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**High oncolytic activity of a double deleted Vaccinia Virus Copenhagen strain against malignant pleural mesothelioma**

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**Key words:** Oncolytic immunotherapy, vaccinia virus, Pleural mesothelioma, thymidine kinase, ribonucleotide reductase

\* T.D and J.N. equally contributed to this work.

## **Abstract**

Malignant pleural mesothelioma (MPM) is a cancer of the pleura that lacks efficient treatment. Oncolytic immunotherapy using oncolytic vaccinia virus (VV) may represent an alternative therapeutic approach for the treatment of this malignancy. Here we studied the oncolytic activity of *VVTK-RR-/GFP* against MPM. This virus is a VV from the Copenhagen strain that is deleted of two genes encoding the thymidine kinase (*J2R*) and the ribonucleotide reductase (*I4L*) and that express the green fluorescent protein (GFP). First we show *in vitro* that *VVTK-RR-/GFP* efficiently infects and kills the twenty two human MPM cell lines used in this study. We also show that the virus replicates in all eight tested MPM cell lines, however with approximately a 10-fold differences in the amplification level from one cell line to another. Then we studied the therapeutic efficiency of *VVTK-RR-/GFP* in NOD Scid mice that bear peritoneal human MPM tumors. One intraperitoneal infection of *VVTK-RR-/GFP* reduces the tumor burden and significantly increases mice survival compared to untreated animals. Thus *VVTK-RR-* may be a promising OV for the oncolytic immunotherapy of MPM.

## Introduction

Malignant pleural mesothelioma (MPM) is an aggressive tumor of the pleura, usually associated with chronic asbestos exposure, mainly during occupational activities. Incidence is increasing and is expected to peak around the year 2020 in western world, and to continue to rise in developing countries <sup>1</sup>. Clinical treatments for MPM including chemotherapy, radiotherapy and surgery, are of limited efficacy. There is an urgent need of new therapeutic approaches.

Oncolytic virotherapy is a therapeutic strategy that is developing rapidly and meets recently its first success with approval of T-vec for treatment of metastatic melanoma <sup>2</sup>. It consists in using oncolytic viruses (OV) that exclusively or preferentially replicate in tumor cells inducing their immunogenic cell death. Several oncolytic vaccinia viruses (VV) are now evaluated in clinical trials for the treatment of different types of cancer <sup>3</sup>. For mesothelioma, different oncolytic VV with inactivation of the thymidine kinase gene (TK) have been studied <sup>4-9</sup>. These virus exerts oncolytic activities due to the high expression of *TK* in proliferating tumor cells that allows viral replication. TG6002 is a VV from the Copenhagen strain that is deleted of the *TK* and the ribonucleotide reductase (*RR*) genes and expresses the suicide gene *FCUI* <sup>10</sup>. As TK, RR is a key enzyme in the supply chain of deoxyribonucleoside triphosphates (dNTPs) for DNA replication and is often overexpressed in cancer <sup>11</sup>. Thus, TG6002 replicates preferentially in tumor cells due to their high TK and RR activities. This OV in combination with 5-fluorocytosine is entering in phase I/II clinical trial for the treatment of recurrent glioblastoma (clinicaltrials.gov: NCT03294486) and gastro intestinal cancer (clinicaltrials.gov: NCT03724071 and NCT04194034).

In this work, we studied a variant of TG6002 that does not contain the *FCUI* gene, but instead the gene encoding Green Fluorescent Protein: *VVTK-RR-/GFP* allowing a monitoring of the viral replication. We measured *in vitro* the oncolytic activity of this OV against a large

panel of 22 MPM human MPM tumor cell lines. We show that they are all lysed by VVTK-RR-*/GFP*, despite differences in the level of viral replication between tumor cell lines. We also show that VVTK-RR-*/GFP* is able to reduce *in vivo* the tumor burden and to increase significantly the survival of NOD Scid Mice bearing peritoneal human MPM tumors. Overall our work shows that TG6002 may be an interesting OV for the treatment of MPM.

## Results

*MPM cell lines are highly sensitive to the oncolytic activity of VVTK-RR-/GFP in vitro.*

First we assessed *in vitro* the oncolytic activity of the VVTK-RR-/GFP against 22 human MPM cell lines established from patient pleural effusion. The GFP gene is under the control of a promoter active during the early and late phase of the viral replication and allow to follow the infection during 4 days by fluorescent video microscopy (Figure 1A and supplemental video 1 to 8). We also measured the fluorescence by flow cytometry 24h after the infection (Figure 1B and 1C).

