ARTICLE - Plasma Cell Disorders

Oncolytic bovine herpesvirus type 1 induces immune microenvironment remodeling and enhances treatment responses in multiple myeloma

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SUPPLEMENTARY INFORMATION

SUPPLEMENTARY METHODS

Cell lines and reagents

Cell lines: The bovine embryo kidney cell line (BS CL-94; BEK) was kindly provided by M. Ferrari (Istituto Zooprofilattico Sperimentale, Brescia, Italy). The Madin-Darby bovine kidney cell line (MDBK; ATCC CCL-22) was obtained from ATCC (Manassas, VA, USA). Cells were maintained in Eagle's Minimal Essential Medium (EMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), sodium pyruvate (1 mM), L-glutamine (2 mM), amphotericin B (0.25 μg/mL), and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) (all from Gibco, Segrate, Italy). The human myeloma cell lines (HMCLs) JJN-3, MM1.S, and OPM-2 were purchased from the Leibniz Institute Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). Cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated 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). The human stromal cell line HS-5 was purchased from ATCC (Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with the same supplements.

The human NK-92 cell line, an interleukin-2 (IL-2)-dependent natural killer cell line, was obtained from ATCC (Manassas, VA, USA). Cells were cultured in α-MEM supplemented with 12.5% heat-inactivated horse serum, 12.5% heat-inactivated FBS, L-glutamine (2 mM), amphotericin B (0.25 μg/mL), and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), and 100 U/mL recombinant human IL-2 (all from ThermoFisher Scientific, Monza, Italy).

All the cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Bovine virus: Bovine herpesvirus type 1 (BoHV-1) wild-type (Cooper strain (Colorado-1), ATCC) was propagated by infecting confluent monolayers of BEK or MDBK cells at a multiplicity of infection (MOI) of 0.1 50% tissue culture infectious doses ($TCID_{50}$) per cell. Cells were maintained in EMEM supplemented with 2% heat-inactivated FBS for 2 h. The medium was then removed and replaced with fresh EMEM containing 10% FBS. When cytopathic effect affected the majority of the cell monolayer (approximately 48 h post-infection), the virus was harvested by freezing and thawing the cells three times and pelleting the virions through a 30% sucrose gradient, as previously described.[29] Virus pellets were resuspended in cold EMEM without FBS. $TCID_{50}$ was determined in BEK or MDBK cells by limiting dilution. For control experiments, BoHV-1 was heat-inactivated by incubation at 95 °C for 1 h.

Drugs: Bortezomib (BTZ; Selleckchem, Munich, Germany), lenalidomide (LENA; Celgene, Milan, Italy), daratumumab (DARA; Janssen Biotech, USA), and elranatamab (ELRA; MedChemExpress, USA) were reconstituted according to the manufacturers' instructions and diluted in culture medium immediately before use.

Patient samples and cell isolation

Bone marrow (BM) aspirates were processed within 4 h of collection. BM mononuclear cells (BMMCs) were obtained from BM aspirates by Ficoll-Hypaque (Bichrome AG, Berlin, Germany) density gradient sedimentation and cultured in RPMI 1640 medium supplemented with 20% heat-inactivated 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). Fresh BMMCs were used to preserve cellular subset integrity and viability, with particular attention to myeloid populations, which are highly susceptible to freeze-thaw-induced perturbations.

$CD138^+$ cells were purified from the BMMCs of multiple myeloma (MM) patients by antibody-mediated positive selection using anti-CD138 magnetic-activated cell separation microbeads

(Miltenyi Biotec, Gladbach, Germany). The purity of immunoselected cells was assessed by flow cytometry analysis using a PE-conjugated anti-CD138 monoclonal antibody. Purified $CD138^+$ cells were seeded and cultured in RPMI 1640 medium supplemented with 20% heat-inactivated 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).

Monocytes were removed from BMMCs by antibody-mediated positive selection using anti-CD14 magnetic-activated cell separation microbeads (Miltenyi Biotec, Gladbach, Germany). The $CD14^-$ fraction (monocyte-depleted BMMCs) was seeded and cultured as described. Depletion efficiency was confirmed by flow cytometry using an anti-CD14 BV786 antibody.

Virus and drug treatments

The HMCLs and the human stromal cell line HS-5 were cultured with or without BoHV-1 for 24 h, 48 h, or 72 h. BoHV-1 was used at 1 and 2 MOI per $1x10^6$ cells. At the end of the experiments, cells were collected for flow cytometry analysis. For transcriptomic analysis, JJN-3 cells were infected with BoHV-1 at 1 MOI and incubated for 24 h. Afterward, the cells were harvested, washed with PBS, and lysed in RLT buffer (Qiagen, Hilden, Germany) for RNA extraction and RNA sequencing.

JJN-3 cells were also treated with BTZ (2, 3, or 5 nM) or LENA (1, 2, or 10 µM), as a single agent or administered concurrently with BoHV-1 (1 MOI) and incubated for 72 h. The MTT assay was used to assess the effect of treatments.

$CD138^+$ purified from BMMCs of MM patients ($0,25x10^6$ cells) were cultured with or without BoHV-1 at 1 and 2 MOI for 48 h, 72 h, or 96 h. After treatment, cells were harvested and analyzed using flow cytometry.

BMMCs from MM patients ($1x10^6$ cells) were cultured with or without BoHV-1 at 1 and 2 MOI for 48 h, 72 h, or 96 h. After treatment, all cells were harvested, washed, and analyzed using flow

cytometry. Cell-free supernatants from the culture were harvested by 8 minutes of centrifugation at 400 × g, aliquoted, and stored at -20 °C until use.

For combination experiments, BMMCs were treated with or without BoHV-1 at 1 and 2 MOI, either alone or in combination with BTZ (2 nM) or LENA (10 µM), both added at the time of infection. To explore combinations with antibody-based therapies, cells were first treated with BoHV-1, and then, 24 h or 48 h after viral exposure, ELRA (0.01 nM) or DARA (10 µg/mL) was administered, respectively. Cells were maintained in culture for a total of 96 h, after which they were harvested and analyzed by flow cytometry.

