UNIVERSITÀ DI PARMA

UNIVERSITA' DEGLI STUDI DI PARMA

DOTTORATO DI RICERCA IN

" Scienze Mediche e Chirurgiche Traslazionali "

CICLO XXXIV

Use of bovine viruses as alternative tools in multiple myeloma oncolytic virotherapy

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Anni Accademici 2018/2019 – 2021/2022

Part of this study has been published:

Marchica V, Franceschi V, Vescovini R, Storti P, Vicario E, Toscani D, Zorzoli A, Airoldi I, Dalla Palma B, Campanini N, Martella E, Mancini C, Costa F, Donofrio G, Giuliani N.

Bovine pestivirus is a new alternative virus for multiple myeloma oncolytic virotherapy. J Hematol Oncol. 2020 Jul 11;13(1):89. doi: 10.1186/s13045-020-00919-w. PMID: 32653014 PMCID: PMC7353805

Abstract

Multiple myeloma (MM) is a hematological malignancy currently incurable. Although many progress have been made in treatment in recent years, MM is still an incurable and refractory disease with poor prognosis and high recurrence rate. Therefore, it is very important to find new treatments. Oncolytic viruses (OVs) represent a new strategy to augment the spectrum of cancer therapeutics. Several studies reported that, in MM, the OVs act through tumor-specific oncolysis and generation of an antitumor immune response. The main viruses that have been studied for MM are human viruses, this approach is highly restricted by pre-existing anti-human virus humoral immunity that neutralizes the anti-tumor effect of OVs. This research project aims to investigate a potential alternative oncolytic strategy using non-human viruses in MM treatment. In particular, we studied two bovine viruses, not pathogenic for humans: the Bovine Herpesvirus type 4 (BoHV-4) and the Bovine Viral Diarrhea Virus (BVDV). Our results indicate that BoHV-4 did not infect MM cell lines and in bone marrow mononuclear cells (BM MNCs) isolated from MM patients BoHV-4 did not affect viability of $CD138^+$ cells but drastically reduced $CD14^+$cells. Investigating on indirect effect through the infection of mesenchymal stromal cells, we found that BoHV-4 did not have an anti-MM oncolytic effect. Interestingly, we found that BVDV exhibits a direct effect on MM cells. In fact, human MM cell lines are selectively sensitive to BVDV treatment with an increase of apoptotic markers and, consequently, of cell death. Furthermore, Bortezomib pre-treatment significantly increased the cytotoxic effect of BVDV on MM cell lines with a synergistic effect. Moreover, BM MNCs isolated from several MM patients treated with BVDV showed a significant selective decrease of the percentage of viable $CD138^+$ MM cells but not of lymphocytes and monocytes.

Finally, the *in vitro* data for BVDV treatment were confirmed in an *in vivo* MM immunodeficient mouse model showing that the treatment significantly reduced the tumoral burden compared to the vehicle. Our innovative approach takes advantage of bovine viruses as BVDV that usually are not pathogenic for humans. Virtually, no MM patients have anti-bovine virus antibodies, allowing an optimal oncolytic anti-MM activity. This research project highlights the possible use of non-human OVs as new anti-MM strategy in particular we showed that BVDV could be a candidate as oncolytic virus for MM treatment. Our study will pave the way for a phase I/II clinical trial to test this promising approach to treat MM patients.

Summary

1. Introduction

1.1 Multiple myeloma and its pathophysiology

Multiple Myeloma (MM) is the most common form of plasma cell dyscrasia, characterized by the accumulation of malignant plasma cells (PCs) in the bone marrow (BM) microenvironment that supports their growth and survival[1,2]. MM can be preceded by premalignant asymptomatic forms as the monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM), that evolve in malignant MM and ultimately to extramedullary MM or plasma cell leukemia (PCL) by multistep genetic and microenvironmental change[3-5]. Moreover, the presence or absence of CRAB criteria (hypercalcemia, renal insufficiency, anemia, and bone disease) differentiates symptomatic from asymptomatic patients[6].

The development of MM depends on a multistep transformation which is characterized by multiple molecular events, such as complex cytogenetic abnormalities as well as the expression of adhesion molecules, the production of cytokines and the interaction with the BM microenvironment cells [7-9].

Some molecular events are already detectable at MGUS level while others arise later, as reported in Figure A [10]. Several studies have highlighted that among genetic alterations chromosomal translocations involving immunoglobulin heavy-chain (IgH) represent an important prognostic value in MM[11]. In particular, t(14;16) and t(4;14) are associated with a poor prognosis, whereas a better prognosis is observed in patients carrying the t(11;14)[12,13]. The heterogeneous alterations that correlate with high risk of progression disease are monosomy or partial deletion

of chromosome 13, loss of the short arm of chromosome 17 (where the tumor-suppressor gene TP53 resides), gain or amplification of chromosomal region 1q21 and hyperdiploidy[14-17].

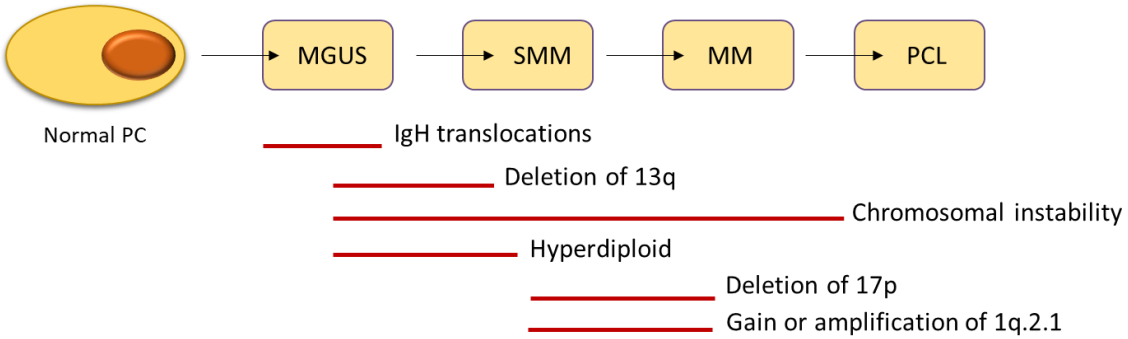

Figure A. Cytogenetic abnormalities in MM. Primary cytogenetic abnormalities occur early when the normal plasma cell transitions to a clonal premalignant stage. Most secondary cytogenetic abnormalities arise later in the disease progression.

In the pathophysiology of MM, alongside the molecular events affecting the PC clone, the alterations of the microenvironment play a critical role, such as in particular, the hyperproduction of cytokines and growth factors, the increase in bone resorption and the angiogenic switch, which distinguish MM from indolent stage of disease[18].

MM cells establish complex interactions both with cellular components of the BM microenvironment, such as mesenchymal stromal cells (MSCs), able to differentiate into osteoprogenitor cells, osteoblasts, osteocytes, osteoclasts, endothelial cells, adipocytes, dendritic cells, macrophages and lymphocytes, and with the extracellular matrix (ECM) composed by molecules such as laminin, collagen, proteoglycans and glycosaminoglycans[19].

The MM cell adhesion occurs through syndecan-1/CD138 and the very late antigen 4 (VLA-4); other molecules as: CD44, which binds osteopontin (OPN), CD56 / NCAM-1, which has a

homotypic bond and CD38, which binds CD31 involved in cell adhesion[20]. The interaction of MM cells to BM MSCs and the ECM, together with the autocrine and paracrine production of numerous cytokines and chemokines, therefore triggers several pathways that result in the upregulation of proteins that activate the cell cycle with anti-apoptotic, pro-proliferative and pro-angiogenic function and regulate the homing of MM cells. The MM interaction with microenvironment determines significant alterations of the microenvironment itself[21]. BM MSCs of MM patients produce higher levels of interleukin (IL)-6 and vascular endothelial growth factor (VEGF) and have alterations in osteoblastic activity with activation of osteoclastic activity[21,22]. Indeed, the bone lesions that characterize patients with MM are due to an alteration of normal bone remodeling which is unbalanced and uncoupled[23]. There are two events that characterize the alteration of bone remodeling: the exalted osteoclastic formation and the reduced osteoblastic formation[23,24]. If the MM cells stimulate osteoclastogenesis, on the other hand osteoclasts promote the growth and survival of MM cells. The increased osteoclastogenesis induced by MM cells creates a vicious circle through the release of several growth factors such as IL-6, chemokine (C-C motif) ligand (CCL) 3, OPN, B cell activating factor (BAFF) and a proliferation inducing ligand (APRIL)[21,23]. Conversely, osteoblasts have a reverse effect on MM cells, the inhibition of osteoblasts activity induced by MM cells promotes tumor growth[21,25].

In addition to bone disease an increase in BM angiogenesis correlated with the BM infiltration, PC proliferative activity and disease status was found in MM[26]. The progression of MM is characterized by a pre-angiogenic phase, corresponding to the indolent forms as SMM and MGUS with slow tumor growth, followed by a pro-angiogenic switch with progressive tumor growth that corresponds to the symptomatic stage of MM[27]. The increased angiogenesis in the BM is mainly

supported by VEGF produced directly by MM cells[27,28]. This cytokine, in turn, stimulates the secretion from the BM MSCs of IL-6 and VEGF itself, which in turn induces the paracrine production of VEGF by the tumor cells[29,30]. Finally, it has been showed that the BM niche besides get involved in the differentiation, migration, proliferation and survival of MM cells, has a crucial role in drug resistance regulate thought release of soluble factors activate intracellular signals[31]. The Figure B reported the interaction between MM cells and BM microenvironment and their main functions.

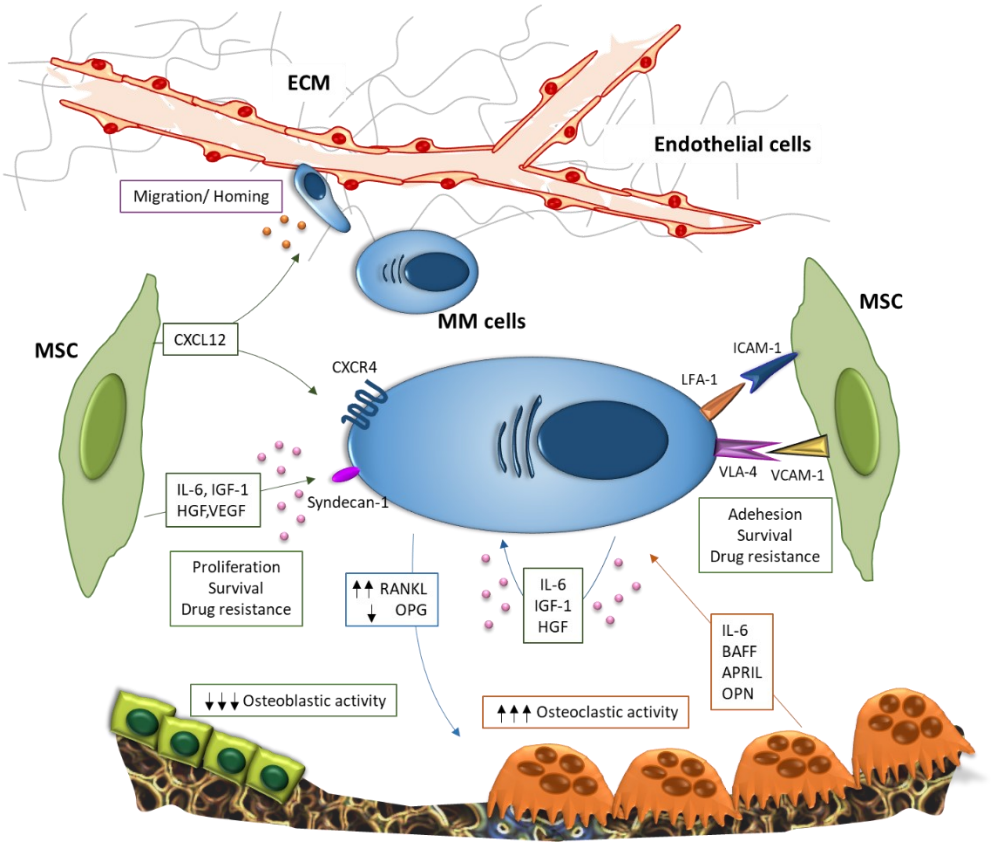

Figure B: MM interaction with BM microenvironment: schematic representation of interaction between MM cells and some BM microenvironment.

1.1.1 Adhesion molecules

The adhesion of MM cells to BM microenvironment cells, together with the autocrine and paracrine production of numerous cytokines and chemokines and the expression of cell-surface molecules play a crucial role in the regulation of MM cells homing[32]. For instance, Syndecan-1 is a transmembrane proteoglycan containing heparan sulfate which mediates the adhesion of MM cells to the ECM through binding to collagen type I[33]. This interaction induces the expression of matrix metalloproteinase 1 (MMP-1) promoting bone resorption and tumor invasion. Other important adhesion molecules involved are[32]:

- β1-integrins: VLA-1 (very late antigen-1), VLA-4, VLA-5
- β2-integrins: LFA-1 (lymphocyte function-associated antigen-1)
- Immunoglobulin superfamily: NCAM (neural cell-adhesion molecule), VCAM-1 (vascular cell -adhesion molecule), ICAM-1 (intercellular cell-adhesion molecule-1), ICAM-2, ICAM-3
- MCP-1 (monocyte chemotactic protein-1)
- CD44
- CD21

Among the adhesion molecules, CD46 is and is considered a possible target for MM therapy, either for virotherapy or for antibody-mediated immunotherapy[34,35]. This molecule is a membrane cofactor protein, known to be expressed by all cell types, except erythrocytes[36,37]. CD46 is over-expressed on human cancer cells, playing an important role in protecting of these cells from the complement mediated lysis[36,38]. Several studies have shown that CD46 is a receptor for the Measles Virus, an oncolytic virus, which infection induces cell death in several tumor cell

lines including in MM cells[39]. Literature data report that CD46 expression is associated with p53 mutational status and that p53 mutated MM cells were highly sensitive to the cytopathic effect of Measles Virus[40].

1.2 Drug therapy for MM

The therapeutic strategy of MM has been direct for many years to the containment of tumoral mass. Significant progress has been made in the treatment of MM in recent years with introduction of new drugs and targeted therapies such as proteasome inhibitors (PIs), Immunomodulatory drugs (IMiDS), monoclonal antibodies (mAbs)[1,41]. These "new drugs" are able to exercise their activity, as well as on the neoplastic clone, also to the BM microenvironment, whose role is fundamental in growth, survival and resistance to conventional chemotherapies of MM cells[42]. These advances have translated into a significant improvement in both patient responses and overall survival[41,43]. However, MM remains an incurable disease with high remission rates and relapse usually occurs with the resistance to chemotherapy[44,45].