We found that all MPM cell lines were sensitive to the infection by VVTK-RR-/GFP. The first wave of green fluorescent tumor cells usually appears between 12h to 15h after the start of the infection. After 24h, the infection spreads to other cells with the apparition of a second wave of green fluorescent tumor cells. Between 24h and 48h after the infection, mobility of tumor cells decreases. This slowing down is quite impressive with Meso11, Meso13 and Meso163 MPM cell lines (supplemental video 2, 3 and 6). After 48h, tumor cells detached from the plastic support and get a round shape probably due to apoptosis induction. Tumor cells then start to burst. Ruptures of the cell membrane provoke rapid loss of the green fluorescence. This secondary necrosis is more or less rapid with cell lines like Meso13 that have lost all the fluorescence by 96h and cell lines that take more time to burst such as Meso34 (supplemental videos 3 and 4). After 4 days, all the 22 MPM cell lines infected by VVTK-RR-/GFP exhibit morbidity that lead to cell death in a few more days.

During the infection, the level of maximum fluorescence varied from one cell lines to another. For instance Meso225 and Meso173 failed to reach high level of fluorescence, whereas Meso13 and Meso34 reach high level of fluorescence (Figure 1B and supplemental videos 3, 4, 7 and 8). Altogether, these results show that human MPM is highly sensitive to VVTK-RR-/GFP infection.

Then we studied the induction of cell death by *VVTK-RR-/GFP* on the 22 MPM cell lines by annexin V-APC/ IP staining 48h after infection. The GFP fluorescence from the infected cells was so strong that it interferes with the IP staining and could not be compensated. However we observed a significant annexin V staining on the 22 MPM cell lines in the presence of *VVTK-RR-/GFP* (figure 2). It confirms the observations made by video microscopy (supplemental videos 1-8) that all tested human MPM cell lines are sensitive to the oncolytic activity of *VVTK-RR-/GFP*.

Then, we measured the production of *VVTK-RR-/GFP* in 8 MPM cell lines 48h post-infection. We were able to detect replication of the virus in all MPM cell lines tested (Figure 3A), especially at the lowest MOI (0.01). Replication was low in three MPM cell lines (Meso11, 163 and 225), medium in three others (Meso13, 45 and 173) and high in the last two (Meso4 and 34). Replication was higher at the lowest MOI (0.01) since it allows more wave of infections than the highest MOI (0.1) that exert a cytopathic effects (Figure 3B). Thus, we found production of infectious viral particles in the 8 tested MPM cell lines highlighting the capacity of this virus to replicate in MPM cells.

#### *In vivo oncolytic activity of VVTK-RR-/GFP.*

To confirm our *in vitro* observations regarding oncolytic activity of *VVTK-RR-/GFP*, we evaluated the virus for the treatment of NOD Scid mice that bear intraperitoneal human Meso163 tumor (Figure 4). In a first experiment on a small number of NOD Scid mice (n=3), we determined that the injection of  $1.10^7$  PFU was not toxic, whereas  $1.10^8$  PFU was lethal (data not shown). In a second experiment, we observed that the intraperitoneal injection of *VVTK-RR-/GFP* to NOD Scid mice that bear Meso163 tumor cells significantly increased the mean survival (MS) compared to mice that received PBS (MS PBS=33days; MS *VVTK-RR-/GFP*=55days; p value=0,0001 (Mantel-cox)) (Figure 4A).

Mice were dissected to evaluate the tumor burden (Figure 4B). Mice that received PBS exhibit numerous metastasis on the abdominal muscular layer, the omentum, the mesentery, the stomach, the peritoneum and the diaphragm. In *VVTK-RR-/GFP* treated animals, we observed not more than three massive tumors that often develop in the omentum and/or near the sternum in all animals. This results suggest that *VVTK-RR-/GFP* succeed to eradicate a lot of metastasis, but failed to control a very small number of tumors that may be the primary tumors.