In vitro blockade of CD111, CD155, and CD138 receptors during BoHV-1 infection

MM1.S ($1x10^6$ cells) were pre-incubated for 1 h with or without 2 µL of each blocking antibody against CD111, CD155, and CD138 (all purchased from BioLegend, San Diego, CA, USA) in dual (CD111 and CD155) or triple (CD111, CD155, and CD138) combinations. Cells were then plated and treated with BoHV-1 at 1 MOI or left untreated. After 48 h, all cells were harvested and analyzed using flow cytometry.

NK cell-mediated cytotoxicity assay

NK-92 cell-mediated cytotoxicity was assessed following pre-treatment of target cells with BoHV-1. JJN-3 cells were infected with BoHV-1 for 24 h, washed extensively to remove residual virus, and then stained with 10nM of calcein-AM (Sigma-Aldrich, St. Louis, MO, USA) for 30 minutes at 37 °C. After incubation, JJN-3 cells were washed and co-cultured with NK-92 effector cells at the indicated effector-to-target (E:T) ratio for 4 h. At the end of co-culture, cells were harvested, stained with 5 µL of anti-CD56 PE-conjugated antibody (Beckman Coulter, Brea, CA, USA) to discriminate NK-92 cells ($CD56^+$) from JJN-3 target cells ($CD56^-$), and analyzed by flow cytometry. Cytotoxicity was determined by quantifying the percentage of JJN-3 cells ($CD56^-$) that were negative for calcein-AM fluorescence, indicating loss of membrane integrity.

Normalized cytotoxicity (%) was calculated using the following formula: [(cytotoxicity in co-culture - spontaneous cytotoxicity in monoculture) / (100 - spontaneous cytotoxicity in monoculture)] × 100.

Cytokine detection by ELISA

Cytokines were measured in the cell-free supernatants of BMMCs treated with or without BoHV-1, as described above. Levels of IFN-α, TNF-α, IFN-γ, IL-6, and IL-1β were determined using commercial ELISA kits (Human IFN-α All Subtype ELISA Kit, Human TNF-α Quantikine HS ELISA Kit, Human IFN-γ Quantikine HS ELISA Kit, Human IL-6 Quantikine HS ELISA Kit, and Human IL-1 beta/IL-1F2 Quantikine HS ELISA Kit; all from R&D Systems, Minneapolis, MN, USA), according to the manufacturers' protocols. Absorbance was measured at 450 nm with wavelength correction at 540 nm using a microplate reader. Cytokine levels were determined from standard curves included in each assay.

Flow cytometry

For all flow cytometry procedures, sample acquisition was performed using a two-laser FACSCelesta cytometer equipped with FACSDiva Software (v8.02; BD Biosciences, Franklin Lakes, NJ, USA), and data were analyzed and plotted using FlowJo Software (v10; BD Biosciences, Franklin Lakes, NJ, USA). Single-fluorochrome compensation was carried out using BD CompBeads and patient-derived BMMCs. Protocols were optimized to minimize inter-patient variability. BD FACSDiva CS&T research beads were used to monitor and adjust photomultiplier tube settings over time. To ensure reproducibility, the same antibody lots were used for all patient samples, and a single operator performed gating and data analysis. All FACS plots were manually inspected to confirm staining quality and data acquisition integrity.

CD111, CD155, and CD138 expression: expression levels of CD111, CD155, and CD138 antigens were determined on HMCLs and HS-5 stromal cells by flow cytometry analysis and expressed as median fluorescence intensity (MFI). In particular, $0.2x10^6$ HMCLs or HS-5 stromal cells were stained in

parallel with either a saturating concentration of anti-CD111 (BioLegend, San Diego, CA, USA), anti-CD155 PE, or anti-CD138 BV421 (both BD Biosciences, Franklin Lakes, NJ, USA) for 30 minutes at 4 °C, protected from light. Cells were then washed with a washing buffer (PBS plus 5% human serum albumin and 5% w/v sodium azide) and directly analyzed by flow cytometry. Before the acquisition, the 7-Amino Actinomycin D (7-AAD) was added according to the manufacturer's instructions. After identification of singlets and exclusion of debris, viable cells were identified as 7-AAD$^-$ events in side scatter (SSC) vs 7-AAD plots. Unstained samples were used to define gating boundaries.

CD111, CD155, and CD138 expression levels on fresh BMMCs were detected by staining 0.5x10^6 cells/tube with a cocktail of saturating concentrations of antibodies in 50 µl of brilliant stain buffer (BD Biosciences, Franklin Lakes, NJ, USA) and 50 µl of washing buffer supplemented with 10% of mouse serum. The antibodies (all, except anti-CD111, purchased from BD Biosciences, Franklin Lakes, NJ, USA) were combined in the two following panels: (1) anti-CD138 BV421, anti-CD38 BV480, anti-CD3 BV605, anti-CD16 BV650, anti-CD56 BV711, anti-CD14 BV786, anti-CD11b FITC, anti-CD111 or anti-CD155 PE, and anti-CD19 PE-CF594; (2) anti-CD138 BV421, anti-CD38 BV480, anti-CD3 BV605, anti-CD16 BV650, anti-CD56 BV711, anti-CD14 BV786, anti-CD34 FITC, anti-CD111 or anti-CD155 PE, and anti-CD19 PE-CF594. Fluorescence-minus-one (FMO) controls were used to define PE gating boundaries. After incubation for 30 minutes at 4 °C protected from light, BMMCs were washed with the washing buffer. Before flow cytometry acquisition, 7-AAD was added according to the manufacturer's instructions. Then, viable cells were identified as 7-AAD$^-$ events, and CD111 or CD155 expression levels were determined on specific gates identifying hematopoietic stem and progenitor cells (HSPCs) (CD34$^+$), T cells (CD3$^+$), NK cells (CD3$^-$CD16$^+$CD56$^+$CD138$^-$), B cells (CD19$^+$), plasma cells (PCs) (CD138$^+$CD38$^+$), monocytes (CD11b$^+$CD14$^{dim/+}$) and non-monocyte myeloid cells (SSC^{high}CD11b$^+$CD14$^-$). The full gating strategy is provided in Supplementary Figure S1A.