The main drugs with anti-MM activity are:

- **Proteasome inhibitors (PIs)**: blocks the proteasome activity, making MM cells sensitive to apoptosis. They include Bortezomib (Bor) is a peptide boronate inhibitor of the proteasome, that exerts its anti-myeloma action at multiple side: inhibit NF-κB activity by blocking the degradation of I-κB, inhibits the adhesion of MM cells to stroma, blocks NF-κB–dependent induction of growth factors, such as IL-6, by stromal cells, inhibit the production of pro-angiogenic factors, abolish the osteoclastogenic stimulation and finally impairs cell cycle and DNA repair [46-48]. Bor is the first drug approved for the treatment of MM patients by targeting

the proteasome[49]. Carfilzomib (Car), is a second-generation proteasome inhibitor, which is currently approved for relapsed/refractory MM [50]. Car is a tetrapeptide epoxyketone, that irreversibly blocks the β5 subunit of the proteasome, responsible for the protein's cellular turnover, with the same affinity of Bor but in an even more specific and potent way[51]. As shown for Bor, Car has a bone anabolic effect via suppression of osteoclast activity and stimulation of osteoblast differentiation[52].

- **Immunomodulatory Drugs (IMiDs®):** IMiDs are a group of new therapeutic agents, analogues of Thalidomide (THAL) a glutamic acid derivative with immunological and immunomodulatory effects. The antitumor action of IMiDs derives mainly from the activity they exercise on immunological surveillance and regulation, on proliferation differentiation-cellular apoptosis and on the BM microenvironment. The two leading compounds are Lenalidomide (LEN) and Pomalidomide (POM). LEN, like THAL, inhibits angiogenesis, induces apoptosis of tumoral cells, inhibits production of inflammatory cytokine and supports cytotoxic activity of natural killer cells (NK) and T cells[53] . POM has strong immunomodulatory abilities and inhibit MM-induced osteoclastogenesis[53].

- **Monoclonal Antibodies (mAb):** is a novel agent with encouraging clinical activity as a single agent and in combination with the drugs actually used in MM treatment. mAb exert their action by targeting selectively specific molecules present on the PCs surface. In particular: Daratumumab (DARA) that targets CD38 a surface protein highly expressed in MM cells, and Elotuzumab (ELO) directed against the SLAMF7 receptor, expressed on normal and malignant PCs. Preclinical studies showed that DARA induces MM cell death through several mechanisms, including complement-dependent cytotoxicity, antibody-dependent cell-

mediated cytotoxicity, antibody-dependent cellular phagocytosis cytotoxicity, apoptosis upon secondary crosslinking and immunomodulatory effects via a decrease in immune suppressive cells. Conversely, ELO has no significant anti-MM activity when given as a single agent to patients with relapsed or refractory multiple myeloma. Nevertheless, when combined with other anti-MM agents improved response and outcome[54].

Currently, the MM standard of care includes a high dose of Melphalan followed by autologous stem cell transplantation in eligible patients and combination therapies with new drugs for induction and maintenance and for non-eligible patients[55,56]. Although the use of these new drugs and transplant techniques has improved progression-free survival and overall survival rates, MM still remains an incurable disease with ~52.3% of MM patients surviving only five years (Myeloma Incidence Statistics. Cancer Research UK. 2016. Available online: https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/myeloma)[57]. Thus, new drugs and therapeutic strategies are needed to overcome drug resistance, to improve the clinical outcome of MM patients and to design personalized approach. Recently, new therapeutic strategies targeting the microenvironment have been developed, opening new perspectives in the cure of the disease including CAR-T cells, drug-conjugate mAbs and bispecific Abs.

1.3 Oncolytic virotherapy

Oncolytic virotherapy is a novel approach for the treatment of tumors that utilizes naturally or engineered viruses to exert an anti-tumor effect. Oncolytic viruses can exert their effect either directly via cell lysis or indirectly by stimulating an immune response within the tumor

microenvironment. These two mechanisms may act in combination and their contribution depends of tumor cell type, characteristics of viral vector, cell mortality and the host immune system[58]. The permissive nature of tumor cells allows the uncontrolled replication of the genetic material and virus propagation. Once the tumor cell has been lysed, the virus releases new viral particles able to infect other malignant cells; furthermore, the release of new viral tumor-related antigens may be recognized and targeted by the immune system. Activation of the immune system together with an increase of the systemic levels of cytokines and leukocytes further improves the anti-tumor systemic effect. Some viruses can act by activating cell apoptosis pathways, others by mediating necrosis. Therefore, successful oncolytic virotherapy requires a fine balance between the two mechanisms of targeting tumor cells.

Different classes of human viruses were examined in preclinical MM models, and the use of several viruses are currently investigated in clinical trials in MM patients. The main classes of human viruses that have been studied for MM include Measles Virus, Reovirus, Adenovirus and Coxsackie Virus (Table a) [59-61]. In particular, the Measles Virus is a negative-strand ribonucleic acid (RNA) virus that belonging to the of Paramyxoviridae family[62]. In the context of MM, the Measles Virus is the most studied and the first to undergo in a phase I clinical trial investigation[63,64]. Measles Virus acts as oncolytic viral therapy by binding to CD46 as receptor on MM cells[65]. Moreover, it was been demonstrated that Measles Virus administered intravenously in MM xenograft models have an antitumorigenic and antineoplastic activity [65]. A clinical study conducted on two patients with MM refractory to conventional therapy and seronegative to Measles Virus, showed a reduction in serum free light chain levels, a reduction of the percentage of malignant PCs and of extramedullary masses in both patients after a single intravenous

administration of the virus, which resulted subsequently in complete remission of the disease which lasted 9 months[66]. In a phase I study it was evaluated the Measles Virus safety and maximum tolerated doses of MM patients with relapsed refractory[67]. The authors confirmed the selectivity of Measles Virus for the tumor and no toxicity. Moreover, only one patient reached complete remission, while the remaining patients reported a decrease of myeloma IgG and a decrease of FLC levels [67].

In addition to monotherapy action several studies reported that oncolytic viruses can be used to enhance the action of other drugs. Indeed, these oncolytic viruses have been investigated pre-clinically also in combination therapy with chemotherapy and/or radiation therapy, and as purging agents during autologous stem cells transplantation[68-70]. In the context of MM, Reovirus is used in combination with drugs currently in use in MM clinical practice, such as Len or Bor in order to induce both cell killing direct effect and an antitumor immune response effect on MM cells [68,69]. These combination treatments could potentially play an important role in enhancing the clinical therapeutic efficacy in patients with MM. However, the use of human oncolytic viruses could be limited by the antiviral immune response of the patients due to vaccination or natural infection. Moreover, the use of human viruses can be associated to the development of a clinically relevant infection in patients with high immune suppression due to the disease and the treatment. For this reason, recently, the attention has focused on some non-human oncolytic viruses that lack pathogenicity in humans but are still capable of destroying human tumor tissue. These viruses exploit the same entry site and mechanism of human viruses to infect and kill tumor cells (Table a) [59,60].

Table a: Oncolytic Viruses in the treatment of Multiple Myeloma

	Oncolytic virus	Genome	Receptors for MM	Mechanism of killing	Combination therapy	Clinicaltrials.Gov identifier
Human	*Measles Virus*	ss(-)RNA	CD46	Lytic viral replication	CP	NCT02192775 NCT00450814
	Reovirus	dsRNA	JAM-A	Lytic viral replication, apoptosis, autophagy	BTZ, Len, Pom, anti-PD-L1	NCT01533194 NCT03015922 NCT02514382 NCT02101944
	Adenovirus	dsDNA	UnK/CAR	Lytic viral replication	N/A	N/A
	Coxsackie virus	ss(+)RNA	ICAM-1, DAF	UnK	N/A	N/A
	HSV	dsRNA	HVEM	Induction of apoptosis	Len	N/A
Non-Human	*Myxoma virus*	dsDNA	UnK	Induction of apoptosis	N/A	N/A
	BVDV	ss(+)RNA	CD46	Induction of apoptosis	BTZ	N/A
	VSV	ss(-)RNA	LDLRs	Lytic viral replication Inhibition of DNA synthesis	BTZ, CP	NCT03017820
	NDV	dsDNA	Sialic Acid	Lytic viral replication, induction of apoptosis	N/A	N/A
	Vaccinia Virus	dsDNA	UnK	Lytic viral replication	N/A	N/A

Abbreviations: HSV: Herpes Simplex Virus; BVDV: Bovine Viral Diarrhea Virus; VSV: Vesicular Stomatitis Virus; NDV: Newcastle Disease Virus. Positive-sense (+) or negative-sense (-), single-stranded (ss) RNA virus or double-stranded (ds) DNA virus. UnK: Unknown; N/A: not applicable; BTZ: Bortezomib; Len: Lenalidomide; CP: cyclophosphamide.

1.4 Bovine Herpesvirus Type-4

Bovine herpesvirus 4 (BoHV-4) is a herpesvirus belonging to Gamma-herpesvirinae subfamily[71]. The infection is usually subclinical but can cause reproductive diseases in cattle such as endometritis, vulvovaginitis and mastitis[72]. BoHV-4 possesses important biological properties: little or no pathogenicity, no oncogenicity, efficient replication and causes cytopathic effect in a

variety of primary cultures and cell lines of various animal species and has surprising trophism towards many tumor cells human[73]. BoHV-4 is characterized by an enveloped icosahedral nucleocapsid containing a double-stranded DNA[74]. BoHV-4 has a B-type genome structure with a 144 ± 6 kb double-stranded DNA consisting of a long unique genome region (LUR) flanked at both ends by tandem repeats called polyrepetitive DNA (prDNA)[74,75]. BoHV-4 has the ability to accommodate large amounts of foreign genetic material within its genome without any appreciable effect on its replication[76]. For its molecular and genetic properties BoHV-4 has been proposed as viral vector for gene delivery and cancer therapy [71,75]. Indeed, it has been reported that BoHV-4 is able to infect some human cell lines inducing apoptosis[76-78]. Redaelli et al. showed a potential oncolytic effect of BoHV-4 on brain tumors. In particular, they demonstrated the ability of BoHV-4 to infect different glioma cell lines, the selectivity of the virus for the tumor cells and ability to induce necrotic cytopathic effect[79]. However, the mechanisms of infection and internalization of BoHV-4 remain still unknown.

1.5 Bovine Viral Diarrhea Virus

Bovine viral diarrhea virus (BVDV) is a Pestivirus of the Flaviviridae family, of which two genotypes (BVD-1 and BVDV-2) and two biotypes (cytopathic and non-cytopathic) are known[80]. BVDV represent a pathogen associated with transient and persistent infections which lead to gastrointestinal, respiratory and reproductive diseases in cattle[81]. Structurally the BVDV virion is composed by a nucleocapsid with icosahedral symmetry containing a small enveloped single-stranded positive sense RNA, and an envelope with four structural proteins (the nucleocapsid C protein and the envelope glycoproteins Erns, E1, and E) and six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) that are required for viral assembly and replication. BVDV entry is

mediated by the interaction of the viral glycoprotein E2 with the cellular transmembrane CD46 receptor. CD46 is known to serve as a binding partner for several human pathogens like Measles Virus[39,82]. It is known that BVDV, in bovine models, induces cell death by apoptosis due to an increase of intracellular viral RNA accumulation[83,84] or due the generation of reactive oxygen species [85]. Moreover, but its oncolytic activity has never been reported in human cancers.

2. Aim of the study

Although MM is still an incurable disease, the oncolytic virotherapy could represent a new strategy to augment the spectrum of anti-MM treatments. However, the main viruses that have been studied for MM are human viruses, this approach is highly restricted by pre-existing anti-human virus humoral immunity that neutralizes the anti-tumor effect of oncolytic viruses. In order to avoid these potential limits of the human viruses, the aim of this study was to investigate the potential oncolytic effect on MM cells of two bovine viruses not pathogenic for humans: BoHV-4 and BVDV. In particular, we evaluated the ability of the two bovine viruses to infect MM cells and induce their cell death in both *in vitro* cell line models and primary human *ex vivo* models. Moreover, we evaluate their therapeutic application in a in vivo subcutaneous mouse model.

3. Materials and Methods

3.1 Cell lines

The human myeloma cell lines (HMCLs) JJN3, OPM2, MM1.S, NCI-H929, the T-acute lymphoblastic leukemia cell line (T-ALL) SKW3-KE37, the B-acute lymphoblastic leukemia cell line (B-ALL) NALM-6, and the lymphoma cell lines GRANTA-519 and RAJI were purchased from Leibniz Institute Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The B-ALL cell line RS4;11 and the T-ALL cell line SUP-T1 were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), amphotericin B (0.25 μg/mL), and antibiotics (100 U/mL penicillin, and 100 μg/mL streptomycin) (ThermoFisher Scientific, Monza, Italy). We also used the human telomerase reverse transcriptase transduced mesenchymal stromal cell line (hTERT-MSCs), kindly provided by Prof. Bussolati (University of Parma, Parma, Italy). These stromal cells were cultured in DMEM (ThermoFisher Scientific, Monza, Italy) with 10% FBS, L-glutamine (2 mM), amphotericin B (0.25 μg/mL), and antibiotics (100 U/mL penicillin, and 100 μg/mL streptomycin).

3.2 Bovine viruses

BoHV-4 was constructed to contain and express red fluorescent protein (RFP) as previously described[73]. Recombinant BoHV-4 and BVDV (strain NADL, ATCC) were propagated by infecting confluent monolayers of bovine embryo kidney [(BS CL-94) BEK] or Madin Darby Bovine Kidney cells [(ATCC: CCL-22) MDBK] at a multiplicity of infection (MOI) of 0.5 50% tissue culture

infectious doses (TCID50) per cell and maintained in MEM (ThermoFisher Scientific) with 2% FBS (ThermoFisher Scientific) for 2 hours (h). The medium was then removed and replaced by fresh MEM containing 10% FBS. When approximately 90% of the cell monolayer exhibited cytopathic effect (CPE) (approximately 72 h post-infection), the viruses were prepared by freezing and thawing cells three times and pelleting the virions through 30% sucrose, as described previously[73]. Viruses pellets were resuspended in cold MEM without FBS. TCID50 were determined in BEK or MDBK cells by limiting dilution.

3.4 Patient's samples

A total cohort of 31 patients (13 males and 18 females) with malignant PC disorders were included in the study: 2 plasma cell leukemia (PCL) (median age 63 years, range 53–73), 29 with active MM including 18 newly diagnosed MM (ND-MM) (median age 74 years; range 52–86) and 11 relapsed MM (R-MM) (median age 73 years; range 59–81). All patients were diagnosed according to the International Myeloma Working Group (IMWG) revised criteria[6]. The main clinical characteristics of all the patients enrolled in the study are summarized in Table 1. BM aspirates were obtained from the iliac crest of patients after informed consent according to the Declaration of Helsinki. Total BM mononuclear cells (MNCs) were obtained from BM aspirates by Ficoll-Hypaque (Bichrome AG, Berlin, Germany) density sedimentation and cultured in RPMI 1640 medium supplemented with 20% FBS, in penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and fungizone antimycotic (2.5 μg/ml); all purchased from ThermoFisher Scientific. This study was approved by local ethic committee institutional review board of Parma (N. 44614).