## Discussion

Altogether these results show that all human MPM cell lines are highly sensitive to the oncolytic activity of *VVTK-RR-/GFP* from the Copenhagen strain. In vitro, the virus is able to replicate and induces cell death that ends up into secondary necrosis. Another study addressed the in vitro sensitivity of six human MPM cell lines to the GLV-1h68, an oncolytic VV from the Lister strain, that is deleted of TK <sup>6</sup>. It reported that all MPM cell lines were sensitive. In a previous study, Mukherjee et al showed that two human MPM cell lines were sensitive to the NYCBOH strain of VV deleted of TK and encoding IL-2 <sup>5</sup>. Herein, we show that 22 out of 22 human MPM cell lines are sensitive. This result also suggests that antiviral type I interferon (IFN I) response seems to fail to protect tumor cells from *VVTK-RR-/GFP* replication. Indeed, we recently showed on the same 22 human MPM cell lines that 15 exhibit defects in the IFN I response that makes them sensitive to oncolytic attenuated measles virus <sup>12</sup>. 7 MPM cell lines were able to control measles virus replication by a functional IFN I response. Among these 7 MPM cell lines, by the titration experiment, we showed that Meso4, 45 and 173 are not able to control *VVTK-RR-/GFP* replication. Thus, either *VVTK-RR-/GFP* replication is not sensitive to the type I IFN response due to virulence factor such as B18R that are able to block the type I IFN response <sup>13</sup>, or there is a failure to detect DNA viruses and thus the *VVTK-RR-/GFP* by these cell lines to induce a type I IFN response.

We also show in this study that a single intraperitoneal injection of *VVTK-RR-/GFP* to immunodeficient mice engrafted with a human MPM cell line increases significantly their survival and reduce the spreading of metastasis. Therapeutic benefits of different VV deleted of the *TK* gene have been reported in human mesothelioma xenograft in mouse models <sup>6, 8</sup>, as well as in syngeneic mesothelioma mouse model <sup>14-16</sup>. The failure of *VVTK-RR-/GFP* to totally cure the mice in our study may be due to the absence of an adaptive immune system that should enhance the oncolytic efficacy by the induction of an antitumor immune response. Indeed, it

has been shown that oncolytic VV induce an immunogenic cell death that allows antigen presenting cells to prime naïve T cell to develop an antitumor immune response <sup>17, 18</sup>. We also reported recently that *VVTK-RR-/GFP* can increase effector cytotoxic CD4+ T cell activation by increasing the HLA class II tumor antigen presentation by tumor cells <sup>19</sup>.

Phase I clinical trials of mesothelioma treatment by oncolytic VV deleted of the *TK* gene and encoding IL-2 have demonstrated that this approach is not toxic, but lack of therapeutic efficacy <sup>4,5</sup>. Other modifications of oncolytic VV may be necessary to increase its efficacy such as the addition of gene encoding CXCL11 <sup>14, 15</sup>. Furthermore, vaccinia viruses can be armed with the suicide gene *FCUI* encoding an enzyme that transforms locally in the tumor the prodrug 5-fluorocytosine into the toxic drug 5-fluorouracile <sup>10, 20</sup>. The virus *VVTK-RR-Fcui+* (TG6002) is now evaluated in phase I/II clinical trial for the treatment of gastro-intestinal cancers in combination with 5-fluorocytosine (Clinicaltrials.gov NCT03724071 and NCT04194034) and of recurrent Glioblastoma (NCT03294486). Given that MPM is resistant to all conventional treatments, oncolytic VV may represent a promising therapeutic approach.

## **Material and methods**

### *Cell Culture*

The 22 human MPM cell lines (from Meso4 to Meso225) were established in our laboratory from pleural effusions collected by thoracocentesis, and genetically characterized <sup>21</sup>. All patients gave their informed consent. All cell lines were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100U/mL penicillin, 100µg/mL streptomycin and 2mM L-glutamine (all reagents from Gibco-Invitrogen) and cultured at 37°C in a 5% CO<sub>2</sub> atmosphere. Cells were routinely checked for Mycoplasma contamination using the Plasmotest™ from InvivoGen.

### *Vaccinia Virus VVTK-RR-/GFP*

Attenuated recombinant vaccinia virus *VVTK-RR-/GFP* was derived from the Copenhagen strain and was deleted in the thymidine kinase (TK) and ribonucleotide reductase (RR) genes and expressed the green fluorescent protein (GFP). GFP is under the control of the p11k7.5 early-late promoter <sup>20</sup>. *VVTK-RR-/GFP* was propagated and titrated in chicken embryo fibroblasts as previously described <sup>22</sup>.