Viability staining and apoptotic assay on cell lines: Untreated or BoHV-1-treated HMCLs and HS-5 stromal cells were stained, according to the manufacturer's instructions, with 7-AAD (BD Biosciences, Franklin Lakes, NJ, USA). After identification of singlets and exclusion of debris, viable and non-viable cells were identified as 7-AAD$^-$ or 7-AAD$^+$ events, respectively, in SSC vs 7-AAD plots. Apoptosis was assessed by the APO2.7 evaluation, which specifically detects 7A6, a 38-kDa mitochondrial membrane antigen expressed during apoptosis. After treatment, cells were collected, stained with a saturating concentration of PE-conjugated APO2.7 antibody (Beckman Coulter, Brea, CA, USA) for 30 minutes at 4 °C protected from light, and analyzed by flow cytometry.

Identification of BMMCs subsets: After treatments, BMMCs were collected, washed, and stained with a cocktail of saturating concentrations of antibodies in 50 µl of brilliant stain buffer (BD Biosciences, Franklin Lakes, NJ, USA) and 50 µl of washing buffer supplemented with 10% of mouse serum. The antibodies (all purchased from BD Biosciences, Franklin Lakes, NJ, USA) were combined in the following panel: anti-CD138 BV421, anti-CD38 BV480, anti-CD3 BV605, anti-CD16 BV650, anti-CD56 BV711, anti-CD14 BV786, anti-CD11b FITC, anti-CD34 PE, and anti-CD19 PE-CF594. After incubation for 30 minutes at 4 °C protected from light, BMMCs were washed with the washing buffer. Before flow cytometry acquisition, 7-AAD was added according to the manufacturer's instructions. PCs were identified as CD138$^+$CD38$^+$. Non-tumor cells, defined as those outside this gate, included HSPCs (CD34$^+$), T cells (CD3$^+$), NK cells (CD3$^-$CD16$^+$CD56$^+$), B cells (CD19$^+$), and myeloid cells (SSC^{high}CD11b$^+$CD14$^{-/+}$) were gated. The full gating strategy is provided in Supplementary Figure S1B.

The BoHV-1 oncolytic effect on PCs was calculated using the following formula: % of CD138$^+$CD38$^+$ cells normalized viability = (% of CD138$^+$CD38$^+$7-AAD$^-$ cells in the treated condition / % of CD138$^+$CD38$^+$7-AAD$^-$ cells in the untreated condition) x 100. In this calculation, the percentage of viable PCs in the untreated condition was set to 100%, and the viability of treated samples was

expressed relative to this value. This calculation was similarly applied to each non-tumor cell population.

Assessment of CD107a and CD69 expression: BMMCs ($1x10^6$ cells) were cultured with or without BoHV-1 at 1 and 2 MOI for 18 h. To assess degranulation, 1 µL of anti-CD107a PE (BD Biosciences, Franklin Lakes, NJ, USA) was added at the beginning of the culture. After treatment, BMMCs were washed and stained with a cocktail of saturating concentrations of antibodies (all purchased from BD Biosciences, Franklin Lakes, NJ, USA) combined in the following panel: anti-CD138 BV421, anti-CD38 BV480, anti-CD3 BV605, anti-CD16 BV650, anti-CD56 BV711, anti-CD14 BV786, anti-CD8 FITC, anti-CD19 PE-FC594. After incubation for 30 minutes at 4 °C protected from light, BMMCs were washed with the washing buffer. Before flow cytometry acquisition, 7-AAD was added according to the manufacturer's instructions. CD107a expression was evaluated on both $CD8^+$ T cells ($CD3^+CD8^+$) and NK cells ($CD3^-CD16^+CD56^+CD138^-$). For activation analysis, CD69 expression was assessed under the same culture conditions on $CD8^+$ T cells ($CD3^+CD8^+$), NK cells ($CD3^-CD16^+CD56^+CD138^-$), and mono/macrophages ($CD14^{dim/+}$). Anti-CD69 PE (BD Biosciences, Franklin Lakes, NJ, USA) was added at the end of the 18-h culture, together with the same antibody panel and staining protocol described above, including 7-AAD labeling before acquisition. The full gating strategy is provided in Supplementary Figure S2.

Bulk RNA sequencing

RNA isolation: Total cellular RNA was extracted from untreated and BoHV-1-treated JJN3 using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA quantity was measured with a NanoDrop One spectrophotometer (ThermoFisher Scientific, Monza, Italy), and RNA integrity was assessed using either the Agilent 2100 Bioanalyzer RNA assay or the TapeStation system (Agilent Technologies, Santa Clara, CA, USA).

Library preparation and sequencing: RNA-seq libraries were prepared using the VAHTS Universal V10 RNA-Seq Library Prep Kit in combination with VAHTS mRNA Capture Beads 2.0 (Vazyme, Nanjing, China), following the manufacturer's instructions (library type: fr-firststrand). Final libraries were quality-checked using the Qubit 3.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) and the Agilent Bioanalyzer DNA assay. Paired-end sequencing (2 × 150 bp) was performed on an Illumina NovaSeq X-Plus platform (Illumina, San Diego, CA, USA).

Bioinformatics analysis: Adaptor sequences and poly(T) tails were trimmed from raw reads using fqtrim (version 0.9.7; http://ccb.jhu.edu/software/fqtrim/). Reads were aligned to the human reference genome (GRCh38) using STAR (v2.7.10a), and gene-level quantification was performed with featureCounts (v2.0.3) using GENCODE v43 annotation. Differential expression analysis was conducted using DESeq2. Genes with a false discovery rate (FDR) < 0.05 were considered significantly differentially expressed. For volcano plot visualization, a more stringent threshold (FDR < 0.0005) and a baseMean > 20 were applied to highlight robust and biologically relevant changes. Specific thresholds used for the volcano plot are detailed in the corresponding figure legend. Gene set enrichment analysis (GSEA) was performed using GSEA software (version 4.4.0, Broad Institute) with predefined gene sets from the Molecular Signatures Database (MSigDB v2024.1). Gene ranking was based on log2 (Fold change) or the ashr shrinkage method implemented in DESeq2. Statistical significance of enrichment was assessed using 10,000 permutations. Gene sets and pathways with FDR < 0.05 were considered significantly enriched. All visualizations were generated using ggplot2 (v3.4.4).

Immunoblot

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) and mouse

monoclonal anti-Vinculin (R&D Systems, Minneapolis, MN, USA) as an internal control. The secondary antibody, peroxidase-conjugated, was anti-mouse (BD Pharmingen, Franklin Lakes, NJ, USA).

