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

Vincenzo Raimondi,[1] Rosanna Vescovini,[1] Paola Storti,[1] Valentina Franceschi,[2] Giulia Pozzi,[1] Camilla Sitzia,[1] Nicolas Thomas Iannozzi,[1] Denise Toscani,[1,3] Benedetta Dalla Palma,[3] Matteo Scita,[3] Mattia Dessena,[1] Sergio Minesso,[2] Stefania Ricci,[1] Fazel Mohammadi,[1] Oxana Lungu,[1] Prisco Mirandola,[1] Gaetano Donofrio[2] and Nicola Giuliani[1,3]

[1]Department of Medicine and Surgery, University of Parma; [2]Department of Medical-Veterinary Science, University of Parma and [3]Hematology, "Azienda Ospedaliero-Universitaria di Parma", Parma, Italy

Correspondence: N. Giuliani
nicola.giuliani@unipr.it

G. Donofrio
gaetano.donofrio@unipr.it

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Abstract

Despite therapeutic advances, multiple myeloma (MM) remains incurable due to the development of drug resistance by malignant plasma cells (PC) and a severe immunosuppressive bone marrow (BM) microenvironment. Oncolytic virotherapy offers the dual benefit of tumor cell lysis and immune activation, but the efficacy of human viruses is often hampered by pre-existing antiviral immunity. Here, we demonstrate that bovine herpesvirus type 1 (BoHV-1), a virus that is non-pathogenic to humans, efficiently infected MM cells, inducing mitochondrial apoptosis and suppressing pro-survival programs, including MYC targets, oxidative phosphorylation, and the unfolded protein response. Infected tumor cells up-regulated NK-activating ligands and down-regulated MHC class I, enhancing susceptibility to NK-mediated cytotoxicity. In patient-derived BM mononuclear cells (BMMC), BoHV-1 selectively reduced malignant PC and immunosuppressive myeloid subsets, while sparing lymphoid populations and hematopoietic progenitors. The infection promoted activation of $CD8^+$ T cells, natural killer cells, and mono/macrophages, driving a shift toward a pro-inflammatory M1-like polarization. Monocyte depletion in BMMC attenuated the BoHV-1 anti-MM effect, confirming their functional contribution. This pronounced immune remodeling was accompanied by an inflammatory cytokine storm dominated by type I/II interferons and key innate immune mediators. Co-treatment of BoHV-1 with either bortezomib or lenalidomide increased anti-MM cytotoxicity. Finally, BoHV-1 up-regulated CD38 on both MM cells and immune effectors, thereby increasing sensitivity to the anti-CD38 daratumumab. These findings establish BoHV-1 as a promising immunovirotherapy agent, effective as a single agent and in combination strategies, by coupling direct oncolysis with broad immune remodeling of the BM microenvironment.

Introduction

Multiple myeloma (MM) is a clonal malignancy of plasma cells (PC) originating within the bone marrow (BM), leading to several clinical complications.[1] Despite advancements in therapeutic approaches, MM remains an incurable disease, as patients experience multiple relapses and eventually develop resistance to conventional anti-MM therapies.[1,2] Immune dysregulation is a feature of MM patients and is involved in the pathophysiology of the disease and therapeutic response.[3] Interactions between MM cells and immune cells within the BM microenvironment are associated with the formation of a milieu favorable to tumor survival and progression.[4] Notably, T cells display transcriptional signatures of exhaustion, whereas natural killer (NK) cells, monocytes, and macrophages exhibit functional impairments that facilitate immune evasion, underscoring the rationale for immunotherapy-based strategies in MM.[5-10] Oncolytic viruses (OV) are immunotherapeutic agents that couple selective tumor cell killing with the induction of systemic anti-tumor immunity.[11] Several human viruses have been explored as oncolytic vectors, with efforts focused on attenuating pathogenicity and enhancing immunogenicity.[12] In MM, measles virus (MV) and reovirus (RV) advanced to early-phase trials but showed limited efficacy: MV-based therapy amplified anti-MM T-cell activity without inducing

objective responses, while RV monotherapy achieved only transient disease stabilization.[13,14] More broadly, the therapeutic promise of human OV is constrained by host immunity, as antiviral responses and pre-existing neutralizing antibodies often limit viral replication and persistence.[15] These challenges have fueled interest in non-human OV, which circumvent pre-existing immunity. Preclinical studies have highlighted vesicular stomatitis virus (VSV) and bovine viral diarrhea virus (BVDV) as promising candidates.[16,17] However, early clinical testing of VSV in relapsed/refractory MM (RRMM) patients yielded only stable disease despite an acceptable safety profile.[16] Collectively, these findings underscore the need for novel OV with distinct biological properties and improved efficacy.[17]

Bovine herpesvirus type 1 (BoHV-1) is a double-stranded DNA virus belonging to the Alphaherpesvirinae subfamily; it is responsible for bovine respiratory disease.[18,19] Structurally, BoHV-1 closely resembles herpes simplex virus 1 and employs similar attachment and entry receptors, including heparan sulfate proteoglycans (HSPG), the nectin-1 (CD111), and the poliovirus receptor (CD155).[20,21] Preclinical studies have shown that oncogenic KRAS signaling can enhance BoHV-1 replication in selected tumor models; however, viral replication is not strictly required for BoHV-1-mediated tumor cell killing.[22]

It is known that BoHV-1 does not productively infect normal human cells;[23,24] instead, it selectively targets immortalized, transformed, and breast cancer-initiating cells.[24,25] Moreover, in a melanoma mouse model, it has been demonstrated that virus replication is not required for oncolytic BoHV-1 immunotherapy effects, suggesting that its anti-tumor activity may be uncoupled from its ability to replicate and induce cytopathic effect.[26]

The oncolytic activity of BoHV-1 has never been explored in hematologic malignancies, but its unique biological and immunological features support it as a promising candidate for oncolytic virotherapy in MM. Here we demonstrate that, besides a direct oncolytic effect, BoHV-1 provides a potent immunovirotherapy that, in combination with anti-MM therapies, may improve overall treatment outcomes.