### *Video microscopy*

One day before infection, MPM cells were seeded in 24-well plates, at a density of 10<sup>5</sup> cells/well. Cells were then infected with *VVTK-RR-/GFP* (MOI=0.1). The time-lapse video microscopy was made using a Leica DMI6000B microscope with a 10x objective. Images were acquired every 15min for 4 days. We used MetaMorph® Microscopy Automation & Image Analysis Software (version 7.8) and Fiji Software for acquisition and analysis <sup>23</sup>.

### *Measurement of MPM cell line infection by VVTK-RR-/GFP*

MPM cell lines were seeded in 12-well plates at a density of  $0.2 \times 10^6$  cells/well in 2ml of culture medium containing VVTK-RR-/GFP at MOI of 0.01 or 0.1. After 24h of culture, cells were harvested for GFP fluorescence measurement. Cells were fixed with phosphate saline buffer (PBS) containing 1% paraformaldehyde during 10min at room temperature and washed with PBS. GFP fluorescence was measured by flow cytometry on a FACS Calibur (BD Biosciences) and analyzed using the BD CellQuest™ Pro software.

### *Measurement of MPM cell death*

To measure MPM cell death induced by VVTK-RR-/GFP, MPM cell lines were cultured and exposed to VVTK-RR-/GFP as for the measurement of infection above. After 48h, some supernatants were collected for measurement of VVTK-RR-/GFP replication below and cells were harvested and labeled with an annexin V-APC/ propidium iodide (IP) labeling kit (BD Pharmingen) according to manufacturer instructions. Cells were then fixed with PBS 1% paraformaldehyde. Annexin V-APC fluorescence was analyzed by flow cytometry as above.

### *Measurement of VVTK-RR-/GFP replication in MPM cell lines*

Plaque-forming unit (PFU) assay was performed on supernatants and cells collected from the 48h cultures of MPM cell lines with VVTK-RR-/GFP as described<sup>22</sup>. The VVTK-RR-/GFP amplification factor was determined by dividing the PFU found in the supernatants by the PFU that were added at the start of the culture.

### *VVTK-RR-/GFP treatment of NOD scid Mice bearing Human MPM Meso163 cell line xenograft*

All *in vivo* experiments complied with European Union regulations on the welfare and use of animals in cancer research. Six week-old female NOD Scid mice were purchased from Centre d'Elevage Janvier (Le Genest-Saint-Isle, France). Twenty mice were challenged intraperitoneally with  $5 \times 10^6$  tumor cells. After 3 weeks, 100 $\mu$ L of PBS were injected in the intraperitoneal cavity of half of the mice. The others received 100 $\mu$ L of PBS containing  $1.10^7$  PFU of VVTK-RR-/GFP intraperitoneally. Mice were followed at least every two days and sacrificed when they became moribund (weight loss, decreased mobility). Mice were then dissected and pictured.

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## Figure Legends

**Figure 1: human MPM cell lines are sensitive to VVTK-RR-/GFP infection.** (A) Meso4, 11, 13, 34, 45, 163, 173 and 225 human MPM cell lines were cultured with VVTK-RR-/GFP at MOI of 0.1 during 96h. Pictures were taken at 0h, 6h, 12h, 24h, 36h, 48h, 60h, 72h and 96h. (B) Meso4, 11, 13, 34, 45, 163, 173 and 225 human MPM cell lines were cultured alone or with VVTK-RR-/GFP at an MOI of 0.01 or 0.1 during 24h. GFP fluorescence was analyzed by flow cytometry. (C) 22 human MPM cell lines were cultured with VVTK-RR-/GFP at MOI of 0.01 and 0.1 during 24h. GFP fluorescence was analyzed by flow cytometry. Histogram represents means  $\pm$  SEM of three independent experiments.

**Figure 2: human MPM cell lines are sensitive to VVTK-RR-/GFP oncolytic activity.** (A) Meso4, 11, 13, 34, 45, 163, 173 and 225 human MPM cell lines were cultured alone or with VVTK-RR-/GFP at MOI of 0.1 during 48h. Cells were labeled with annexin V-APC and propidium iodide. Annexin V-APC fluorescence was analyzed by flow cytometry. Grey histograms represent cells cultured alone and white histogram cells cultured with VVTK-RR-/GFP. (B) 22 human MPM cell lines were cultured with VVTK-RR-/GFP at MOI of 0.01 and 0.1 during 48h. Cells were labeled with annexin V-APC and propidium iodide. Annexin V-APC fluorescence was analyzed by flow cytometry. Histogram represents means  $\pm$  SEM of three independent experiments.