Statistical analysis

Data from cell lines are presented as mean ± standard deviation (SD) and were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test. Data from primary cells are shown as median with interquartile range (IQR) and were analyzed using the Friedman test, followed by Dunn's multiple comparisons test, as data were paired. The number of patient samples used for different experiments is specified in each figure legend. Correlation coefficients were calculated using the Spearman rank correlation method. All statistical tests were two-sided, with a significance threshold set at $p < 0.05$. Statistical significance is indicated as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. Data analysis and graphical representations were performed using GraphPad Prism, version 10.1.1 (GraphPad Software Inc., La Jolla, CA, USA).

Supplementary Table

Supplementary Table S1. Main characteristics of MM patients.

PATIENT ID	GENDER	AGE (years)	STAGE	Ig SUBTYPE	LIGHT CHAIN ISOTYPE	% PCs (BMA)	PREVIOUS LINES OF THERAPY*	R-ISS	CYTOGENETIC RISK
MM-1	F	71	ND	IgG	λ	7,5%		I	HR
MM-2	M	57	ND	Light chain	κ	18,0%		I	SR
MM-3	F	82	ND	IgA	κ	19,6%		I	SR
MM-4	F	75	ND	IgA	κ	40,0%		II	SR
MM-5	M	59	ND	IgG	κ	23,0%		I	SR
MM-6	M	57	ND	Light chain	λ	52,0%		III	HR
MM-7	F	66	ND	IgG	λ	53,0%		II	SR
MM-8	F	86	ND	IgG	κ	35,0%		III	HR
MM-9	F	63	ND	Light chain	λ	43,0%		II	SR
MM-10	M	69	ND	IgA	κ	32,0%		II	HR
MM-11	M	73	ND	IgG	κ	34,0%		II	HR
MM-12	M	81	ND	IgA	λ	17,0%		III	HR
MM-13	F	46	ND	IgG	κ	17,0%		I	HR
MM-14	M	58	ND	IgG	λ	42,0%		III	HR
MM-15	F	83	ND	IgG	κ	29,0%		I	SR
MM-16	M	47	ND	IgA	κ	17,0%		I	SR
MM-17	F	85	ND	IgG	κ	23,0%		III	HR
MM-18	M	94	ND	IgG	κ	15,0%		II	SR
MM-19	F	64	ND	IgG	κ	10,0%		II	HR
MM-20	F	82	ND	IgG	λ	13,0%		I	SR
MM-21	F	47	ND	IgA	κ	10,0%		I	HR
MM-22	F	77	ND	Non-secretory		6,0%		II	HR
MM-23	F	48	ND	IgA + BJ	κ	22,0%		III	HR
MM-24	M	67	ND	IgG	κ	24,0%		I	SR
MM-25	M	77	ND	IgG	κ	22,0%		II	HR
MM-26	M	48	ND	IgG	κ	20,0%		II	SR
MM-27	F	48	ND	IgG	κ	30,0%		II	SR
MM-28	F	83	ND	IgA	λ	16,0%		I	HR
MM-29	M	80	RR	IgG	κ	20,4%	2	I	SR
MM-30	M	74	RR	IgA	λ	50,0%	1	III	HR
MM-31	M	77	RR	IgG	λ	20,0%	1	II	SR
MM-32	F	83	RR	IgG	λ	20,0%	1	I	SR
MM-33	F	74	RR	IgG	κ	68,0%	3	III	SR
MM-34	M	79	RR	IgA	κ	10,0%	3	II	SR

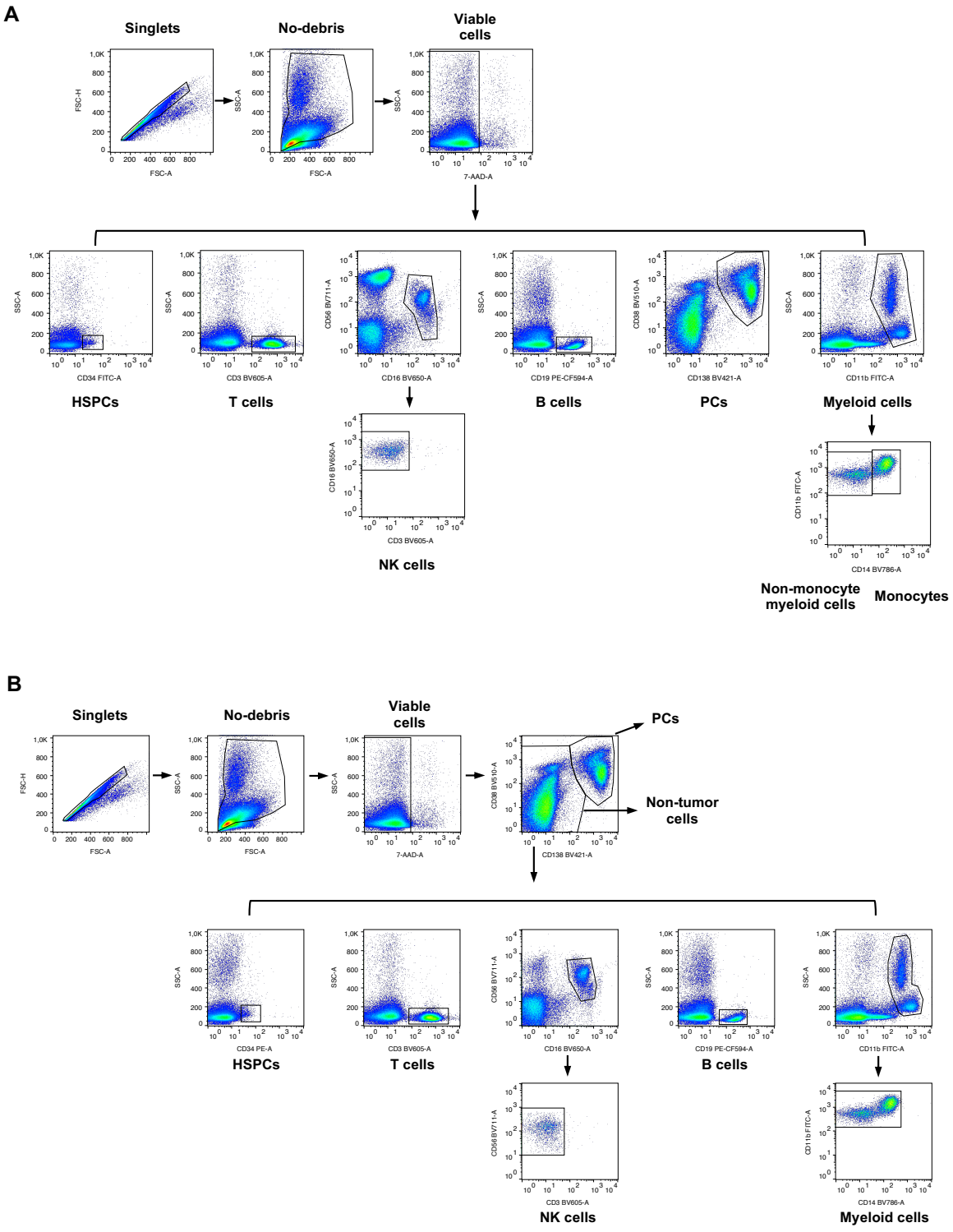
MM-35	M	72	RR	IgA	λ	30,0%	3	III	HR
MM-36	M	76	RR	IgG	κ	14,0%	6	II	HR
MM-37	M	64	RR	IgA	κ	40,0%	3	II	HR
MM-38	F	58	RR	IgG	λ	10,0%	1	II	HR
MM-39	M	68	RR	IgA	κ	53,0%	2	III	SR