Methods

Cell lines and reagents

All bovine and human cell lines, along with their respective culture conditions, were maintained as described in the *Online Supplementary Methods*.

Ethics approval and consent to participate

Patient samples were obtained after informed consent in accordance with the Declaration of Helsinki. This study was approved by the Area Vasta Emilia Nord ethics committee of the Regional Health Service, Italy (protocol code: 69/2023/TESS/UNIPR; CE:21/03/2023).

Patient samples and cell isolation

A total cohort of 39 consecutive patients with MM was included in the study: 28 newly diagnosed MM (NDMM) (median age 68 years; range 46-94) and 11 RRMM (median age 74 years; range 58-83). All patients were diagnosed according to the International Myeloma Working Group (IMWG) revised criteria.[27] Cytogenetic risk was defined according to the IMS/IMWG 2024 consensus recommendations.[28] The main characteristics of all the patients enrolled in the study are summarized in *Online Supplementary Table S1*. Patients' BM mononuclear cells (BMMC) were isolated by Ficoll density gradient centrifugation. CD138$^+$ PC were purified by positive magnetic selection, and monocytes were depleted using anti-CD14 antibody-conjugated magnetic microbeads. Detailed protocols for cell isolation and culture conditions are provided in the *Online Supplementary Methods*.

Virus propagation, infection, and drug treatments

BoHV-1 wild-type (strain Cooper, ATCC) was propagated as previously described.[29] Human myeloma cell lines (HMCL), HS-5 stromal cells, patient-derived CD138$^+$ PC, and BMMC were treated with BoHV-1 at 1 and 2 MOI. BMMC were also treated with heat-inactivated BoHV-1 (2 MOI). Cell-free supernatants were collected from BMMC cultures for subsequent analyses. Combination treatments with bortezomib (BTZ), lenalidomide (LENA), daratumumab (DARA), and elranatamab (ELRA) were performed either concurrently with BoHV-1 infection or sequentially. Detailed protocols for virus propagation, infection, and drug treatments are provided in the *Online Supplementary Methods*.

In vitro blockade of CD111, CD155, and CD138 receptors

Blocking experiments targeting CD111, CD155, and CD138 putative BoHV-1 receptors were performed as described in the *Online Supplementary Methods*.

Natural killer cell-mediated cytotoxicity assay

NK-92-mediated cytotoxicity against BoHV-1-pretreated JJN-3 cells was evaluated by flow cytometry using calcein-AM staining, as detailed in the Online Supplementary Methods.

Cytokine detection by ELISA

Levels of IFN-α, TNF-α, IFN-γ, IL-6, and IL-1β in cell-free BMMC supernatants were measured by ELISA, as detailed in the *Online Supplementary Methods*.

Flow cytometry

Immunophenotyping, viability, apoptosis, degranulation, and activation marker analyses were performed by multiparametric flow cytometry using standardized protocols and antibody panels, as detailed in the *Online Supplementary Methods*. Gating strategies and data analysis procedures are provided in *Online Supplementary Figures S1* and *S2*.

Bulk RNA sequencing

Total RNA was extracted from untreated and BoHV-1-treated JJN-3 cells, and RNA-seq libraries were prepared and sequenced on an Illumina NovaSeq platform. Reads were processed, aligned to the human genome (GRCh38), and quantified using standard bioinformatics pipelines. Differential gene expression analysis was performed using DESeq2, and gene set enrichment analysis (GSEA) was conducted as detailed in the *Online Supplementary Methods*. Further methodological details, including software versions, quality control, and thresholds, are provided in the *Online Supplementary Methods*.

Immunoblot

Immunoblotting analyses were performed as described in the *Online Supplementary Methods*.

Statistical analysis

Data are presented as mean ± standard deviation (SD) for cell lines and as median with interquartile range (IQR) for primary cells. Parametric and non-parametric tests were applied as appropriate, including one-way ANOVA with Tukey's test and the Friedman test with Dunn's correction. Correlations were assessed by Spearman's method. All statistical tests were two-sided. $P<0.05$ was considered statistically significant. Further details are provided in the *Online Supplementary Methods*.

Results

BoHV-1 infection elicits intrinsic apoptosis and suppresses survival pathways in human myeloma cell lines

To investigate the susceptibility of malignant PC to BoHV-1, we profiled the surface expression of canonical viral attachment and entry receptors, CD111 and CD155, across a panel of HMCL. Given the role of HSPG in viral attachment, we also assessed the expression of syndecan-1 (CD138), a predominant HSPG and well-known hallmark of PC. Flow cytometry analysis revealed high, albeit heterogeneous, expression of CD111 and CD155 across JJN-3, MM1.S, and OPM-2 cells, accompanied by uniformly elevated CD138 levels, as expected, delineating a receptor landscape compatible with BoHV-1 entry (Figure 1A).