3.5 Viruses and drug treatments

3.5.1 Viruses treatments

The HMCLs, T-ALL, B-ALL and hTERT-MSCs cells were treated with BoHV-4 and BVDV and maintained at 37 °C in a 5% CO2 atmosphere, for 24, 48, and 72 h. For in vitro experiments, we used 1 MOI of viruses/1 × 10^6 cells. For BVDV experiments the same conditions were performed with or without 0.05% trypsin-EDTA (ThermoFisher Scientific) incubation and after treatments all cells were collected for Multiplex PCR analysis.

BM MNCs from patients were cultured with or without BoHV-4 or BVDV for 72 h and maintained in at 37 °C in a 5% CO2 atmosphere. After treatment, all cells were collected for flow cytometry analysis, PCR analysis, and western bot analysis.

3.5.2 Co-culture experiments

For BoHV-4 experiments, confluent and adherent hTERT-MSCs were co-cultured with JJN3 or MM1.S (ratio 1:5) and treated or not with BoHV-4 (1 MOI). After 48 hours of co-culture, cells were collected for flow cytometry analysis.

3.5.3 Drug treatments

For BVDV experiments, the HMCL JJN3 cells were also pre-treated with Bor (2.5 nM) or vehicle for 24 h. Bor was purchased from Selleckchem (Munich, Germany). The drug was reconstituted following the manufacturer's protocol and diluted in the cell culture medium just before the use. Following drug washout with PBS, cells were counted and infected with BVDV for 24, 48, and 72 h. At the end of experiments, cells were collected for flow cytometry analysis. For combination

index experiments, JJN3 cells were pre-treated with Bor at different concentrations (0.125–8 nM) for 24 h, washed out with PBS and incubated in 96-well plates with or BVDV at several viral titers (0.0625–4 MOI) or the combination of the 2 drugs (2:1) or vehicle for 48 h. MTT assay was assessed to calculate the effect of combination of the 2 drugs. The combination index analysis was performed using CompuSyn software version 1 (http://combosyn.com/).

3.6 Flow cytometry

3.6.1 Viability staining and apoptotic assay on cell lines

The HMCLs, T-ALL, B-ALL, lymphoma cells and hTERT-MSCs cells were stained, according to manufacturer's instructions, with 7-Amino Actinomycin D (7-AAD) purchased from BD Biosciences (Franklin Lakes, NJ, USA). Viable and non-viable cells were identified as 7-AAD-negative or 7-AAD-positive events, respectively, in dot plots of SSC vs. 7-AAD.

Apoptosis was assessed by the APO2.7 assay, which specifically detects 7A6, a 38-kDa mitochondrial membrane antigen expressed during apoptosis. After treatment, cells were collected, stained with saturating quantity of PE-conjugated APO2.7 antibody (Beckman Coulter, Marseille, France), and analyzed by flow-cytometry.

3.6.2 Identification of MM cells in co-culture with hTERT-MSCs

After BoHV-4 treatment, hTERT-MSCs were acquired to assess cell viability with 7-AAD staining and cell infection with evaluation of RFP expression. The gating strategy included: a first FSC and SSC gating to remove debris and cell fragments, a subsetting live gating based on 7-AAD negative expression and then the evaluation of RFP^+ cells.

After 48 hours of BoHV-4 treatment, co-culture system of hTERT-MSCs with MM cells were stained with saturating quantities of anti-CD38-APC (purchased from BD Bioscience and R&D) to identify JJN3 or MM1.S. Before the acquisition, 7-AAD was added according to manufacturer instructions. The used gating strategy, included a first FSC and SSC gating to remove debris and cell fragments, a subsetting live gating based on 7-AAD negative expression and the evaluation of RFP^+ cells performed on $CD38^+$ cells.

3.6.3 Identification of BM MNCs subsets

After treatments, BM MNCs were collected and stained with saturating quantities of antibodies (purchased from BD Bioscience) combined in the following two panels: (1) anti-CD14-FITC, anti-CD138-PE, and anti-CD19-APC; (2) anti-CD56-FITC, anti-CD138-PE, and anti-CD3-APC. Before the acquisition, 7-AAD was added to staining panels according to manufacturer instructions. The gating strategy to evaluate the percentage of viable MM cells, T lymphocytes, B lymphocytes, monocytes, and NK cells included a first FSC and SSC gating to identify the cells of interest, based on the relative size and complexity of the cells, while removing debris and cell fragments, and a subsetting live gating based on 7-AAD negative expression. Next, we identified MM cells as CD138+, T lymphocytes as CD3+, B lymphocytes as CD19+, monocytes as CD14), and NK cells as CD56+CD138−, respectively.

For BVDV experiments, the oncolytic effect on MM cells was calculated using the following formula: % of CD138+ cells mortality = 1−(% of CD138+ 7-ADD− in BVDV condition/CD138+ 7-AAD− in control condition) × 100. In all flow cytometry procedures, the acquisition and analysis

of samples were performed on a two-laser FACSCalibur instrument (BD Biosciences) using CellQuest software (BD Biosciences).

3.6.4 CD46 expression

Expression levels of the CD46 antigen were determined on HMCLs, B and T-ALL, lymphoma cells, and on BM MNCs obtained from MM patients by flow cytometry analysis and expressed as median fluorescence intensity (MFI). In particular, to evaluate the expression of CD46, 0.2×10^6 HMCLs or B-ALL, T-ALL and lymphoma cell lines were stained with a saturating quantity of anti-CD46 PerCP (Thermofisher Scientific) for 30 min at 4 °C protect from light. Cells were then washed with a cell wash solution (PBS plus 5% human serum albumin and 5 w/V sodium azide) and directly analyzed by flow cytometry.

CD46 expression levels on fresh BM MNCs were detected by staining 0.5×10^6 cells/tube with saturating quantities of antibodies (all, except anti-CD46, purchased from BD Bioscience, Franklin Lakes, NJ, USA) combined in the following two panels: (1) anti-CD56 FITC, anti CD138 PE, anti-CD46 PerCP, and anti-CD3 APC; (2) anti-CD14 FITC, anti-CD138 PE, anti-CD46 PerCP, and anti-CD19 APC. After incubation for 30 min at 4 °C protected from light, BM MNCs were washed with the cell wash solution and analyzed by flow cytometry. Unstained samples were employed for gating controls. Concerning flow cytometry gating strategy, the analysis included a forward (FSC) and side (SSC) scatter gating to identify the cells of interest based on the relative size and complexity of the cells, while removing debris and cell fragments. In BM MNCs analysis, CD46 expression levels were determined on specific gates identifying: T lymphocytes ($CD3^+$), B lymphocytes ($CD19^+$), monocytes ($CD14^+$), NK cells ($CD56^+CD138^-$), and MM cells ($CD138^+$).

3.7 Reverse transcriptase PCR amplification and nested multiplex PCR

For BVDV experiments total cellular RNA was extracted from cells using RNeasy total RNA isolation kit (Qiagen; Hilden, Germany) following the manufacturer's instructions, and then quantified using a NanoDrop™ One (ThermoFisher Scientific). For the RNA viral gene NS5B detection, reverse transcription (RT) and PCR were combined in a single step as previously described[86].

Primary PCR was performed using the following specific primer pairs:

SENSE A: 5'-AAGATCCACCCTTATGA(A/G)GC-3'

ANTISENSE A: 5'-AAGAAGCCATCATC(A/C)CCACA-3'

The product of the primary PCR was used in nested PCR. The multiplex primers used for nested PCR are the following:

BVDV-1: 5'-TGGAGATCTTTCACACAATAGC-3'

MULTISENSE: 5'-GCTGTTTCACCCAGTT(A/G)TACAT-3'

For internal sample quality control, a volume of 1 µg of RNA was reverse-transcribed, in accordance with the manufacturer's protocol. Qualitative PCR were performed using the following specific primer pairs for GAPDH:

F: 5'-CAACGGATTTGGTCGTATTG-3'

R: 5'-GGAAGATGGTGATGGGATTT-3'

Products were electrophoresed on a 1.5% agarose gel (ThermoFisher Scientific) and stained with gel red (Biotium, Hayward, USA).

3.8 Western blot

For BVDV experiments the cytosolic extracts were obtained using a commercial kit (Active Motif, Carlsbad, CA, USA) following the manufacturer's protocol. For immunoblotting, the following antibodies were used: mouse monoclonal anti-caspase 3 antibody (Active Motif, Carlsbad, CA, USA), rabbit monoclonal anti-Mcl-1 antibody (Cell Signaling, Leiden, Netherlands), rabbit monoclonal anti-Bcl-2 antibody (Cell Signaling, Leiden, Netherlands), and mouse monoclonal anti-β-actin (Sigma-Aldrich, Milan, Italy) as internal control. The secondary antibodies peroxidase conjugated were anti-mouse (BD Pharmingen, Franklin Lakes, NJ, USA) and anti-rabbit (Cell Signaling). Protein bands were quantified using ImageJ software (U.S. National Institutes of Health, Bethesda, MA, USA).

3.9 *In vivo* mouse studies

For BVDV experiments two different groups of six severe combined immunodeficiency/non-obese diabetic (NOD/SCID) mice (4 to 6 weeks old) were housed under specific pathogen-free conditions and were injected subcutaneously with 5×10^6 of JJN3. When plasmacytomas have become palpable, BVDV or saline solution was injected intratumorally twice a week for 2 weeks. All procedures were performed according to the National and International current regulations. Tumor growth was monitored at different time points and, 3 weeks after cell inoculation, mice were killed and tumor mass, spleens, and peripheral blood were collected for immunohistochemical staining and western blot analysis. Maximum length, thickness, and width of the tumor masses were measured with a caliper, and tumor volume was calculated according to the following formula: $0.523 \times \text{length} \times \text{width}^2$. Plasmacytomas obtained from tumors removed

from mice were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. Moreover, plasmacytomas lysates were used to perform the western blot analysis. This study was approved by the Italian Ministry of Health review board (Italy).

3.10 Statistical analysis

Data were expressed as mean ± SD. ANOVA and two-tail Student's t tests or Kruskal-Wallis and Mann-Whitney tests were used, and p values < 0.05 were considered statistically significant. GraphPad Prism 8™ (GraphPad Software Inc., La Jolla, CA, USA) was used for all the statistical analyses.

4. Results

4.1 BoHV-4 is unable to infect and kill myeloma cells

Firstly, we treated HMCLs and B-ALL, T-ALL, lymphoma cell lines (defined as non-MM cells) with BoHV-4 for 24, 48 and 72 hours in order to evaluate its potential oncolytic effect. The infection efficiency, in terms of RFP expression and the cell mortality, evaluated as percentage of 7-AAD+ cells, were verified by flow cytometry analysis. In the Table 2 we reported the mean±SD% of dead cells of HMCLs treated with BoHV-4 vs untreated cells. Unfortunately, as reported in Figure 1A for HMCLs and 1B for non-MM cells, the percentages of both RFP+ cells and 7-AAD+ cells, were not significantly different in treated cells as compared to controls at all time of cultures. Thus, BoHV-4 was unable to infect and consequently to kill HMCLs or non-MM cells. In order to confirm these evidences with primary MM cells, we performed *ex vivo* experiments on BM MNCs. In particular, we treated BM MNCs obtained from 3 MM patients for 72 hours with BoHV-4 and flow cytometry analysis confirmed previous result. As shown in Figure 2A MM cells, identified as $CD138^+$ cells, were not susceptible to BoHV-4 infection (p=ns). Concomitantly, as reported in Figure 2B, we observed a significant reduction of $CD14^+$ cells after BoHV-4 treatment (p=0.039). These data overall suggest that BoHV-4 does not exert a specific anti-MM oncolysis and conversely it has a myeloablative effect on BM MNCs.

4.2 BoHV-4 does not act through MSCs-mediated delivery

It has been known that the oncolytic viruses can also act through the infection of microenvironment cells that work as vectors to release them into the tumor[87]. Since the MM is

dependent on the BM microenvironment[19], firstly we checked the oncolytic effect on hTERT-MSCs and subsequently we performed co-culture experiments of MM cells with hTERT-MSCs in the presence of BoHV-4.

After 48 hours of treatment, 46.5 % of hTERT-MSCs resulted RFP^+ cells indicating the infection by BoHV-4 and we observed an increase of mortality cells evaluated as % of 7-AAD^+ cells (% of 7-AAD^+ CNT *vs* BoHV-4: 0.92 *vs* 30.6) as reported in Figure 3A.

In parallel, we assessed co-culture system of hTERT-MSCs and 2 HMCLs, JJN3 and MM1.S, treated with BoHV-4 for 48 hours. We found that MM cells identified as $CD38^+$ cells did not express RFP (% of RFP^+cells: JJN3=0.63% and MM1.S=0%) (Figure 3B and 3C). Furthermore, we did not observe any effect on MM cells mortality (% of 7-AAD^+CNT *vs* BoHV-4: JJN3 12.2 *vs* 18.3; MM1.S 7 *vs* 9.3). Overall, these results indicate that BoHV-4 since able to infect hTERT-MSCs cannot act indirectly on MM cells through MSCs-mediated delivery.

4.3 BVDV treatment selectively leads to HMCLs death

Subsequently, we focus on potential oncolytic effect of BVDV, the second bovine virus taken into account in our study. First of all, we analyzed the expression levels of CD46, the cellular receptor for BVDV entry, on HMCLs and B-ALL, T-ALL, lymphoma cell lines by flow-cytometry. In line with literature data, all cell lines were CD46 positive [37]. Interestingly, we observed that MM cells express higher levels of CD46 (Figure 4A) (median MFI_{CD46} value 523.74) than non-MM cells (median MFI_{CD46} value 161.61) (Figure 4B), suggesting that MM cells could be more susceptible to BVDV effect.

To verify our hypothesis, the BVDV oncolytic effect was assessed on the same MM and non-MM cell lines after 24, 48 and 72 hours of treatment. The infection efficiency, in terms of viral genes expression, was checked after 24 hours by nested multiplex PCR. As reported in Figure 4C and Figure 4D the presence of BVDV was observed both in MM cells and non-MM cells, respectively. Subsequently, flow cytometry analysis on HMCLs treated with BVDV reported a significant increase of cell mortality, as percentage of 7-AAD$^+$ cells, already after 24 hours of infection for JJN3, OPM2 and MM1.S, and after 48 hours for NCI-H929 (Figure 4E). In the Table 3 we reported the mean±SD% of dead cells of HMCLs treated with BVDV vs untreated cells. The increase of cell mortality after BVDV treatment was not observed in non-MM cells, denoting that the lytic effect of BVDV is specific for MM cells (Figure 4F).