***** Applicable only to patients with relapsed disease

Abbreviations: MM: multiple myeloma; ND: newly diagnosed; RR: relapsed/refractory; F: female; M: male; Ig: immunoglobulin; BMA: bone marrow aspirate; R-ISS: revised international staging system; HR: high-risk; SR: standard-risk.

SUPPLEMENTARY FIGURES AND FIGURE LEGENDS

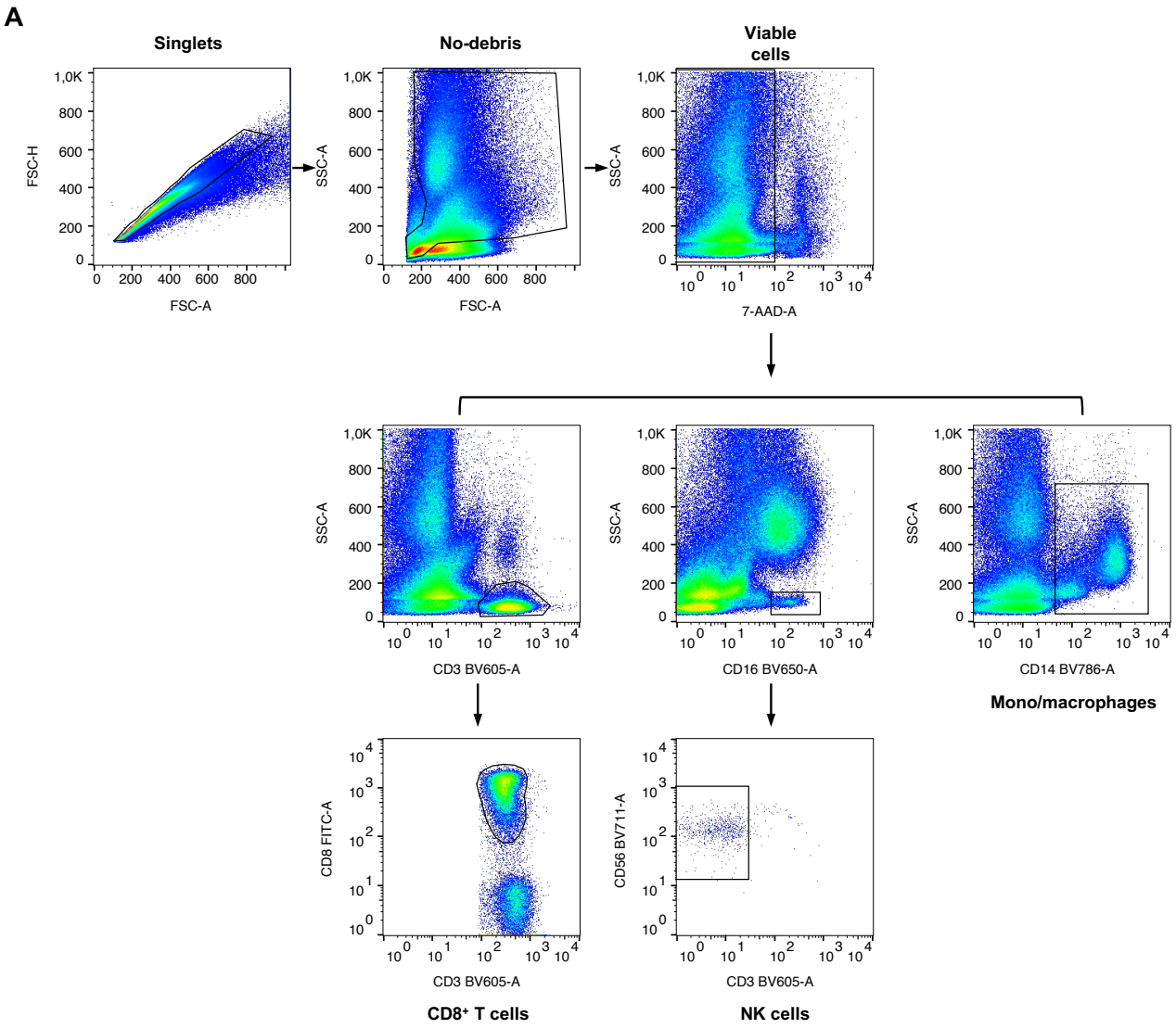
Supplementary Figure S1



Supplementary Figure S1. Flow cytometry gating strategies for BM subpopulation analysis.