To functionally evaluate BoHV-1 treatment, the HMCL were infected at 1 and 2 MOI, and cell mortality was monitored via 7-AAD staining. All HMCL, including OPM-2 cells (KRAS

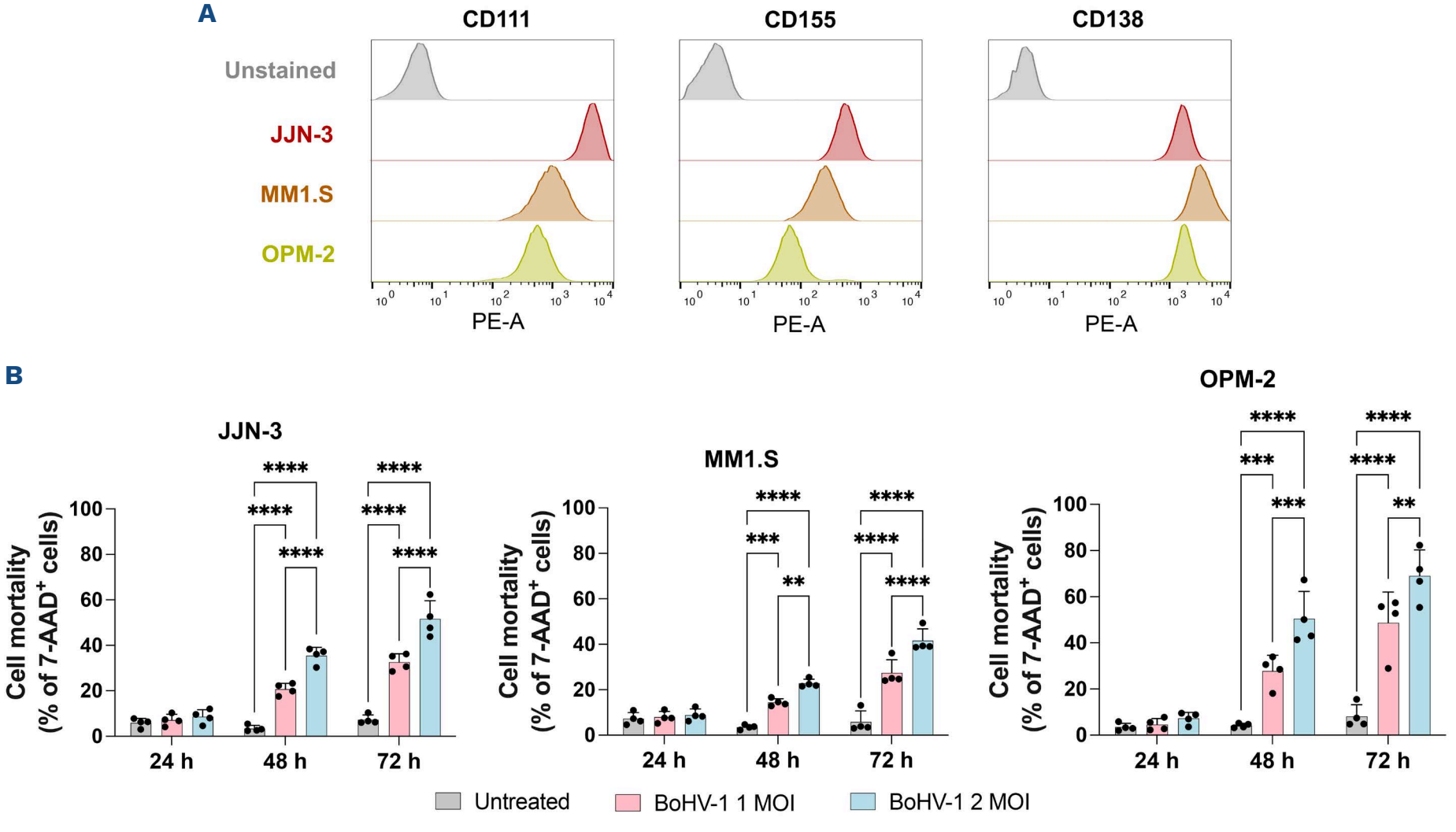


Figure 1. BoHV-1 efficiently targets human myeloma cell lines and induces cytotoxicity. (A) Flow cytometry histograms showing the expression levels of CD111, CD155, and CD138 in JJN-3, MM1.S, and OPM-2 cells. (B) Assessment of cell mortality in JJN-3, MM1.S, and OPM-2 following BoHV-1 infection at 1 and 2 MOI for 24 hours (h), 48 h, and 72 h. Each dot represents an independent experiment (N=4); bars indicate mean ± standard deviation. Statistical significance was determined using two-way ANOVA with Tukey's multiple comparisons test. **$P<0.01$, ***$P<0.001$, ****$P<0.0001$.

wild-type), displayed a progressive, MOI- and time-dependent increase in cell death, with significant cytotoxicity at 48 hours (h) and further exacerbation at 72 h post infection (Figure 1B), consistent with a robust cytopathic effect.

To confirm the involvement of BoHV-1 attachment and entry receptors, MM1.S cells were pre-incubated with blocking antibodies targeting CD111, CD155, and CD138 antigens. The dual blockade of CD111 and CD155 significantly reduced BoHV-1-induced cytotoxicity, and this effect was further enhanced by concurrent blockade of CD138 (*Online Supplementary Figure S3A*), suggesting that BoHV-1 entry is at least partially dependent on the coordinated expression of these surface receptors.

Next, we assessed whether BoHV-1-induced cytotoxicity was mediated by apoptosis. Flow cytometric analysis revealed a time-dependent increase in apoptotic cells at 48 h and 72 h post infection (Figure 2A), corroborated by immunoblot detection of cleaved pro-caspase-3 at 48 h (Figure 2B), indicating activation of the intrinsic apoptotic cascade.

To investigate the broader transcriptional response to BoHV-1, we performed bulk RNA sequencing of JJN-3 cells infected at 1 MOI for 24 h. Differential expression analysis identified 1,075 up-regulated and 216 down-regulated transcripts (False Discovery Rate [FDR] <0.0005) (Figure 2C). GSEA using the MSigDB Hallmark gene sets revealed significant enrichment of apoptosis, p53 signaling, TNFα signaling via NF-κB, and inflammatory response (Figure 2D), aligning with phenotypic evidence of cell death and inflammatory activation.

