoma cell lines by a MUC1(950-956) specific CD8+ T cell clones and the NY-ESO-1 specific T cell clone does not recognize MV encoding cherry protein (MVch) infected tumor cell line.*** The HLA-A\*0201+ melanoma cell line M199 was cultured alone (NI), with MV, MVch or MVny (MOI=1) during 48h. Cells were collected and a part of M199 was pulsed with the NY-ESO-1 (157-165) or MUC1(950-959) peptide for one hour and washed. Cells were co-cultured with the CD8+ T cell clone M117.167 or N5.14 for 5h in presence of brefeldin A and then fixed and stained for CD8 and intracellular IFN-g. N5.14 clone is specific of HLA-A\*0201/MUC1(950-959) complex. Fluorescence was analyzed by flow cytometry.



**Figure S3: MVny does not modify HLA-DP and HLA-A\*0201 expression by M88 and M199 cell lines respectively.** M88 and M199 cell lines were cultured alone or with MV or MVny (MOI=1) for 48h. (A) Surface expressions of HLA-DP on M88 and HLA-A $\mu$ 0201 by M199 were measured by flow cytometry. RMFI is indicated on histograms. (B) Results (means + SD) of the relative mean of fluorescence intensity (RMFI) from four independent experiments. RMFI were obtained by the ratio of the mean fluorescence of cells stained by the specific mAb by the mean fluorescence of cells stained with the isotype control. RMFI were normalized to non-infected MFI. RMFI were normalized to non-infected cells RMFI.



**Figure S4: Type I interferons induced IFN $\gamma$  production by CD4 $^{+}$  and CD8 $^{+}$  T cell clones.** (A) NY67, the NY-ESO-1-specific CD4 $^{+}$  T cell clone was treated or not (NT) with IFN- $\alpha$ 2a (1000IU/mL) and IFN- $\beta$ 1 (1000IU/mL) combined or not with Ruxolitinib (2 $\mu$ M) during 5h. Cells were fixed permeabilized and stained with fluorescent conjugated anti-CD4 and anti-IFN $\gamma$  specific monoclonal antibodies. Fluorescence was analysed by flow cytometry. (B) HLA-DP4 $^{+}$  DCs were uninfected (Ni), or infected with MV or MVny for 48h (MOI 1). A part of Ni DC were pulsed with 10 $\mu$ M of NY-ESO-1 peptide. NY67 was then cultured with these DCs for 5h in presence of Ruxolitinib (2 $\mu$ M), a JAK1/2 inhibitor that inhibits IFNAR signalling and brefeldin A. Cells were fixed, permeabilized and stained with fluorescent conjugated anti-CD4 and anti-IFN- $\gamma$  specific monoclonal antibodies. Fluorescence was analysed by flow cytometry. (C) M117.167, the NY-ESO-1-specific CD8 $^{+}$  T cell clone was treated or not (NT) with IFN $\alpha$ 2a (1000IU/mL) and IFN $\beta$ 1 (1000IU/mL) combined or not with Ruxolitinib (2 $\mu$ M) during 5h. Cells were fixed permeabilized and stained with fluorescent conjugated anti-CD8 and anti-IFN $\gamma$  specific monoclonal antibodies. Fluorescence was analysed by flow cytometry. (D) HLA-A2 $^{+}$  DCs were uninfected (Ni), or infected with MV or MVny for 48h (MOI 1). A part of Ni DC were pulsed with 10 $\mu$ M of NY-ESO-1 peptide. M117.167 was then cultured with these DCs for 5h in presence of Ruxolitinib (2 $\mu$ M), a JAK1/2 inhibitor that inhibits IFNAR signalling and brefeldin A. Cells were fixed, permeabilized and stained with fluorescent conjugated anti-CD8 and anti-IFN- $\gamma$  specific monoclonal antibodies. Fluorescence was analysed by flow cytometry.