A) Gating strategy for the identification of BM populations in fresh BMMCs stained for CD111 or CD155. Singlets were first gated, followed by the exclusion of debris. Among the no-debris population, viable cells (7-AAD^{-}) were gated. From viable cells, HSPCs (CD34^{+}), T cells (CD3^{+}), NK cells (CD3^{-}CD16^{+}CD56^{+}CD138^{-}), B cells (CD19^{+}), PCs (CD138^{+}CD38^{+}), monocytes (CD11b^{+}CD14$^{dim/+}$), and non-monocyte myeloid cells (SSC^{high}CD11b^{+}CD14^{-}) were identified. PE gates were set using FMO controls. **B)** Gating strategy for BMMC populations after treatment. Singlets were gated, followed by the exclusion of no-debris. Among the no-debris population, viable cells (7-AAD^{-}) were gated. Viable cells were stratified into tumor cells, defined as PCs (CD138^{+}CD38^{+}), and non-tumor cells. Within non-tumor cells, HSPCs (CD34^{+}), T cells (CD3^{+}), NK cells (CD3^{-}CD16^{+}CD56^{+}), B cells (CD19^{+}), and myeloid cells (SSC^{high}CD11b^{+}CD14$^{-/+}$) were identified.

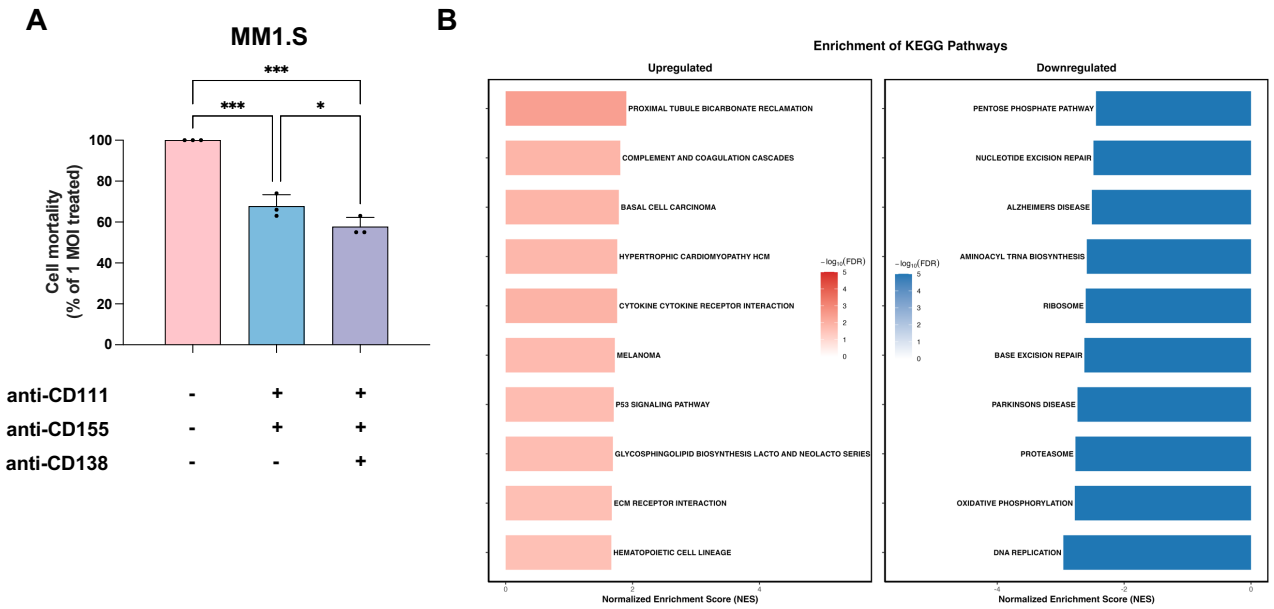
Supplementary Figure S2



Supplementary Figure S2. Gating strategy for the assessment of CD107a and CD69 expression on BM immune effector cells.

Singlets were first gated, followed by the exclusion of debris. From the no-debris cells, viable cells (7-AAD^-) were gated. Within viable cells, $CD3^+CD8^+$ T cells, NK cells ($CD3^-CD16^+CD56^+CD138^-$), and mono/macrophages ($CD14^{dim/+}$) were identified.