Conversely, gene sets related to proliferation and metabolic fitness were significantly down-regulated, including MYC targets, oxidative phosphorylation, and the unfolded protein response (UPR), a pathway critical for proteostasis in immunoglobulin-producing cells (Figure 2D). KEGG pathway analysis supported these findings, highlighting extensive transcriptional remodeling during BoHV-1 infection (*Online Supplementary Figure S3B*).

BoHV-1 targets malignant plasma cells in the primary bone marrow microenvironment

To validate our findings in HMCL in the primary BM setting, we evaluated the expression of BoHV-1 attachment and entry receptors in BMMC from MM patients. Representative flow cytometry histograms of CD111, CD155, and CD138 expression in the major immune cell subsets from one patient are shown in *Online Supplementary Figure S4A*. Cohort-wide analysis confirmed that CD111 and CD155 were most abundantly expressed on myeloid cells (both non-monocyte myeloid cells and monocytes), followed by PC and hematopoietic stem and progenitor cells (HSPC), with no detectable expression in T cells, NK cells, or B cells (Figure 3A). As expected, the expression of CD138 was consistently restricted to the PC compartment (Figure 3A). Given that stromal cells provide the structural backbone of hematopoietic and PC niches,[30] yet are challenging to identify within BMMC, we used the stromal cell line HS-5 to assess the expression of BoHV-1 attachment and entry receptors in non-hematopoietic cells. Flow cytometric analysis revealed minimal surface expression of CD111 and CD155 (*Online Supplementary Figure S4B*), suggesting a restricted capacity for viral infection.

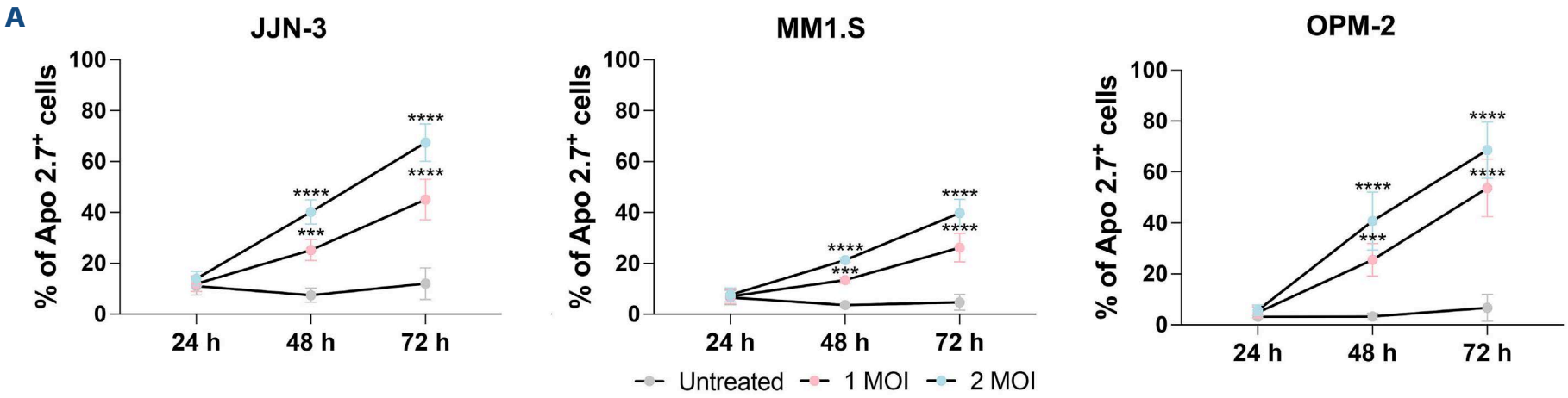
To assess the susceptibility of primary PC to BoHV-1 infection, the $CD138^+$ cells purified from patient-derived BMMC were infected. A significant time- and dose-dependent reduction in viability was observed at 72 h and 96 h post infection (Figure 3B), consistent with a direct cytopathic effect of the virus on MM cells. Subsequently, we investigated the effect of BoHV-1 on total BMMC from MM patients at 48 h, 72 h, and 96 h post infection. As shown in a representative case (*Online Supplementary Figure S4C*), BoHV-1 treatment induced a progressive reduction in the percentage of MM cells. Across the cohort, a significant time- and dose-dependent decrease in $CD138^+CD38^+$ cell viability was observed compared to untreated samples, with median viabilities of 79% and 73% at 48 h, 59% and 48% at 72 h, and 52% and 35% at 96 h for 1 and 2 MOI, respectively (Figure 3C). Treatment with heat-inactivated BoHV-1 did not affect PC viability in BMMC, indicating that cytotoxicity requires intact viral structural proteins (*Online Supplementary Figure S4D*).

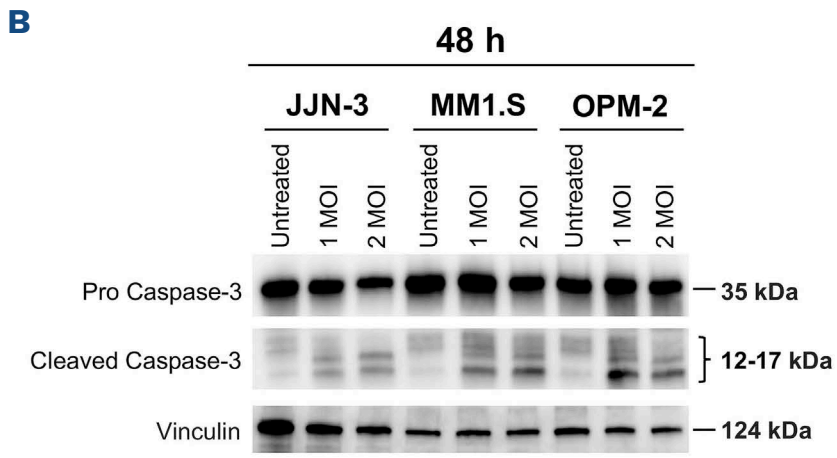
To determine whether the efficiency of BoHV-1-induced cytotoxicity correlated with tumor burden, we analyzed the association between baseline MM cells percentage in untreated BMMC and post-infection viability. No significant correlation was observed (r = -0.1532) (Figure 4A), suggesting that viral activity is independent of initial PC load. Furthermore, the extent of BoHV-1-mediated reduction in BM PC viability was comparable between NDMM and RRMM patients (Figure 4B) and across standard-risk (SR) and high-risk (HR) cytogenetic groups (Figure 4C).