Moreover, to better investigate the mechanism of BVDV infection, we treated JJN3, SUPT-1, GRANTA-519 and NALM-6 cell lines with 1 MOI of BVDV for 48 hours. At the end of the culture period, in order to remove the virus attached to the cellular surface, cells were collected with or without trypsin incubation. Focusing on BVDV treated cells, we found that BVDV viral gene expression was not detectable in non-MM cell lines after trypsin incubation (Figure 4G). On the other hand, we observed the expression of BVDV viral gene in MM cells with and without trypsin incubation. These results suggest that BVDV binds to both MM and non-MM cells but is able to enter only in MM cells.

4.4 BVDV triggers apoptosis in HMCLs

In order to further evaluate the cytotoxic effect of BVDV we analyzed the expression of apoptotic markers. HMCLs treated with BVDV showed a significant increase of APO2.7 expression after 48 and 72 hours of infection as compared to controls, as showed in Figure 5A. The mean±SD% of

APO2.7 expression in HMCLs treated with BVDV *vs* untreated cells was reported in Table 4. In the Figure 5B we reported a representative experiment of APO2.7 staining on MM cells treated with BVDV for 24, 48 and 72 hours. These results demonstrate that BVDV treatment increases the percentage of APO2.7^{+} cells over the time. Furthermore, we found that the BVDV treatment of JJN3 and OPM2 cells for 48 hours leads to the activation of caspase-3 and the down-regulation of the anti-apoptotic proteins BCL-2 and MCL-1 (Figure 5C and 5D). Conversely, in non-MM cell lines we did not find any differences in terms of APO2.7 expression between BVDV-treated cells and control conditions (Figure 6). All these experiments showed that BVDV treatment reduced selectively the viability of MM cells by activating the apoptotic pathway.

4.5 Bortezomib pre-treatment increases the oncolytic effect of BVDV in HMCLs

Because it has been reported that Bor increases the efficacy of several human oncolytic viruses in MM and other tumoral models[88-90] therefore, we tested MM cell death combining Bor and BVDV treatments. As reported in a representative sample in Figure 7A, the Bor (2.5nM) pre-treatment of JJN3 cells for 24 hours enhances the cytotoxic *in vitro* effect of BVDV, increasing MM cell death over time. We observed a statistically significant decrease of cell viability after 24 and 48 hours of BVDV treatment after Bor pre-treatment (mean±SD% of 7-AAD^{+} dead cells: 24 hours BVDV 15.22±1.4 *vs* Bor+BVDV 18.47±1, p=0.009; 48 hours BVDV 35.06±3.8 *vs* Bor+BVDV 62.88±6.4, p=0.0003), reaching the highest mortality rates after 72 hours (mean±SD% of 7-AAD^{+} dead cells: 72 hours BVDV 72.04±4.8 *vs* Bor+BVDV 87.25±7.3, p=0.013) (Figure 7B).

Using Chou–Talalay analyses, we examined the drugs interaction between Bor and BVDV. JJN3 cells were pre-treated with various doses of Bor (0.125 nM-8nM) for 24 hours following the infection with different MOIs of BVDV for 48 hours (0.0625-4 MOI). Viability data were then

utilized to calculate the CI by the Compusyn program, in which CI<1 indicates synergistic interaction and CI=1 is additive. Our data showed that the combination of Bor and BVDV (at 2:1 *ratio,* respectively) synergistically killed MM cells. A synergistic effect was obtained for concentrations of Bor lower than 1.9 nM and of BVDV lower that 0.58 MOI, as shown for JJN3 in Figure 7C. An additive effect was obtained for concentrations of Bor 1.9 nM and BVDV 0.58 MOI.

4.6 Primary MM CD138$^+$cells are susceptible to oncolytic activity of BVDV

We analyzed the CD46 expression levels on the different BM subpopulations, as MM cells, monocytes, T, B and natural killer (NK) lymphocytes. As expected, CD46 was expressed by all BM MNCs, but there was a marked heterogeneity in terms of the intensity of expression. In all samples the flow-cytometry analysis showed that the MFI of CD46 was higher on MM cells (CD138$^+$) (median MFI_{CD46} value 1269.8, range 704.16-5149.88) in comparison with other subpopulations such as monocytes (CD14$^+$), T lymphocytes (CD3$^+$), NK (CD138$^-$CD56$^+$) and B lymphocytes (CD19$^+$). Figure 8A reported the CD46 expression analysis on fresh BM MNCs from one representative MM patient (CD138$^+$ MFI_{CD46} =2232.43; CD14$^+$ MFI_{CD46} =1345.57; CD3$^+$ MFI_{CD46} =552.32; CD56$^+$CD138$^-$ MFI_{CD46} =463.46; CD19$^+$ MFI_{CD46} =273.84). Subsequently, we investigated the BVDV *ex vivo* effect in 29 patients with active MM and from 2 patients with PCL after 72 hours of treatment. As shown in a representative analysis of one MM patient (Figure 8B), the BM MNCs treated with BVDV display a decrease of percentage of CD138$^+$, while the other subpopulations remain unchanged.

Analyzing our total cohort of BM MNCs from MM patients, we found a significant decrease of both the percentage of CD138$^+$ cells (Figure 9A) *(*p< 0.0001) and of the MFI_{CD138} (Figure 9B) (p< 0.0001) after BVDV treatment compared to the control. Furthermore, considering patients with

newly diagnosed MM and relapsed MM, we found that the BVDV-related mortality of $CD138^+$ was not significantly different between two groups (Figure 9C). Also, between refractory patients to Bor or Lenalidomide (Len) treatment we did not observe significantly differences in term of mortality of $CD138^+$ cells (Figure 9D).

We also reported that the percentage of $CD14^+$ cells increased after BVDV treatment ($p< 0.0001$) (Figure 9E), also in terms of MFI_{CD14} ($p< 0.0001$) (Figure 9F). Interestingly, we found that the percentage of $CD3^+$, $CD19^+$ and $CD56^+$ cells, evaluated in a subset of our sample's cohort after BVDV treatment, did not change (Figure 9G, 9H and 9I). These results suggest that the BVDV oncolytic effect was limited to MM cells, potentially associated to a monocyte activation and did not affect lymphocyte populations.

4.7 BVDV reduces tumor growth *in vivo* in NOD/SCID MM mouse model

Based on these *in vitro* results, we next evaluated the effect of BVDV treatment in an *in vivo* mouse model subcutaneously injected with JJN3 cells. Tumor volume measurements performed during treatment (at 4, 6, 10, 13 and 16 days after cells injection) showed a progressive reduction of tumor growth in mice treated with BVDV compared to controls (Figure 10A). At the end of the experiment, we found that mice treated with BVDV showed a significant reduction of tumor masses as compared with untreated mice (p=0.04) in terms of tumor volumes (Figure 10B). A significant reduction of the tumors size was confirmed after plasmacytoma explant and hematoxylin-eosin staining, as shown for 2 representative mice in Figure 10C. Interestingly, 3 mice out of 6 of the BVDV group showed a complete reduction and disappearing of tumor masses at the time of the mice sacrifice. The presence of BVDV has been assessed by multiplex PCR in all tumor masses treated with the bovine virus, where the plasmacytomas was still present at the

end of the experiment as reported in Figure 10D. Finally, the IHC analysis performed on tumor masses showed that the mice treated with BVDV presented necrotic tumor area as compared to control (Figure 10E). Furthermore, we analyzed the protein levels of active-caspase 3 and β-actin by western blot on *ex vivo* plasmacytoma lysates from a representative mouse treated with BVDV or saline solution. Interestingly, we observed the activation of Caspase-3 only in mouse treated with BVDV, showing that the reduced tumor mass is associated to apoptotic death of tumor cells (Figure 10F).

5. Discussion

Oncolytic virotherapy is an emerging therapeutic approach for MM as for other cancers [91,92]. Most of the data published reported the use of human Measles Virus to kill MM cell[93]. More recently, other virus as Reovirus, Myxoma and Adenovirus were reported to have oncolytic activity in MM cells[94-96].

Measles Virus interacts with CD46 to enter into MM cells and to induce a cytopathic effect[34]. Actually, different Measles Virus constructs have been administrated to patients with MM in clinical trials with encouraging results[66,67]. However, one of the main concerns regarding the use of Measles Virus as well as of other human virus is the presence of preexisting neutralizing anti-virus antibodies in cancer patients related to previous immunity. Indeed, the cases reported of an anti-MM effect of Measles Virus administration had undetectable circulating anti-Measles Virus antibodies. Because the vaccination anti-Measles Virus is a worldwide necessary procedure to protect from Measles Virus infection, alternative approaches for oncolytic virus therapy should be found[97]. The Mayo Clinic approach considers different modalities to overcome the blocking

activity of anti-Measles Virus antibodies including pre-therapy transiently depletion of anti-Measles Virus antibodies, the use of Measles Virus-infected cell carriers to deliver the virus evading anti-Measles Virus antibodies and the use of engineering oncolytic Measles Virus not recognized by anti-Measles Virus antibodies[97]. Despite these promising approaches, an alternative and innovative strategy for virotherapy could be the use of non-human virus. In this study, we tested this hypothesis, investigating the possible oncolytic activity on human MM cells of two bovine viruses not pathogenic for humans: the BoHV-4 and the BVDV. Firstly, we tested BoHV-4 a bovine virus known to have an oncolytic activity on solid tumors, but we did not find any significant cytopathic effect against both HMCLs and primary MM cells obtained from patients.

The use of oncolytic viruses as well as a direct approach can also provide an indirect approach. In particular, different cell types have been studied and proposed as vectors to delivery oncolytic viruses to the tumor site[87,98] . Among these, it has been reported that MSCs support the replication of oncolytic virus and can transfer infectivity to tumors[98]. In line with literature data, we checked the potential indirect oncolytic effect of BoHV-4 thought the MSCs-delivery. Despite we found that BoHV-4 had a direct oncolytic effect on hTERT-MSCs, we did not observe an indirect effect on MM cells in terms of both cell viability and infection. These results in addition to the myeloablative effect observed among BM MNCs treated with BoHV-4 suggest that this virus is not a promising candidate in anti-MM virotherapy.

Conversely, our data obtained using BVDV both *in vitro* and *in vivo* experiments indicate, for the first time, the oncolytic activity of BVDV against tumor cells, in particular MM cells.

Firstly, we focus on virus entry: BVDV it is known to bind CD46 to enter into cells, as showed for the Measles Virus. CD46 is known to be expressed by all cell types, except erythrocytes[36,38,99]. Ong H.T. et al showed that even though CD46 is ubiquitously expressed at low levels on all nucleated cells it is expressed, quantitatively, at higher levels on MM cells compared to all other cellular populations in the BM [34] and it is considered a possible target either for virotherapy or for antibody-mediated immunotherapy[35,40]. It was reported that Measles Virus infection induced cell death of several cancer cell lines other than MM cells [100] and that its efficacy was correlated to the level of CD46 expression by tumor cells[101]. In addition, it was recently reported that, in MM cells CD46 expression was associated to p53 mutational status and that P53 mutated MM cells were highly sensitive to Measles Virus cytopathic effect[40]. Other authors reported a relationship between CD46 expression and the presence of 1q gain amplification in MM cells[35]. In our study firstly, we confirmed the expression profile of CD46 on both HMCLs and primary MM cells and then we demonstrated the cytopathic activity of BVDV. This effect was independent by the presence of p53 mutational status of HMCLs and was attenuated by nutlin3a as reported by others[40]. Interestingly, we show that other cell lines, as acute leukemia and lymphoma, did not respond to the oncolytic effect of BVDV, despite their CD46 expression and BVDV ability to bind these cell lines. Overall, our results indicate that CD46 expression by tumor cells is necessary for the attachment of BVDV, but it is not sufficient to turn cells susceptible to infection and to achieve the oncolytic effect of BVDV, thus suggesting the involvement of other mechanisms. Literature data reported that Heparan sulfate family, including CD138, hallmark of MM, acts as a cellular receptor for BVDV binding to the host cells[102]. Our hypothesis is that other receptor/co-receptor, as CD138, could be involved in the mechanism of virus internalization into MM cells.

Several authors have reported that BVDV induces apoptosis in mammalians cells associated with the caspase-9 and caspase-8 activation that ultimately results in caspase-3 cleavage[83,103,104]. In line with literature data, our data show the cleavage of the effector caspases-3 in BVDV-treated MM cells. The activation of cellular caspase-3 on MM cells clearly correlated with the cytopathic BVDV-induced changes, suggesting a direct oncolytic effect of BVDV on MM cells mediated by apoptosis. In addition, beside caspase-3 activation, we found a significant downregulation of the BCL-2 and MCL-1 protein expression in BVDV-treated MM cells. As known BCL-2 proteins particularly MCL-1 are critically involved in the survival of MM cells[105-107]. However, appropriate studies will be necessary to clarify which transcriptional profile of MM cells as compared to other lymphoid cells is associated to the permissive role for BVDV in MM cells.

Data obtained on HMCLs were then confirmed in a large number of primary BM samples. Interestingly we found that BVDV was able to induce a cytopathic effect independently by the type of primary sample tested either at the diagnosis or at the relapse. In all the BM samples tested, we found that $CD138^+$ cells were only cell type susceptible to the oncolytic activity of BVDV, as demonstrated by the unchanged viability of $CD14^+$, $CD3^+$, $CD19^+$ and $CD56^+$ cells after BVDV treatment, despite their CD46 expression. These data interestingly suggest the lack of toxicity of the potential BVDV-based oncolytic virotherapy among BM cells. Along with the reduction of $CD138^+$ viable cells, our results show that MM cells treated with BVDV displayed a significantly decrease in CD138 surface expression, thus suggesting its involvement in BVDV internalization. Moreover, these observations are in line with literature data showing a progressive loss of surface expression of CD138 on primary MM cells undergoing apoptosis.[108] Interestingly, we did not find any difference on BVDV effect between MM patients resistant or

not to therapy and in addition between patients resistant to Bor or Len. These data suggested that the BVDV activity was independent to the presence of drug resistance in MM patients.

Based on the evidence of the oncolytic activity of BVDV on MM cells, following, we checked whether anti-MM drugs might improve the BVDV activity. Bor is a widely used proteasome inhibitor known to induce apoptosis through caspase-8 and caspase-9 signaling which further leads to caspase-3 activation in multiple myeloma cells[109]. Furthermore, several studies reported Bor ability to increase the oncolytic activity of different virus, as Adenovirus and Reovirus, in MM[69,110]. In line with these observations, we showed that Bor pre-treatment significantly increase the oncolytic effect of BVDV with a synergistic effect due to the activation of the same apoptotic signaling, caspase-3-mediated.