Supplementary Figure S3

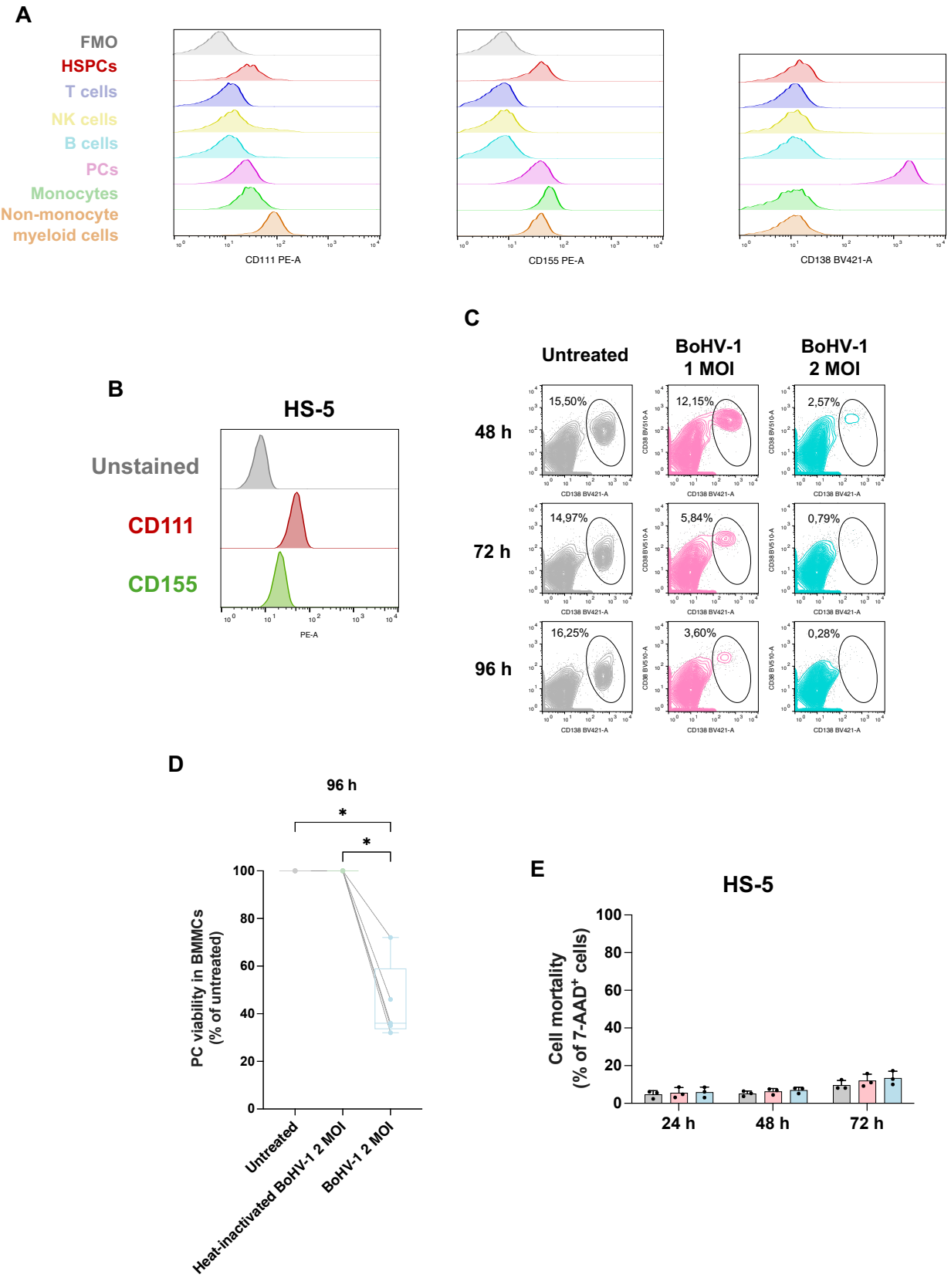


Supplementary Figure S3. BoHV-1 receptor blockade and transcriptional profiling of BoHV-1-infected HMCLs.

A) Relative cell mortality of MM1.S cells pre-treated with CD111, CD155, and CD138 blocking antibodies and infected with BoHV-1 (1 MOI, 48 h), vs infected cells without blockade. Bars indicate mean ± SD; each dot represents an independent experiment (n=3). Statistical analysis was performed using two-way ANOVA with Tukey's multiple comparisons test.

B) KEGG pathway enrichment analysis of JJN-3 cells following BoHV-1 infection (1 MOI, 24 h), showing the top 10 significantly upregulated and downregulated pathways. KEGG pathways were considered significantly enriched at FDR < 0.05. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Supplementary Figure S4

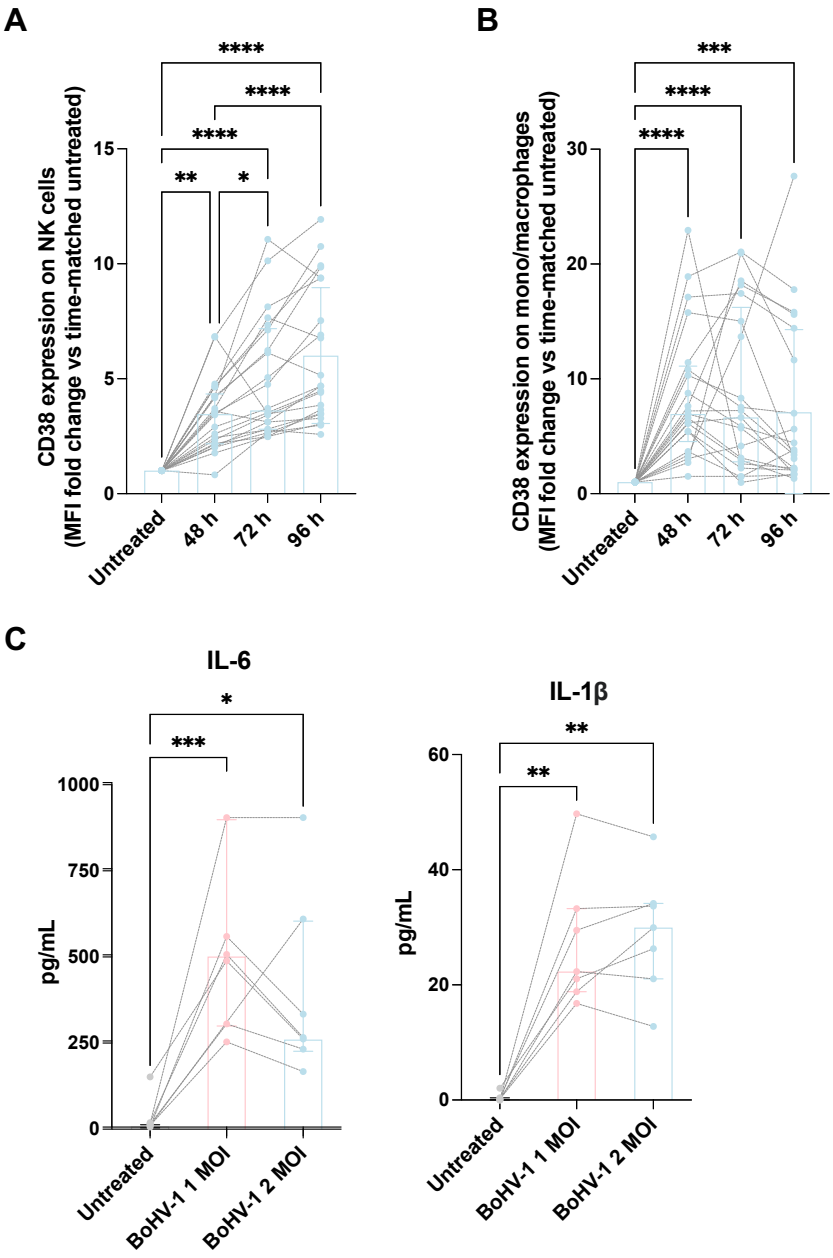


Supplementary Figure S4. BoHV-1 entry receptor expression and cell viability in MM patient cells and HS-5 stromal cells.

A) Flow cytometry histograms from one representative MM patient, showing the expression levels of CD111, CD155, and CD138 on HSPCs, T cells, NK cells, B cells, PCs, monocytes, and non-monocyte myeloid cells. **B)** Flow cytometry histograms showing the CD111 and CD155 expression levels on HS-5 stromal cells. **C)** Representative flow cytometry dot plots showing the percentage of viable PCs within total BMMCs from one MM patient, untreated or BoHV-1-treated (1 and 2 MOI) for 48 h, 72 h, and 96 h. **D)** Relative viability of PCs in total BMMCs from MM patients (n=5) treated with BoHV-1 (heat-inactivated 2 MOI and 2 MOI), for 96 h vs the untreated samples. Each dot represents an individual patient. Bars and boxplots indicate medians with IQR; lines connect paired samples where applicable. **E)** Assessment of cell mortality (as 7-AAD$^+$ cells) in HS-5 stromal cells following BoHV-1 infection at 1 and 2 MOI for 24 h, 48 h, and 72 h.