To further characterize the impact of BoHV-1 beyond the malignant compartment, we analyzed its effect on non-tumor populations within the BMMC. 96 h post infection, BoHV-1 treatment resulted in a significant reduction of the percentage of myeloid cells, which was coupled to a significant increase of T, NK, and B percentages (Figure 4D). Notably, the percentage of HSPC remained unchanged (Figure 4D). In line with receptor expression data, BoHV-1 exposure did not affect the viability of HS-5 stromal cells (*Online Supplementary Figure S4E*), indicating that the non-hematopoietic stromal population is not susceptible to virus-induced cytotoxicity.

BoHV-1 induces activation and cytotoxicity of immune effector cells and promotes immunogenic reprogramming of multiple myeloma cells

It has been well established that OV exert anti-tumor effects both through direct oncolysis and by activating anti-tumor immune responses. Based on this, we inves-





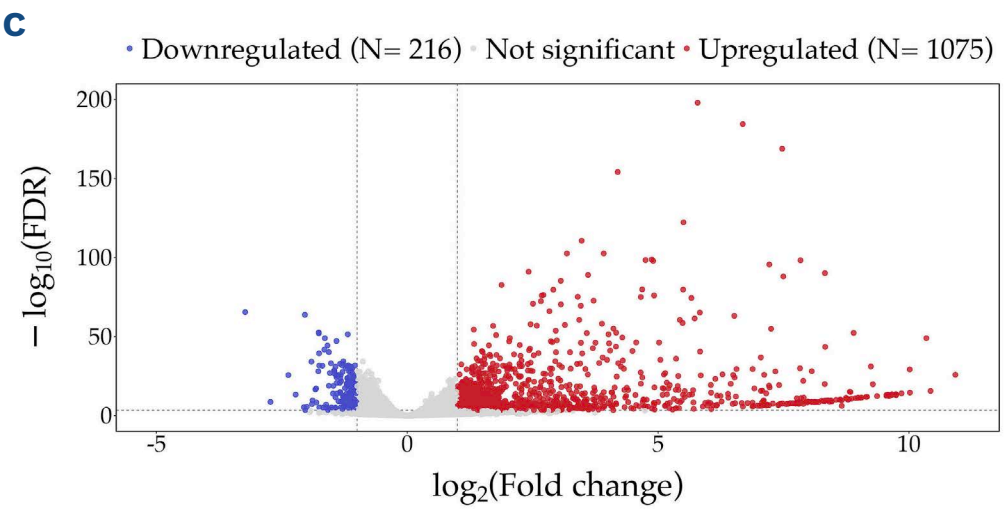
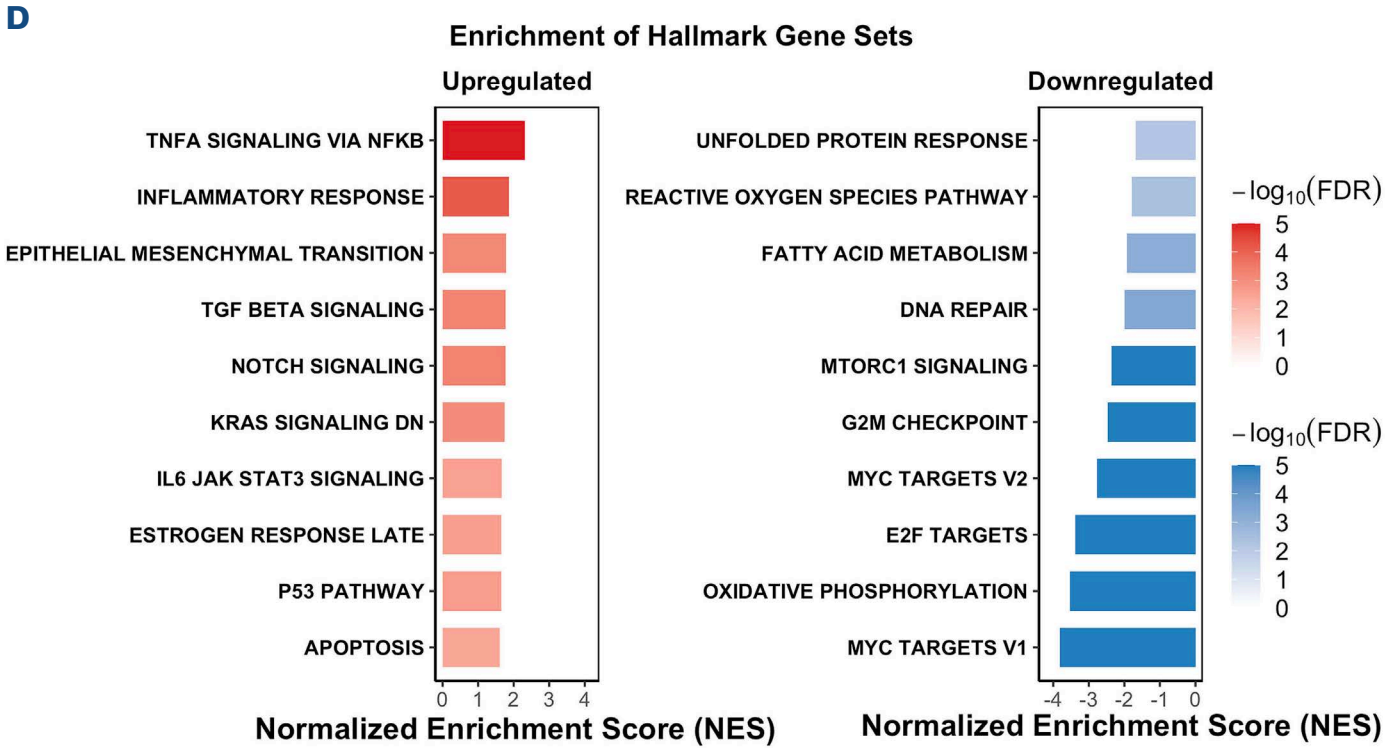