**Figure 3: human MPM cell lines are permissive to VVTK-RR-/GFP replication.** Meso4, 11, 13, 34, 45, 163, 173 and 225 human MPM cell lines were cultured with VVTK-RR-/GFP at MOI of 0.01 and 0.1 during 48h. Culture supernatants and cells were then collected and presence of VVTK-RR-/GFP infectious particle was measured by PFU assay. (A) Histogram represents the total PFU found in cells and supernatants. (B) Histogram represents the

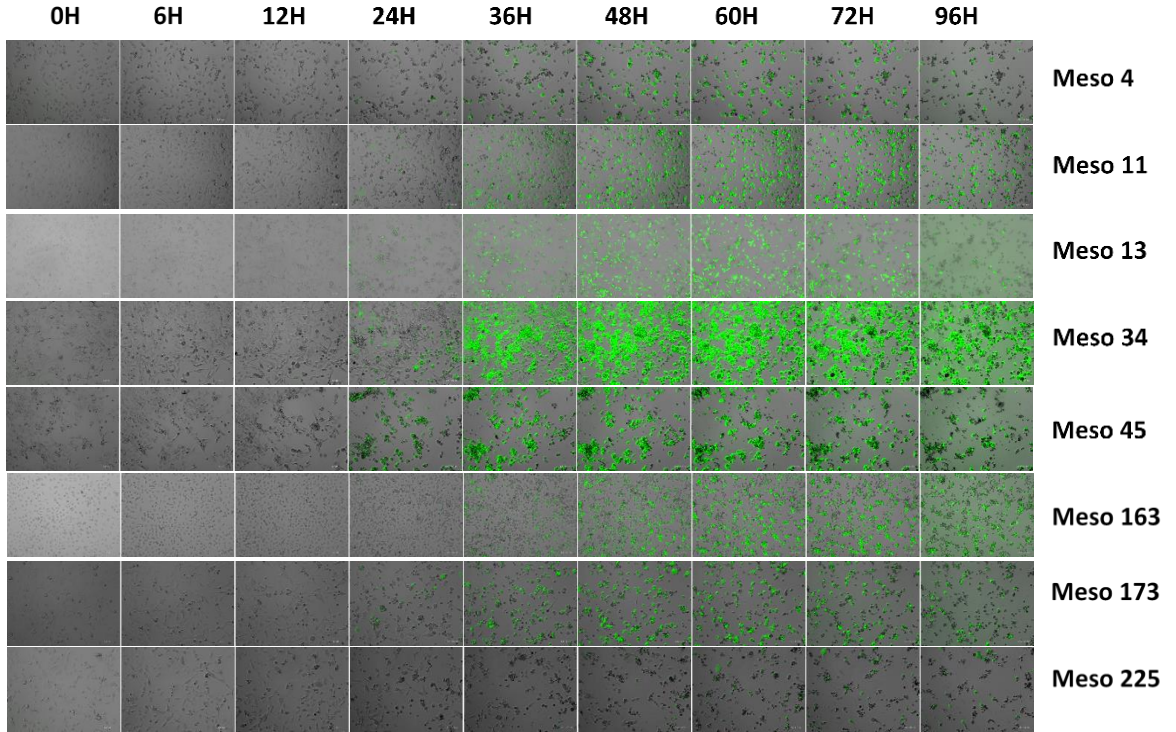
amplification factor determined by dividing the total produced PFU by the PFU used at the start of the experiments.