Each dot represents an independent experiment (n=3); bars indicate mean ± SD. Statistical analyses were performed using the Friedman test with Dunn's multiple comparisons test **(D)** and two-way ANOVA with Tukey's multiple comparisons test (**E)**. *$p < 0.05$

Supplementary Figure S5

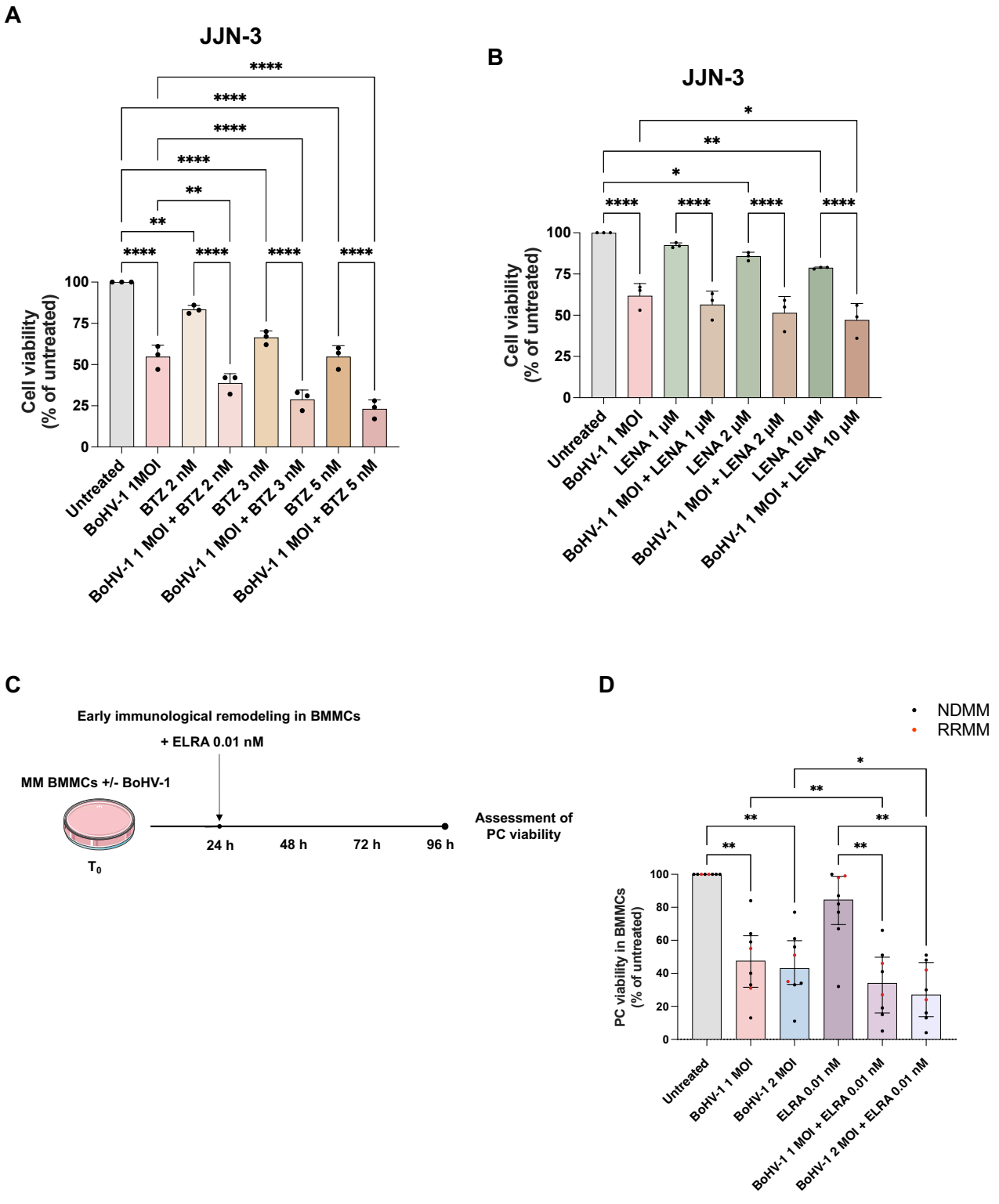


Supplementary Figure S5. Temporal modulation of CD38 expression in NK cells and mono/macrophages and cytokine induction upon BoHV-1 infection.

Relative CD38 expression on **A)** NK cells and on **B)** mono/macrophages among BMMCs treated with BoHV-1 (2 MOI) at 48 h, 72 h, and 96 h compared to time-matched untreated samples (results pooled from 22 patients). **C)** IL-6 and IL-1β levels in the cell-free supernatants of MM patients' BMMCs (n=7) treated with or without BoHV-1 (1 and 2 MOI for 48 h).

Each dot represents an individual patient. Bars and boxplots indicate medians with IQR; lines connect paired samples where applicable. Statistical analyses were performed using the Friedman test with Dunn's multiple comparisons test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

Supplementary Figure S6



Supplementary Figure S6. Experimental design and functional evaluation of BoHV-1–based combination treatments.

A) Relative viability of JJN-3 cells 72 h after treatment with BoHV-1 (1 MOI), BTZ (2, 3, or 5 nM), alone or in combination. **B**) Relative viability of JJN-3 cells 72 h after treatment with BoHV-1 (1 MOI), LENA (1, 2, or 10 μM), alone or in combination. **C)** Experimental design for BoHV-1 and ELRA combination. **D)** Relative viability of PCs from MM patients' BMMCs 96 h after treatment with BoHV-1 (1 and 2 MOI), ELRA (0.01 nM), alone or in combination (n=8).

Each treatment condition was compared with the untreated control. Data from JJN-3 cells are presented as mean ± SD from three independent experiments. Statistical analysis was performed using repeated-measures one-way ANOVA with Tukey's multiple comparisons test. Data from patient-derived BMMCs are presented as median and IQR from paired experiments. Black dots represent patients with NDMM, while red dots represent those with RRMM. Statistical analysis was performed using the Friedman test with Dunn's correction. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$