Figure 2. BoHV-1 triggers apoptotic and transcriptional reprogramming in human myeloma cell lines. (A) Percentage of APO2.7⁺ apoptotic cells in JJN-3, MM1.S, and OPM-2 cells at the indicated time points following BoHV-1 infection (1 and 2 MOI). Data represent mean ± standard deviation from four independent experiments. Statistical significance was determined using two-way ANOVA with Tukey's multiple comparisons test. (B) Immunoblot analysis of pro- and cleaved caspase-3 in JJN-3, MM1.S, and OPM-2 cells treated with or without BoHV-1 (1 and 2 MOI) for 48 hours (h). Vinculin served as a loading control. (C) Volcano plot of differentially expressed genes in JJN-3 cells infected with BoHV-1 (1 MOI, 24 h) compared to untreated cells. Genes were considered significantly up-regulated if log2(-Fold change) > 1 and False Discovery Rate (FDR) <0.0005 (red), significantly down-regulated if log2(-Fold change) < -1 and FDR <0.0005 (blue), and non-significant genes in gray. (D) Gene set enrichment analysis showing the top 10 significantly enriched up-regulated and down-regulated hallmark gene sets in JJN-3 cells infected with BoHV-1 (1 MOI, 24 h). Hallmark gene sets were considered significantly enriched at FDR <0.05. ***P<0.001, ****P<0.0001.



tigated how BoHV-1 impacts effector cells within the BM microenvironment of MM patients.

In CD8⁺ T cells, BoHV-1 treatment led to a significant up-regulation of the early activation marker CD69 (Figure 5A) and CD107a (Figure 5B), a degranulation marker associated with cytolytic function. These findings are consistent with