Finally, to confirm the *in vitro* data, we tested the oncolytic activity of BVDV in an *in vivo* mouse model. We used a NOD/SCID mouse model to focus on the direct cytopathic effect of BVDV on MM cells using a subcutaneous route of administration of the virus. This preclinical model showed a significant *in vivo* anti-MM effect with a progressive reduction of tumor growth in mice treated with BVDV. In particular, we found that the reduced tumor mass is associated to caspase-3-mediated apoptotic death of tumor cells, confirming the *in vitro* and *ex vivo* data. Interestingly, we lack to find the presence of the virus in the vital organs as the heart and the lung indicating the high specificity of the BVDV for MM cells and the lack of toxicity.

6. Conclusions

In conclusion, our results confirmed the use of bovine viruses as alternative strategy for anti- MM virotherapy, although not all bovine viruses can be suitable for oncolytic therapy. For the first

time we demonstrate an oncolytic activity of BVDV a bovine virus non-pathogenic for human being showing that the BVDV oncolytic activity is specific for MM cells. Our data suggest that the use of BVDV is a possible alternative to human virus for an oncolytic approach in MM treatment. This study gives the rational to design clinical approach for the use of BVDV in patients with MM.

7. References

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8. Tables

Table 1: Clinical characteristics of patients

DIAGNOSIS	STAGE	ISS	GENDER	AGE	LIGHT CHAINS	%PC BOM	HIGH RISK
MM-1	ND	III	M	76	l	90%	NO
MM-2	ND	II	F	73	l	60%	NO
MM-3	ND	II	F	52	k	70%	NO
MM-4	ND	II	M	85	k	30%	YES
MM-5	ND	III	M	80	k	70%	NO
MM-6	ND	III	F	72	l	70%	YES
MM-7	ND	III	F	74	k	18%	NO
MM-8	ND	II	M	74	k	40%	NO
MM-9	ND	III	M	57	l	80%	
MM-10	ND	III	F	71	k	100%	YES
MM-11	ND	I	F	80	k	30%	NO
MM-12	ND	III	M	79	k	25%	NO
MM-13	ND	II	F	67	k	60%	NO
MM-14	ND	II	M	77	l	30%	
MM-15	ND	III	F	86	l	80%	NO
MM-16	ND		F	57	k	25%	NO
MM-17	ND	III	F	74	k	70%	YES
MM-18	ND	I	F	53	K	30%	YES
MM-19	R	III	F	81	k	40%	YES
MM-20	R	III	M	78	l	40%	YES
MM-21	R	I	M	59	k	20%	
MM-22	R	I	M	65	k	60%	NO
MM-23	R	III	M	78	k	85%	YES
MM-24	R	-	F	79	l	30%	NO
MM-25	R	III	F	72	l	90%	
MM-26	R	III	F	79	k	80%	YES
MM-27	R	I	M	69	l	50%	NO
MM-28	R	I	F	72	k	50%	NO
MM-29	R	III	M	73	k	25%	YES
PCL-1	D	III	F	53	k	90%	NO
PCL-2	R	III	F	73	l	90%	SI

Abbreviations: MM: Multiple Myeloma; ND: Newly Diagnosed; R: Relapsed; F: female; M: male; ISS: International Staging System; %PC BOM: percentage of plasma cells evaluated by bone biopsy; HIGH RISK: defined by presence of deletion of 17P and or traslocation (t) of (4;14) and or t(14;16)

Table 2: Statistical analysis on HMCLs treated with BoHV-4

		Mean±SD % of dead cells BoHV-4 *vs* CNT	*p* value
JJN3	24 hours	23.2±11.6% vs 22.1±11.3%	*ns*
	48 hours	25.8±10.8% vs 23.5±10.1%	*ns*
	72 hours	43.3±1.9% vs 36.7±3.5%	*ns*
MM1.S	24 hours	11.3±5% vs 12.4±5.5%	*ns*
	48 hours	10.5±%0.9 vs 10.95±0.8%	*ns*
	72 hours	22.5±3.2% vs 18.6±5.6%	*ns*

Abbreviations: BoHV-4: Bovine Herpes Virus type 4; CNT: control (untreated cells); SD: standard deviation

Table 3: Statistical analysis on HMCLs treated with BVDV

		Mean±SD % of dead cells BVDV *vs* CNT	*p* value
JJN3	24 hours	18.5±2.5% vs 5.3±1%	0.00005
	48 hours	44.2±9.8% vs 11.3±1.5%	0.0002
	72 hours	54.3±12.8% vs 21.5±1.9%	0.002
OPM2	24 hours	28±1% vs 9±1.4%	<0.00001
	48 hours	48±5.6% vs 13±2.7%	0.00002
	72 hours	58±1.7% vs 22±1.8%	<0.00001
MM1.S	24 hours	9±1.8% vs 4±1.7%	0.012
	48 hours	12±%3 vs 5±1.2%	0.005
	72 hours	20±1.7% vs 4±0.5%	<0.00001
NCI-H929	24 hours	10±0.01 vs 8±0.01	0.16553
	48 hours	26±2.6% vs 10±1.2%	0.00004
	72 hours	76±2.5% vs 10.5±0.5%	<0.00001

Abbreviations: BVDV: Bovine Viral Diarrhea Virus; CNT: control (untreated cells); SD: standard deviation

Table 4: Statistical analysis of APO2.7 expression in HMCLs treated with BVDV

		Mean±SD % of APO2.7 expression BVDV vs CNT	*p* value
JJN3	48 hours	32.8±6.1 vs 13.02±2.3	0.0009
	72 hours	51.35±6.8 vs 17.4±9.7	0.001
OPM2	48 hours	12.6±2.5 vs 4.5±0.6	0.0007
	72 hours	39.7±7.2 vs 6.2±1.8	0.0001
MM1.S	48 hours	16.4±1 vs 8.4±1.5	0.0001
	72 hours	23.4±1.7 vs 10.4±1.5	0.00003
NCI-H929	48 hours	30.5±3.3 vs 10.5±0.5	0.00002
	72 hours	85.3±3 vs 10.2±0.35	<0.0001

Abbreviations: BVDV: Bovine Viral Diarrhea Virus; CNT: control (untreated cells); SD: standard deviation

9. Legend of Figures

Figure 1*: in vitro* BoHV-4 treatment of HMCLs and non-MM cells

A) Mean ± SD of the percentage of RPF^+ cells and mean ± SD of the percentage of 7-AAD^+ cells in JJN3 and MM1.S after 24, 48 and 72 hours of treatment with BoHV-4 (1 MOI). **B)** Mean ± SD of the percentage of RPF^+ cells and mean ± SD of the percentage of 7-AAD^+ cells in GRANTA, Supt1 and NALM6 after 24, 48 and 72 hours of treatment with BoHV-4 (1 MOI). (CNT= control, untreated cells).

The graphs represent the mean percentage of three independent experiments for each cell line evaluated by flow-cytometry analysis.

Figure 2*: ex vivo* BoHV-4 treatment of BM MNCs

A) Representative dot plots of flow cytometry analysis showing the percentage of viable $CD138^+$ cells from one MM patient after 72 hours of BoHV-4 (1 MOI) treatment compared to untreated control. The graph on the right represents the individual values of percentage of $CD138^+$ cells among BM MNCs of 3 patients untreated or treated with BoHV-4**. B)** Representative dot plots of flow cytometry analysis showing the percentage of viable $CD14^+$ cells from one MM patient after 72 hours of BoHV-4 treatment compared to untreated control. The graph on the right represents the individual values of percentage of $CD14^+$ cells among BM MNCs of 3 patients untreated or treated with BoHV-4. The *p* value was calculated by paired t test (CNT= control, untreated cells).

Figure 3*: in vitro* BoHV-4 treatment of hTERT-MSCs alone and in co-culture with HMCLs

A) Representative dot plots of flow cytometry analysis showing the percentages of non-viable 7-AAD^+ cells and the RFP^+ cells among hTERT-MSCs after 48 hours of BoHV-4 treatment. **B)**

Representative dot plots of flow cytometry analysis showing the percentages of non-viable 7-AAD$^+$ cells and the RFP$^+$ cells among JJN3 **(B)** or MM1.S **(C)** cells, identified as CD38$^+$ cells, in co-culture with hTERT-MSCs after 48 hours of BoHV-4 treatment. (CNT= control, untreated cells).

Figure 4: Expression levels of CD46 and oncolytic effect of BVDV on several hemopoietic cancer cell lines

Representative histogram plots of flow cytometry showed CD46 expression levels on: **A)** four HMCLs (JJN3, NCI-H929, MM.1S and OPM2) and **(B)** two T-ALL lines as SKW3-KE37, SUP-T1, two B-ALL lines as NALM-6, RS4;11 and two B-cell lymphomas lines as GRANTA-519, RAJI. The graphs represent the CD46 median fluorescence intensity (MFI). The picture shows the presence of BVDV in MM cell lines **(C)** and in non-MM cell lines **(D)** evaluated by Nested multiplex PCR after 24 hours of BVDV (1 MOI) treatment. GAPDH was used as internal quality control. **E)** The histograms represent the percentage of 7-AAD$^+$cells after 24, 48 and 72 hours of treatment with BVDV (1 MOI). We reported the mean± SD percentage of dead cells, as 7-AAD$^+$ cells, of four independent experiments on JJN3, MM1.S, OPM2 and NCI-H929 and **(F)** three independent experiments of non-MM cells (SKW3-KE37, SUP-T1, NALM-6, RS4;11, GRANTA-519, RAJI); *p* values were calculated by two-tailed student's t test. (*p<0.05, **p<0.01, ***p<0.001) (CNT= control, untreated cells). **G)** The picture shows the expression of NS5B-BVDV gene evaluated by Nested multiplex PCR on JJN3, SUPT-1, GRANTA-519 and NALM-6 after 48 hours of BVDV (1 MOI) treatment, with cells collected with (BVDV TRYP) or without (BVDV) trypsin incubation. GAPDH was used as internal quality control.

Figure 5: The cytotoxic *in vitro* effect of BVDV on HMCLs

A) Mean ± SD of the percentage of Apo 2.7^+ cells in JJN3, MM1.S, OPM2 and NCI-H929 after 24, 48 and 72 hours of treatment with BVDV (1 MOI). The graphs represent the mean percentage of Apo2.7^+ cells of four independent experiments for each cell line evaluated by flow-cytometry. **B)** Representative histogram plots of flow cytometry showing the percentages of NCI-H929 cells positive for the apoptotic marker APO2.7, after 24, 48 and 72 hours of BVDV (1 MOI) treatment or in the control condition. **C)** Pro- and Active-Caspase 3 expression was evaluated by Western blot in JJN3 and OPM2 cells treated with or without BVDV (1 MOI) for 48 hours. β-actin was used as loading control and JJN3 treated with high doses of Bor as positive control (Cnt+). The histogram represents the protein bands intensity quantified using ImageJ software reported as arbitrary unit normalized by the loading control. **D)** Western blot of Bcl-2 and Mcl-1 expression on JJN3 and OPM2 cells treated for 48 hours with or without BVDV (1 MOI). β-actin was used for loading control and RPMI-8226 cells line as positive control for both protein (Cnt+). The histograms represent the protein bands intensity quantified using ImageJ software reported as arbitrary unit normalized by the loading control. The *p* values were calculated by two-tailed student's t test. (**p*<0.05, ***p*<0.01, ****p*<0.001) (CNT= control, untreated cells)

Figure 6: APO2.7 expression on non-MM cells

Representative histogram plots of flow cytometry analysis showing the percentages of NALM-6, SKW3-E37 and GRANTA-519 cells positive for the apoptotic marker APO2.7, after 24, 48 and 72 hours of BVDV (1 MOI) treatment compared to control.

Figure 7: Pre-treatment with Bor increases the susceptibility of JJN3 to BVDV oncolytic activity

A) Representative dot plots of flow cytometry analysis shown the percentages and morphology of viable (7-AAD$^-$, red gate) and non-viable (7-ADD$^+$, green gate) JJN3 cells after 24, 48 and 72 h of BVDV treatment (1 MOI), with or without 24h of pre-treatment with Bor (2.5 nM). **B)** The histograms represent the statistical analysis of four independent experiments of JJN3 cells pre-treated with Bor (2.5 nM) for 24 hours and followed by BVDV treatment (1 MOI) for 24 (left panel), 48 (central panel) and 72 (right panel) hours respectively. The *p* values were calculated by two-tailed student's t test. (*p<0.05, **p<0.01, ***p<0.001) (CNT= untreated cells). **C)** JJN3 cells were treated with increasing doses of Bor (from 0.125 to 8 nM), increasing doses of BVDV (from 0.0625 to 4 MOI), or the combination of the 2 drugs (2:1) or vehicle. After 48 h, cell viability was assessed, and the data were analyzed as % of the value obtained with the cells treated with vehicle. Combination index analysis was then performed using CompuSyn software. Isobologram for ED50 represents means ± SEM of 3 experiments with 5 determinations each.

Figure 8: Expression levels of CD46 and *ex vivo* effect of BVDV on BM MNCs subpopulations

A) Flow cytometry histograms of one representative MM patient, showing the expression levels (MFI) of CD46 on monocytes (CD14$^+$), T lymphocytes (CD3$^+$), B lymphocytes (CD19$^+$), NK cells (CD56$^+$CD138$^-$) and MM cells (CD138$^+$). **B)** Representative dot plots of flow cytometry analysis show the percentage of viable cells on BM subpopulations obtained from one MM patient after 72 hours of BVDV (1 MOI) treatment compared to untreated control.

Figure 9: *Ex vivo* oncolytic activity of BVDV on CD138$^+$ primary cells

The graphs represent the individual values of percentage **(A)** and MFI (**B**) of CD138$^+$ cells obtained from BM MNCs of 31 patients treated with BVDV (1 MOI) for 72 hours and in the untreated

control**. C)** The scatter plot displays the $CD138^+$ cells mortality between BM MNCs from 18 patients with newly diagnosed MM (MM ND) and BM MNCs from 11 relapsed MM (MM R) patients; the analysis was performed as described in Material and Methods section. The *p* value was calculated by Mann-Whitney test (ns= not significant). **D)** The scatter plot shown the $CD138^+$ cells mortality between BM MNCs from 5 patients refractory to Bor treatment and BM MNCs from 5 Len-refractory patients; the analysis was performed as described in Material and Methods section. The *p* value was calculated by Mann-Whitney test (ns= not significant). The graphs show the percentage **(E)** and MFI (**F**) of CD14+ cells obtained from BM MNCs of 31 patients treated with BVDV (1 MOI) for 72 hours and in the untreated control. The graphs represent the individual values of the percentage of CD3 positive cells **(G)** panel) obtained from BM MNCs of 20 patients, the percentage of CD19 positive cells **(H)** obtained from BM MNCs of 16 patients and the percentage of CD56 positive cells **(I)**obtained from BM MNCs of 16 patients. All BM MNCs were treated with BVDV for 72 hours or untreated as control condition. Paired sample are linked by a line. The *p* value was calculated by Wilcoxon's test (CNT= control, untreated cells).