**Figure 4: one intraperitoneal injection of VVTK-RR-/GFP increases survival of NOD scid mice engrafted with the human MPM cell line Meso163 and decreases tumors formations.**

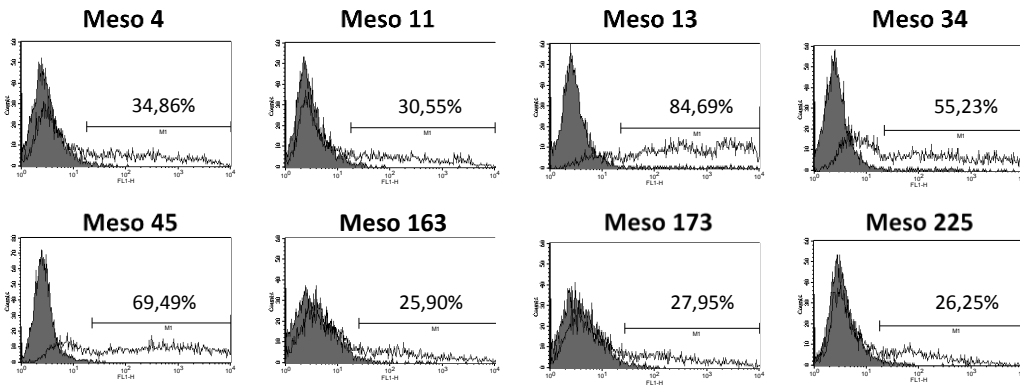
NOD scid mice were challenged intraperitoneally with  $5 \cdot 10^6$  Meso163 human MPM cells. After 3 weeks, at day 0, PBS or VVTK-RR-/GFP ( $1 \cdot 10^7$  PFU) was injected intraperitoneally. (A) Graph represents survival of NOD scid mice. (B) Autopsies of a representative NOD scid mice engrafted with the human MPM cell line Meso163 and treated with PBS or VVTK-RR-/GFP. Arrows indicates tumors.