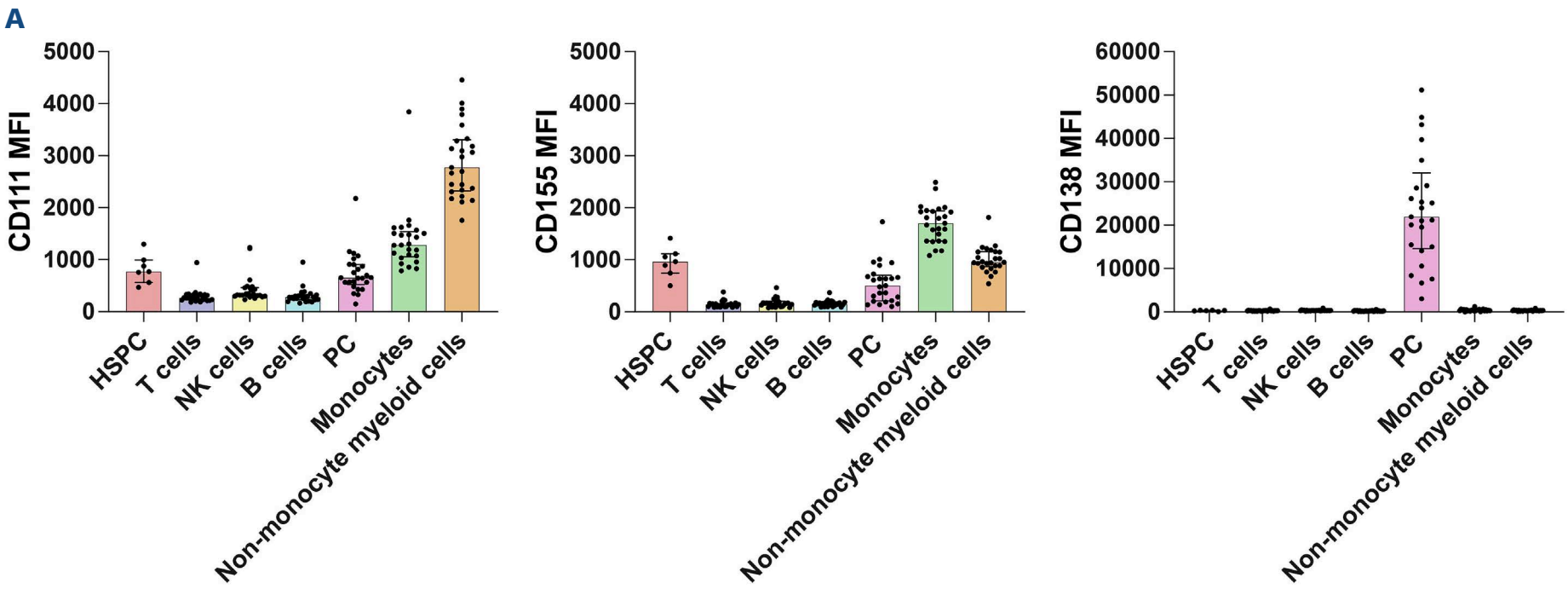


Figure 3. BoHV-1 induces cytotoxicity in malignant plasma cells within patient-derived bone marrow cells. (A) Scatter plots show CD111, CD155, and CD138 median fluorescence intensity (MFI) across bone marrow mononuclear cell (BMMC) populations (N=25 patients per population; N=7 for hematopoietic stem and progenitor cells [HSPC]). (B) Relative viability of purified multiple myeloma (MM) CD138⁺ plasma cells (PC) (N=6), infected with BoHV-1 at 1 and 2 MOI for 48 hours (h), 72 h, and 96 h *versus* untreated samples. (C) Relative viability of PC in total BMMC from MM patients treated with BoHV-1 (1 and 2 MOI), for 48 h, 72 h, and 96 h *versus* the untreated samples. Results were pooled from 32, 30, and 24 patients, respectively. Each dot represents an individual patient. Bars and boxplots indicate medians with Interquartile Range; lines connect paired samples where applicable. Statistical analyses were performed using two-way ANOVA with Tukey's multiple comparisons test (B) and Friedman test with Dunn's multiple comparisons test (C). **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

an initial functional priming of CD8⁺ T cells upon viral exposure, observed at early time points.

Natural killer cells displayed a similarly enhanced activation profile, with a robust increase in expression of CD69 (Figure 5C), CD38 (Figure 5D), and CD107a (Figure 5E), indicative of heightened metabolic activity and cytotoxic function. This increase in CD38 expression progressed over 48 h, 72 h, and 96 h post BoHV-1 exposure (*Online Supplementary Figure S5A*), in parallel with the time-dependent reduction in PC viability.

We next examined whether BM NK-cell activation correlated with immune-related transcriptional changes in tumor