Figure 10: BVDV treatment inhibits tumoral growth in MM NOD/SCID mouse model

(A) Scatter plot represents the tumor mass (mm^2) after 4, 6, 10, 13 and 16 days of intratumoral treatment with BVDV (blue dots) or PBS (CNT, violet dots) in mice with palpable plasmacytoma.Data are reported as individual values (plots) and the median range (bars). (**B**) Box plot graph reports the volumes of tumor mass collected after mice sacrifice in the untreated control condition and in the BVDV group. Values are reported as median volume and the range. *P* values were calculated by Mann-Whitney test. **(C)** Representative hematoxylin and eosin staining (top) and photographs of removed tumor masses (bottom) from one representative

mouse of the control group and one of the BVDV-treated group (original magnification, 1x). **(D)** The picture shows the presence of BVDV in all the tumor treated of which was possible the collection after the end of the experiment (n°3), evaluated by Nested multiplex PCR. GAPDH was used as internal quality control. **(E)** Hematoxylin and eosin in staining of one tumor from the control group and one tumor from the mice treated with BVDV highlighting the tumor necrosis in the BVDV group (original magnification 20x). **(F)** Western blot of active-caspase 3 expression on plasmacytomas lysates obtained from one mouse treated with saline solution (Mouse CNT) or one mouse treated with BVDV (Mouse BVDV). β-actin was used as loading control and JJN3 treated with high doses of Bor as positive control (Cnt+). The histogram represents the protein bands intensity quantified using ImageJ software reported as arbitrary unit normalized by the loading control.

10. Figures

Figure 1

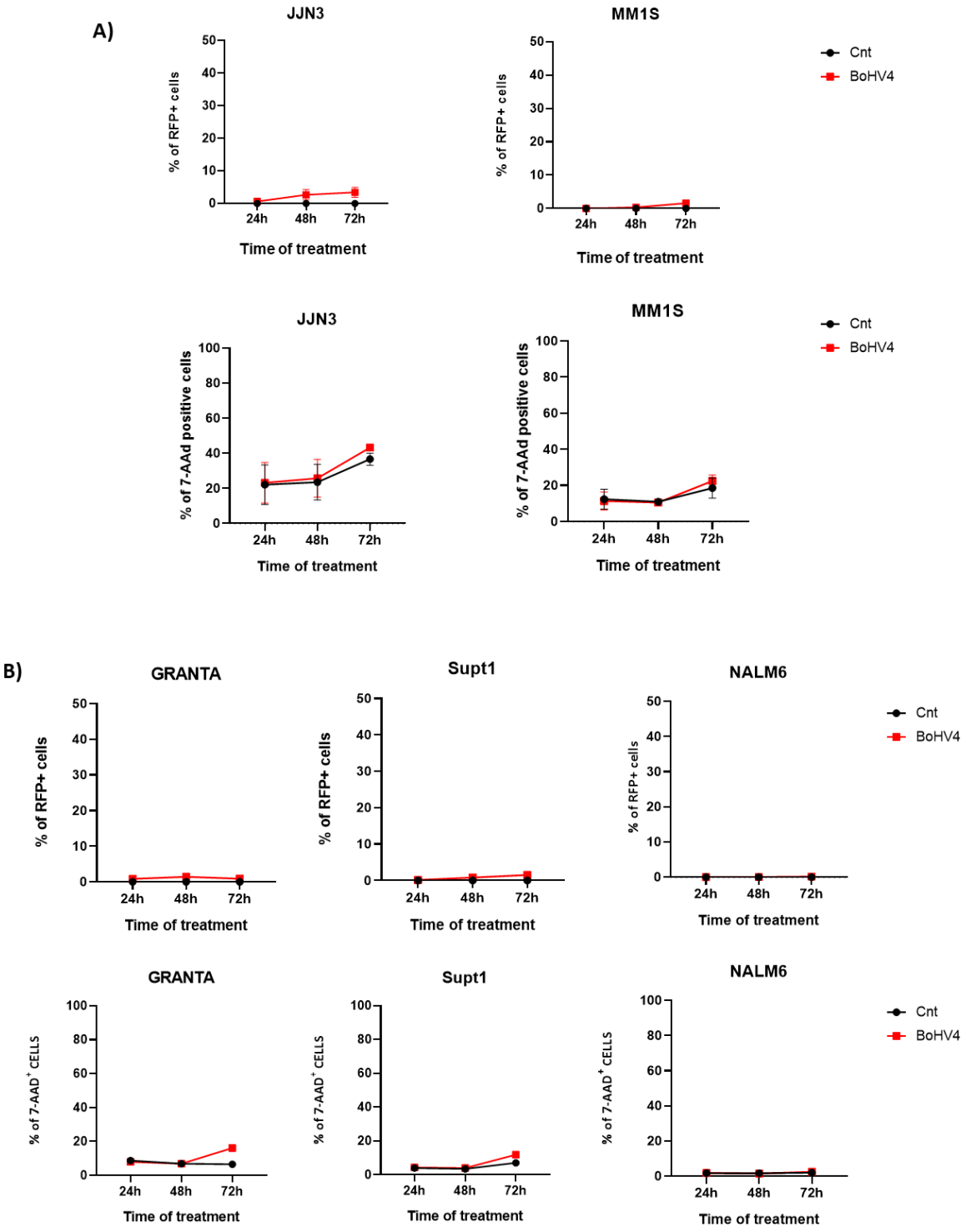


Figure 2

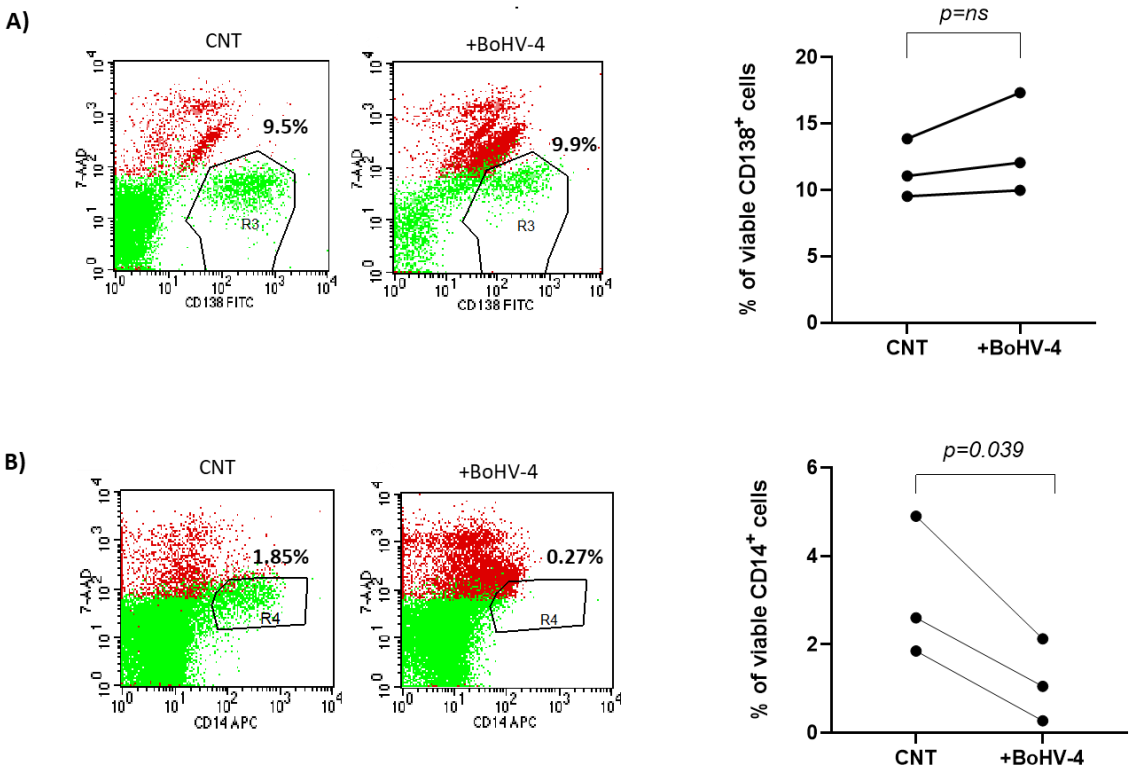

A)
CNT
+BoHV-4
9.5%
9.9%
R3
7-AAD
CD138 FITC
p=ns
% of viable CD138+ cells
CNT
+BoHV-4
B)
CNT
+BoHV-4
1.85%
0.27%
R4
7-AAD
CD14 APC
p=0.039
% of viable CD14+ cells
CNT
+BoHV-4

Figure 3

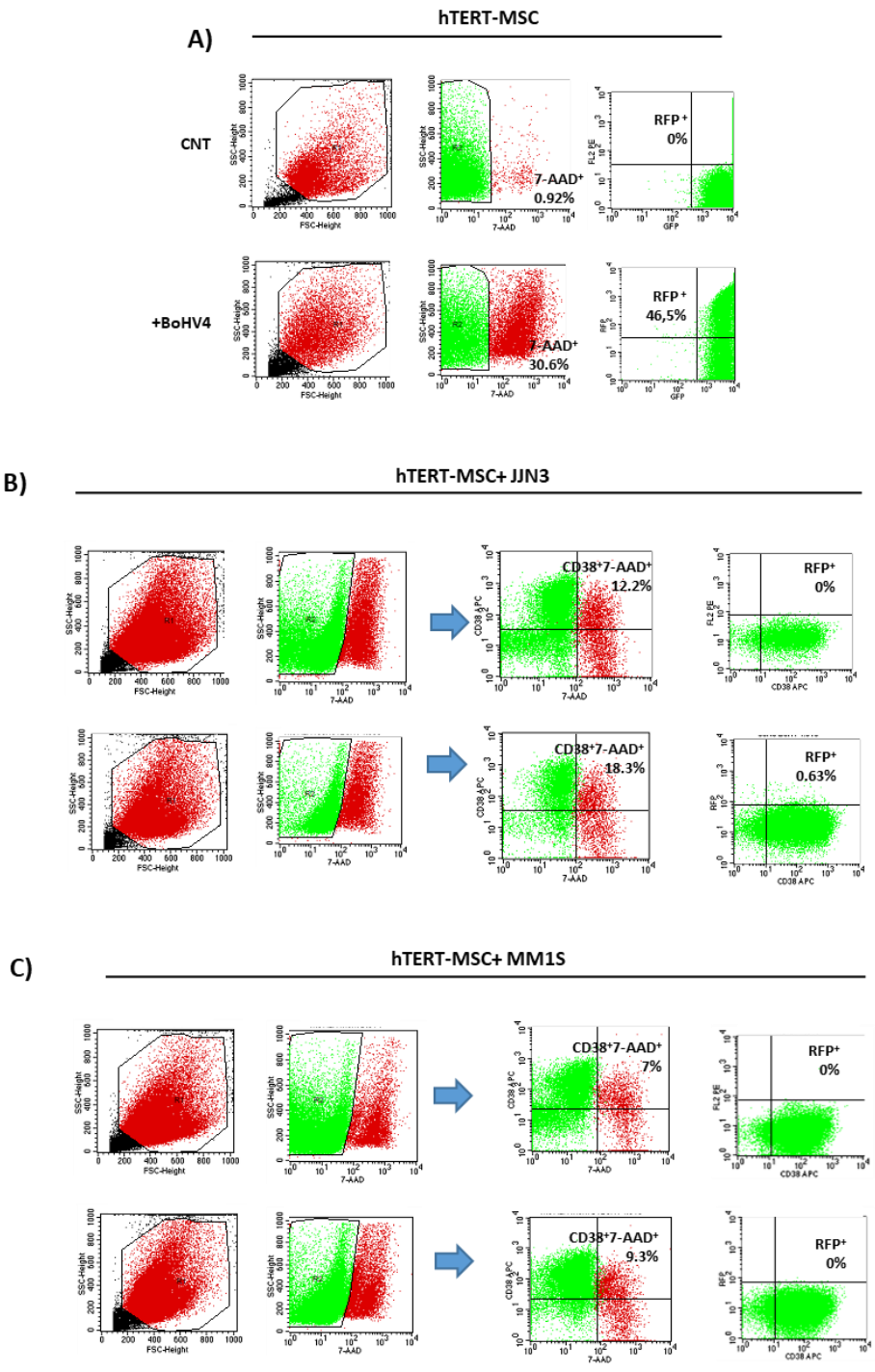

A)
hTERT-MSC
CNT
+BoHV4
7-AAD+ 0.92%
RFP+ 0%
7-AAD+ 30.6%
RFP+ 46,5%
B)
hTERT-MSC+ JJN3
CD38+7-AAD+ 12.2%
RFP+ 0%
CD38+7-AAD+ 18.3%
RFP+ 0.63%
C)
hTERT-MSC+ MM1S
CD38+7-AAD+ 7%
RFP+ 0%
CD38+7-AAD+ 9.3%
RFP+ 0%