**Figure 1**

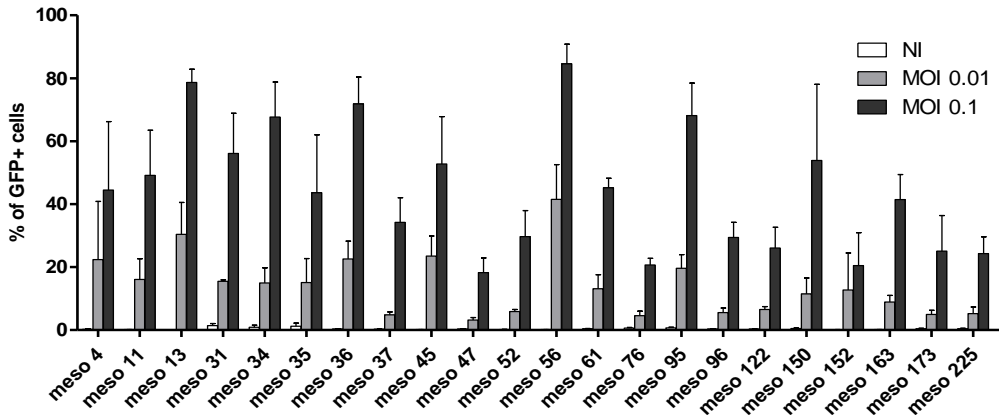
**A**



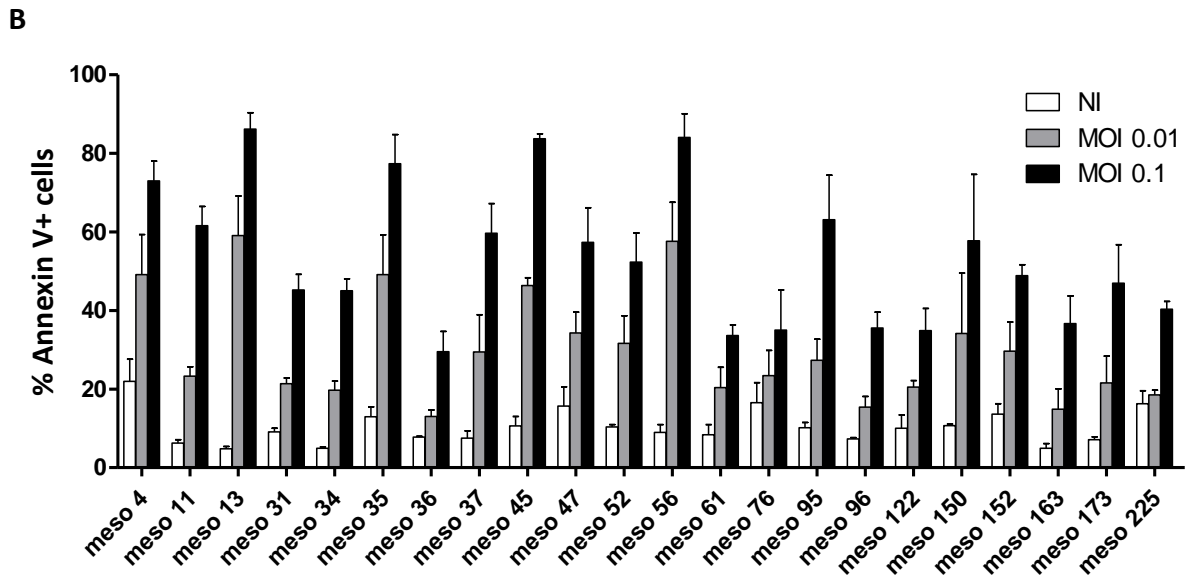
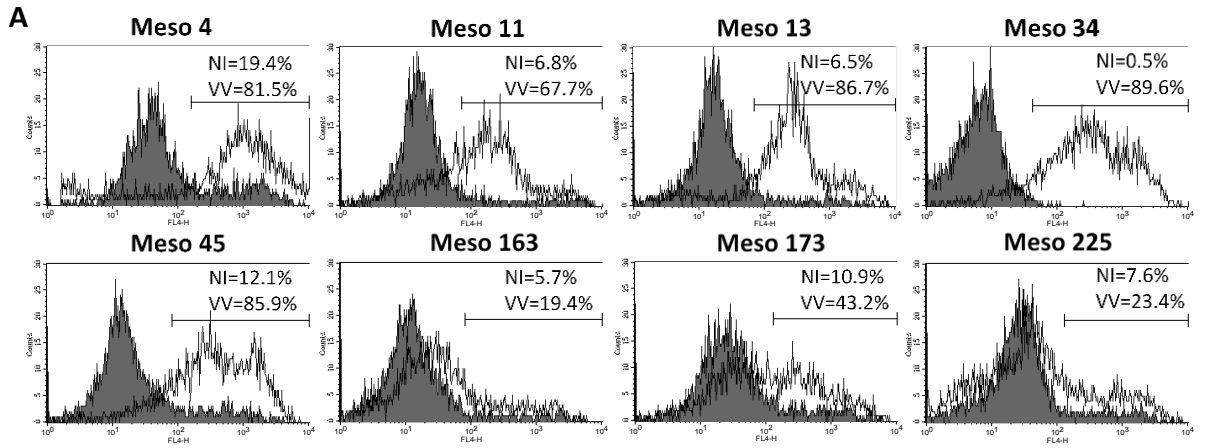
**B**



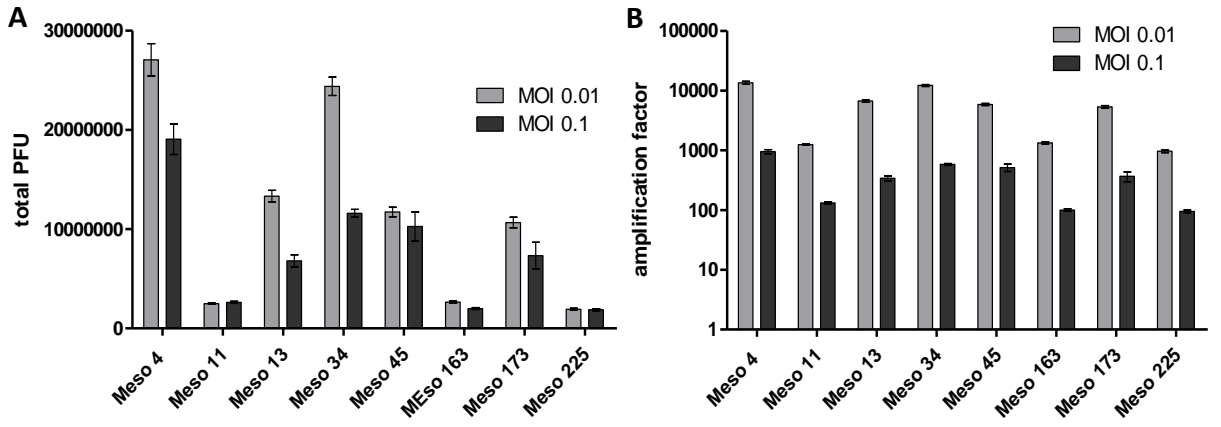
**C**



**Figure 2**



**Figure 3**



**Figure 4**