Figure 4

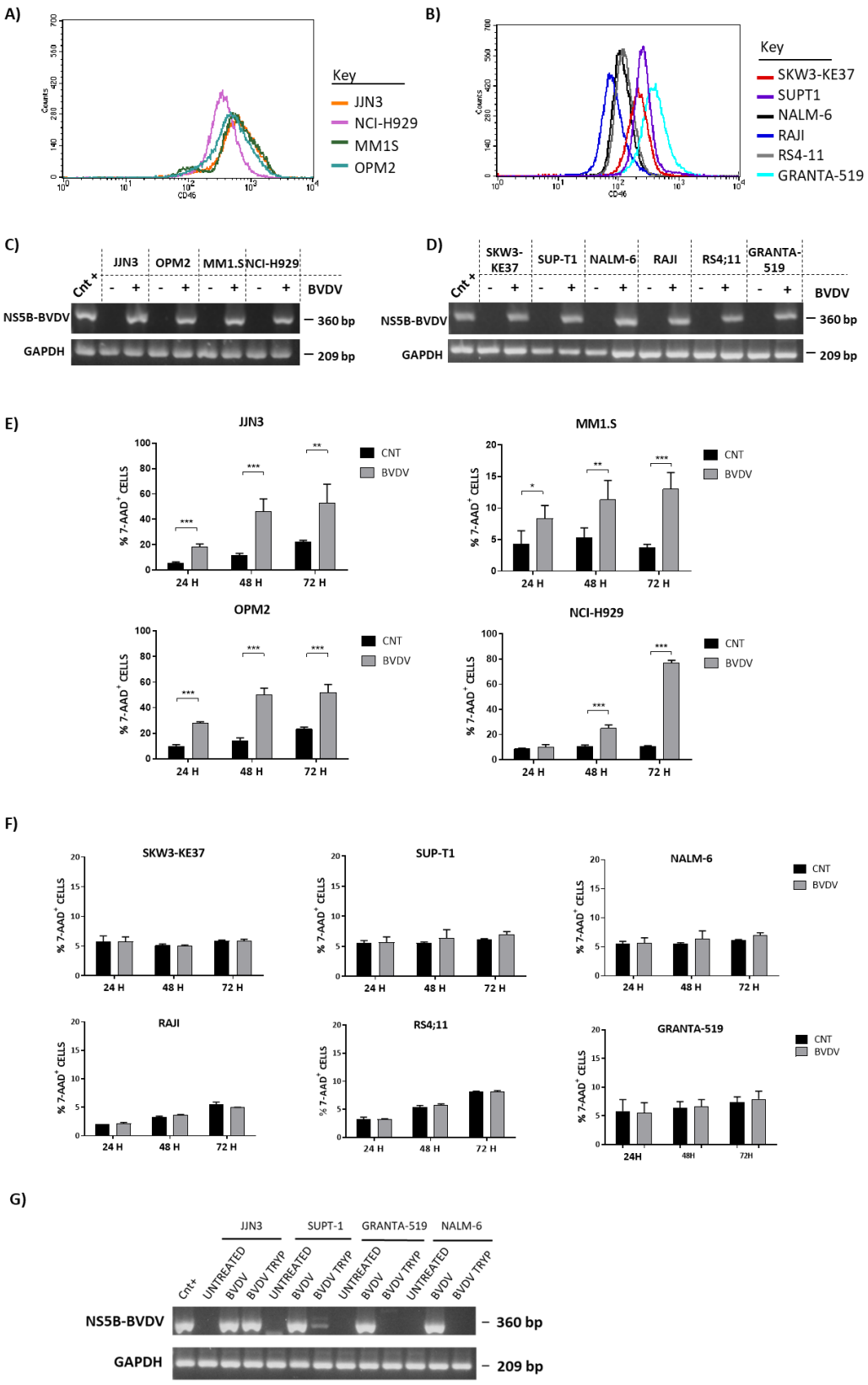

A)
Key
JJN3
NCI-H929
MM1S
OPM2
B)
Key
SKW3-KE37
SUPT1
NALM-6
RAJI
RS4-11
GRANTA-519
C)
JJN3
OPM2
MM1.S
NCI-H929
Cnt +
BVDV
NS5B-BVDV
360 bp
GAPDH
209 bp
D)
SKW3-KE37
SUP-T1
NALM-6
RAJI
RS4;11
GRANTA-519
Cnt +
BVDV
NS5B-BVDV
360 bp
GAPDH
209 bp
E)
JJN3
MM1.S
OPM2
NCI-H929
% 7-AAD+ CELLS
CNT
BVDV
24 H
48 H
72 H
F)
SKW3-KE37
SUP-T1
NALM-6
RAJI
RS4;11
GRANTA-519
% 7-AAD+ CELLS
CNT
BVDV
24 H
48 H
72 H
G)
JJN3
SUPT-1
GRANTA-519
NALM-6
Cnt+
UNTREATED
BVDV
BVDV TRVP
NS5B-BVDV
360 bp
GAPDH
209 bp

Figure 5

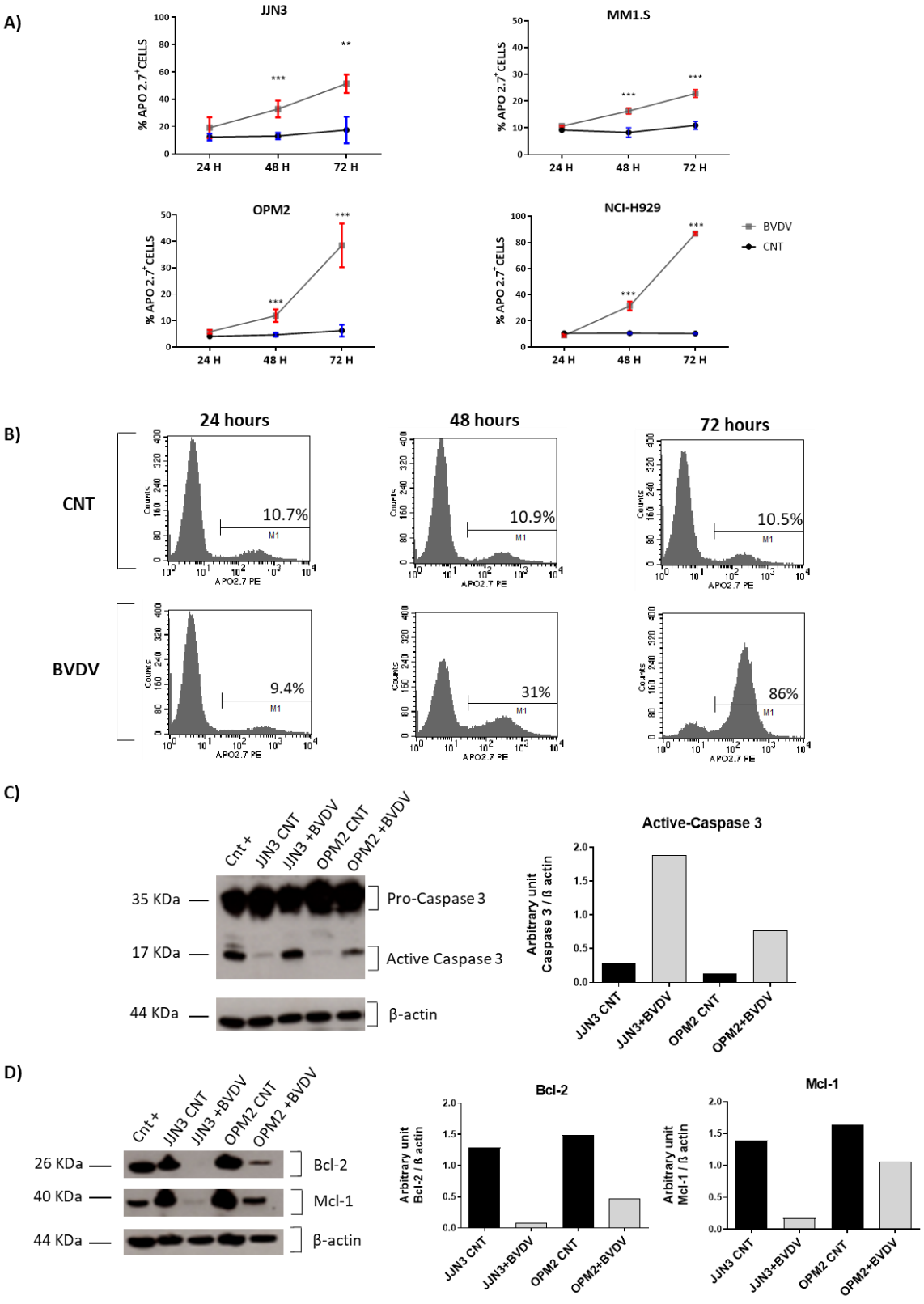

A)
JJN3
MM1.S
OPM2
NCI-H929
% APO 2.7+CELLS
24 H
48 H
72 H
BVDV
CNT
B)
24 hours
48 hours
72 hours
CNT
BVDV
10.7%
10.9%
10.5%
9.4%
31%
86%
M1
APO2.7 PE
Counts
C)
Cnt+
JJN3 CNT
JJN3 +BVDV
OPM2 CNT
OPM2 +BVDV
35 KDa
17 KDa
44 KDa
Pro-Caspase 3
Active Caspase 3
β-actin
Active-Caspase 3
Arbitrary unit Caspase 3 / ß actin
JJN3 CNT
JJN3+BVDV
OPM2 CNT
OPM2+BVDV
D)
26 KDa
40 KDa
44 KDa
Bcl-2
Mcl-1
β-actin
Arbitrary unit Bcl-2 / ß actin
Arbitrary unit Mcl-1 / ß actin

Figure 6

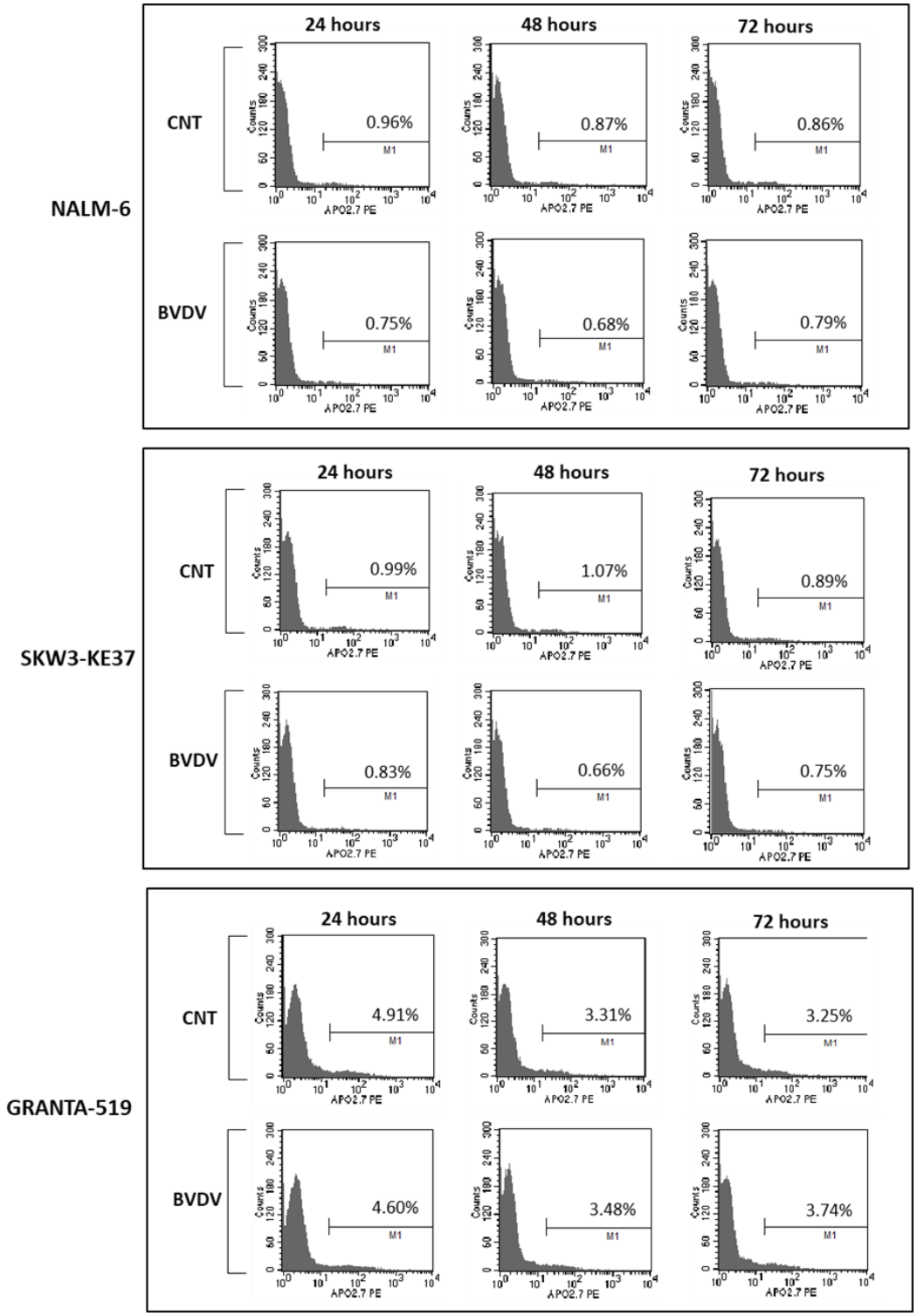

24 hours
48 hours
72 hours
NALM-6
CNT
0.96%
0.87%
0.86%
BVDV
0.75%
0.68%
0.79%
SKW3-KE37
CNT
0.99%
1.07%
0.89%
BVDV
0.83%
0.66%
0.75%
GRANTA-519
CNT
4.91%
3.31%
3.25%
BVDV
4.60%
3.48%
3.74%
M1
APO2.7 PE
Counts

Figure 7

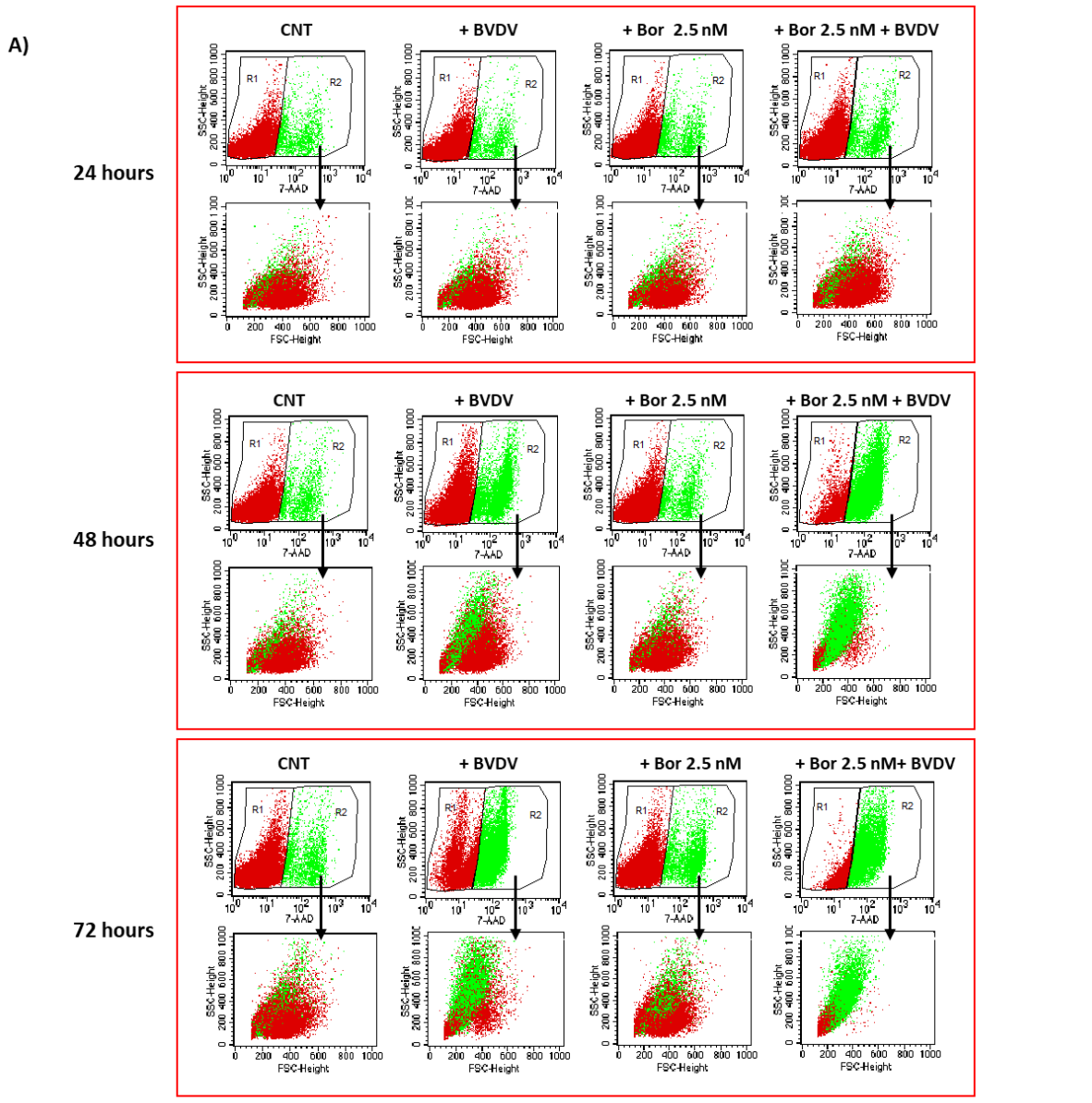

A)
CNT
+ BVDV
+ Bor 2.5 nM
+ Bor 2.5 nM + BVDV
24 hours
48 hours
72 hours
R1
R2
7-AAD
SSC-Height
FSC-Height
B)
24 hours
48 hours
72 hours
% 7-AAD POSITIVE CELLS
CNT
+ BVDV
+ Bor 2,5 nM
+ Bor 2,5 nM + BVDV
C)
BVDV (MOI)
Bortezomib (nM)

Figure 8

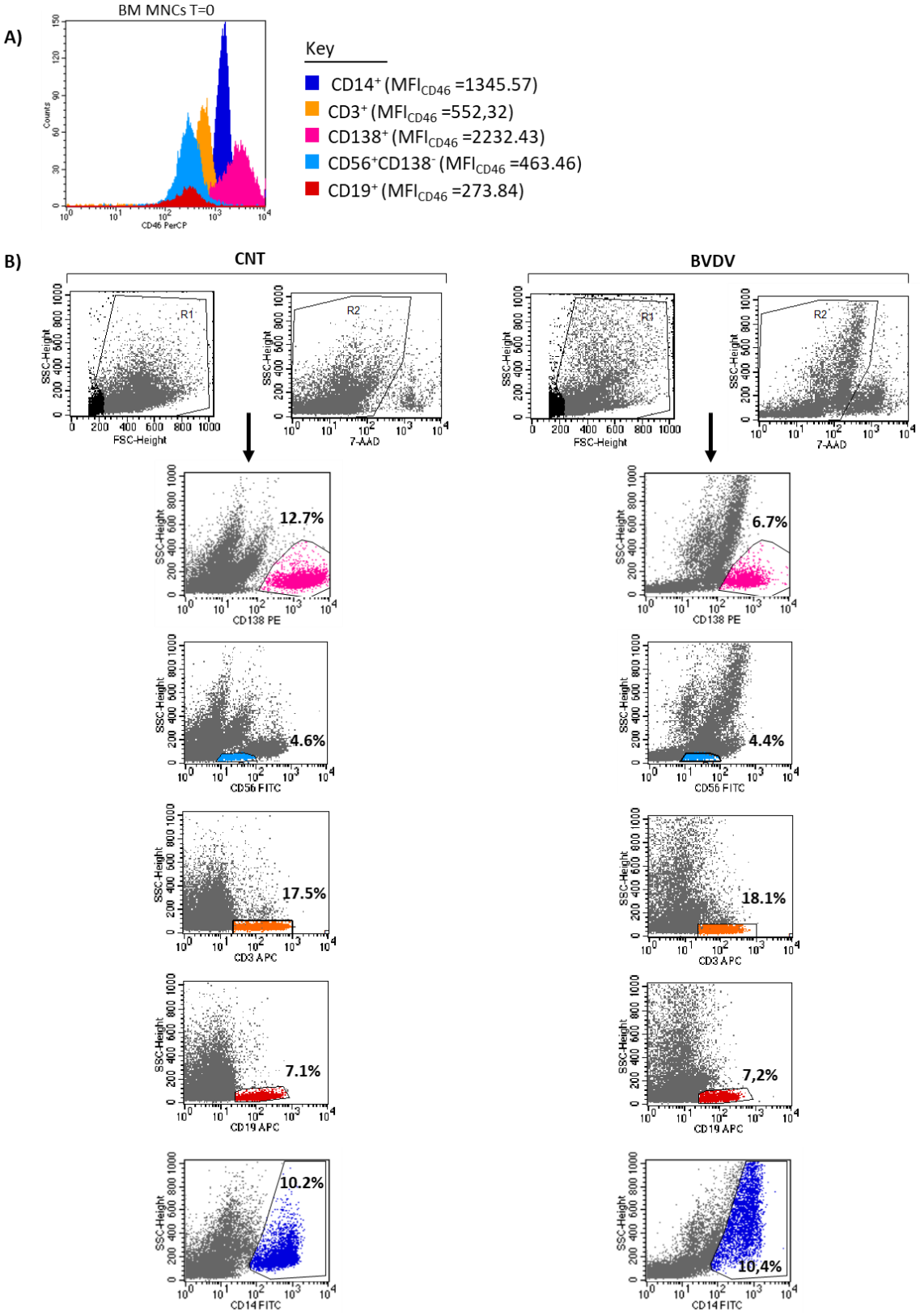

A)
BM MNCs T=0
Counts
CD46 PerCP
Key
CD14+ (MFICD46 =1345.57)
CD3+ (MFICD46 =552,32)
CD138+ (MFICD46 =2232.43)
CD56+CD138- (MFICD46 =463.46)
CD19+ (MFICD46 =273.84)
B)
CNT
BVDV
SSC-Height
FSC-Height
R1
R2
7-AAD
12.7%
6.7%
CD138 PE
4.6%
4.4%
CD56 FITC
17.5%
18.1%
CD3 APC
7.1%
7,2%
CD19 APC
10.2%
10,4%
CD14 FITC

Figure 9

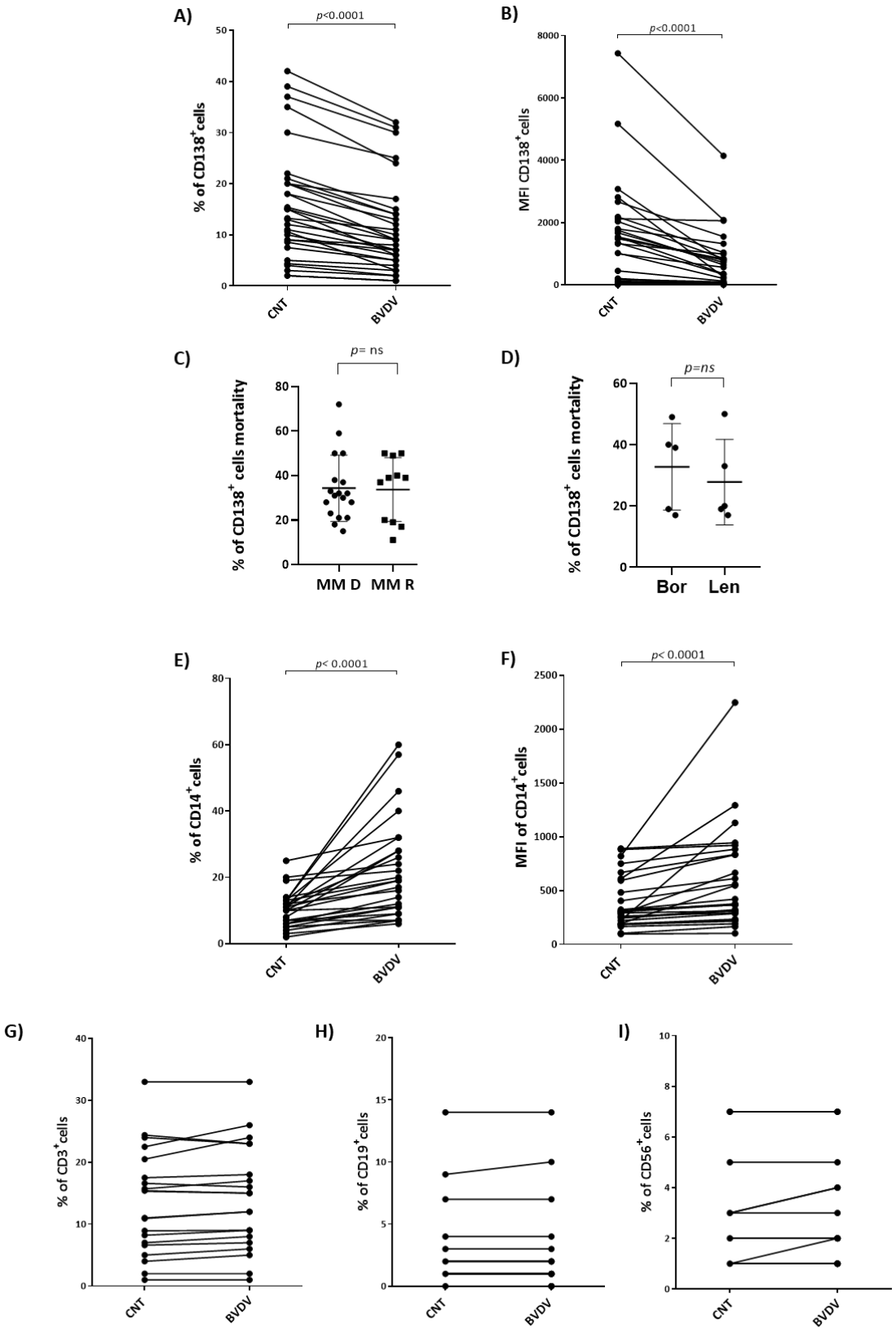
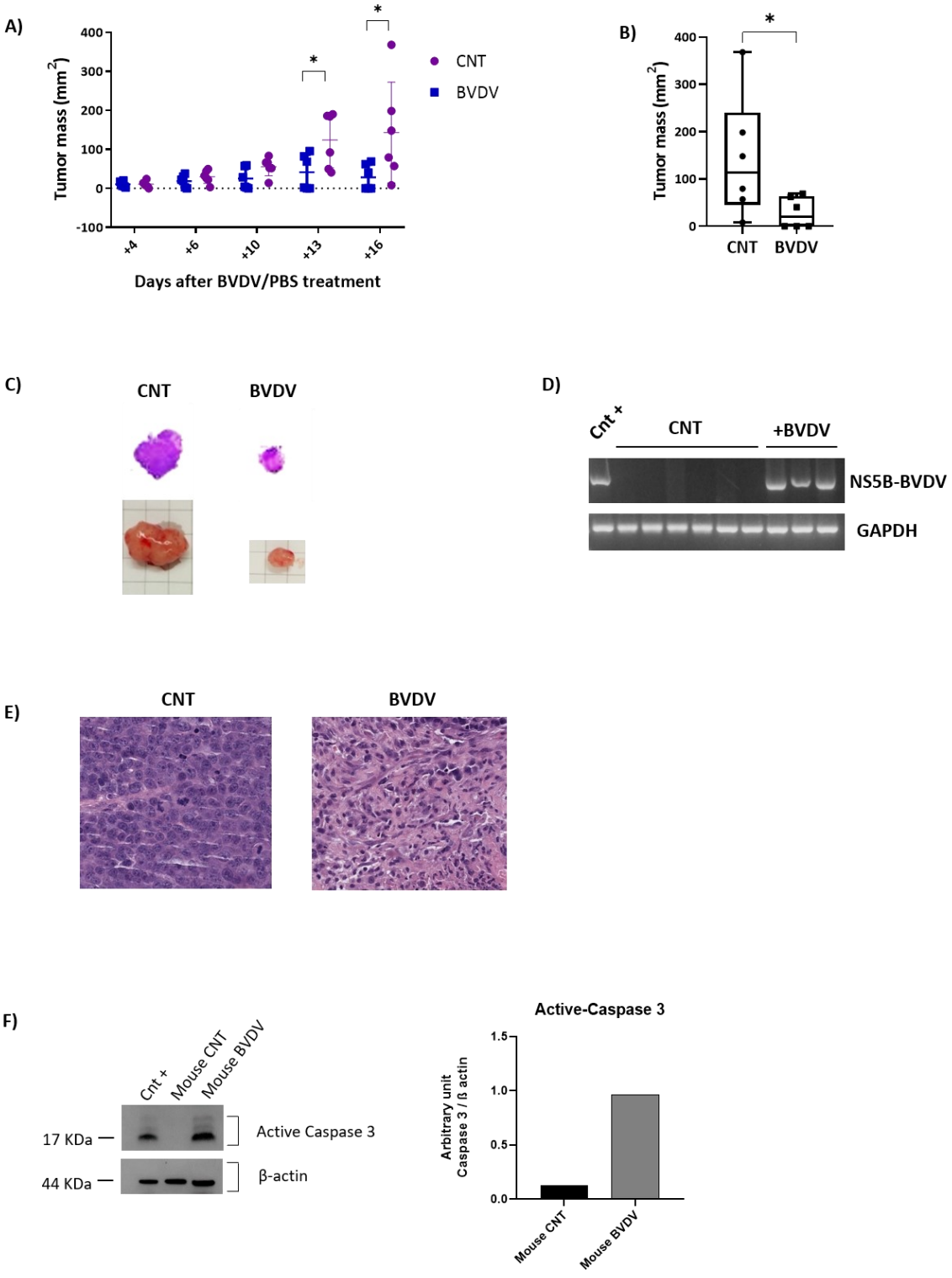

A)
p<0.0001
% of CD138+cells
CNT
BVDV
B)
p<0.0001
MFI CD138+cells
CNT
BVDV
C)
p= ns
% of CD138+ cells mortality
MM D
MM R
D)
p=ns
% of CD138+ cells mortality
Bor
Len
E)
p< 0.0001
% of CD14+cells
CNT
BVDV
F)
p< 0.0001
MFI of CD14+cells
CNT
BVDV
G)
% of CD3+cells
CNT
BVDV
H)
% of CD19+cells
CNT
BVDV
I)
% of CD56+cells
CNT
BVDV

Figure 10


A)
Tumor mass (mm2)
400
300
200
100
0
-100
+4
+6
+10
+13
+16
Days after BVDV/PBS treatment
CNT
BVDV
*
*
B)
Tumor mass (mm2)
400
300
200
100
0
CNT
BVDV
*
C)
CNT
BVDV
D)
Cnt +
CNT
+BVDV
NS5B-BVDV
GAPDH
E)
CNT
BVDV
F)
Cnt +
Mouse CNT
Mouse BVDV
17 KDa
44 KDa
Active Caspase 3
β-actin
Active-Caspase 3
Arbitrary unit
Caspase 3 / ß actin
1.5
1.0
0.5
0.0
Mouse CNT
Mouse BVDV