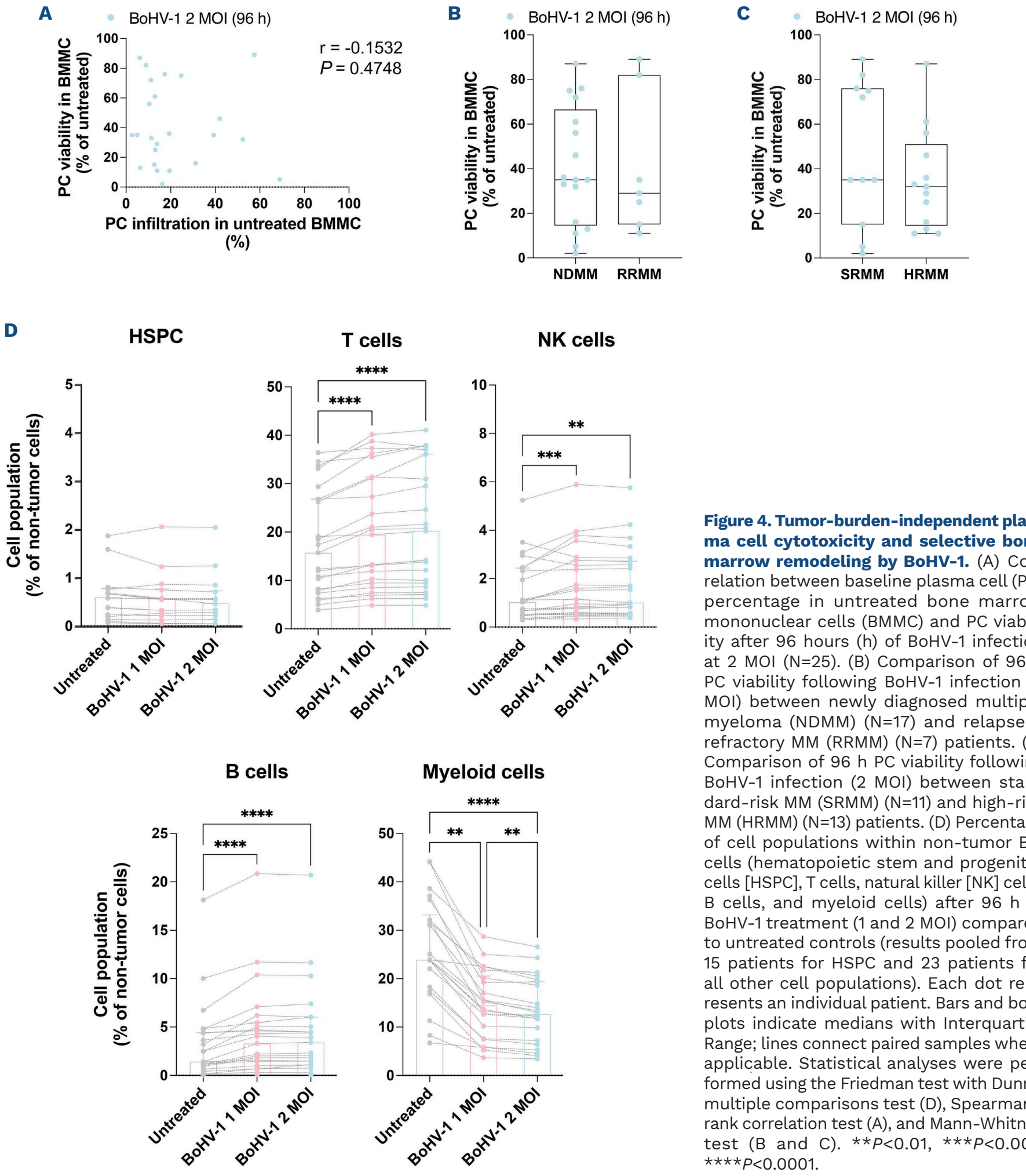


Figure 4. Tumor-burden-independent plasma cell cytotoxicity and selective bone marrow remodeling by BoHV-1. (A) Correlation between baseline plasma cell (PC) percentage in untreated bone marrow mononuclear cells (BMMC) and PC viability after 96 hours (h) of BoHV-1 infection at 2 MOI (N=25). (B) Comparison of 96 h PC viability following BoHV-1 infection (2 MOI) between newly diagnosed multiple myeloma (NDMM) (N=17) and relapsed/refractory MM (RRMM) (N=7) patients. (C) Comparison of 96 h PC viability following BoHV-1 infection (2 MOI) between standard-risk MM (SRMM) (N=11) and high-risk MM (HRMM) (N=13) patients. (D) Percentage of cell populations within non-tumor BM cells (hematopoietic stem and progenitor cells [HSPC], T cells, natural killer [NK] cells, B cells, and myeloid cells) after 96 h of BoHV-1 treatment (1 and 2 MOI) compared to untreated controls (results pooled from 15 patients for HSPC and 23 patients for all other cell populations). Each dot represents an individual patient. Bars and boxplots indicate medians with Interquartile Range; lines connect paired samples where applicable. Statistical analyses were performed using the Friedman test with Dunn's multiple comparisons test (D), Spearman's rank correlation test (A), and Mann-Whitney test (B and C). $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$.

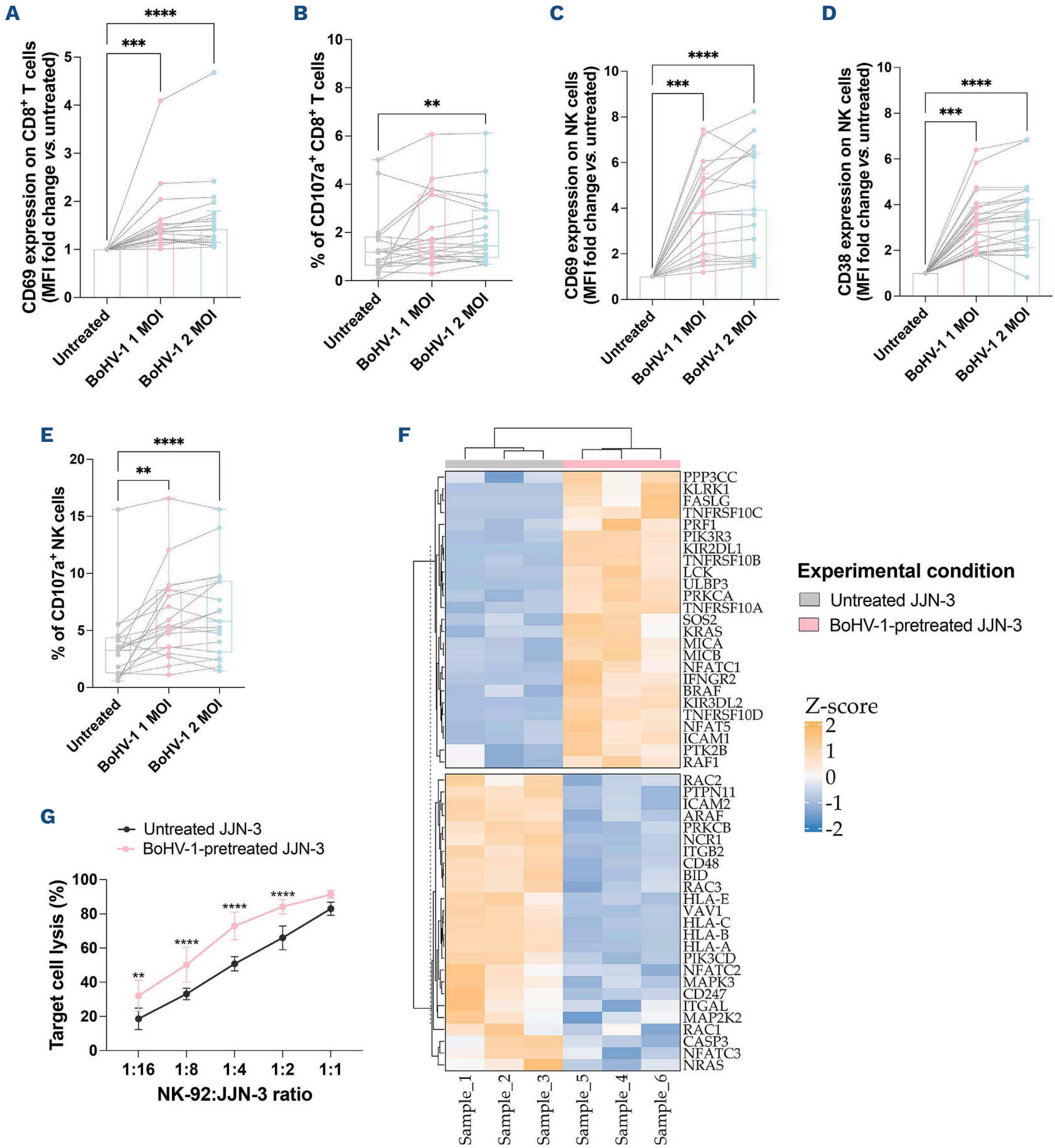


Figure 5. BoHV-1 enhances the activation of immune effector cells and promotes the expression of immunogenic markers in multiple myeloma cells. (A) Relative CD69 expression on CD8⁺ T cells from multiple myeloma (MM) patients' bone marrow mononuclear cells (BMMC) (N=18) treated with BoHV-1 (1 and 2 MOI) for 18 hours (h) versus the untreated samples. (B) Percentages of CD107a⁺ on CD8⁺ T cells among BMMC (N=18) after 18 h of treatment. (C) Relative CD69 expression on natural killer (NK) cells among BMMC at 18 h and (D) relative CD38 expression on the same population at 48 h following BoHV-1 treatment (1 and 2 MOI), compared to untreated samples (results pooled from 18 patients for CD69 and 26 patients for CD38). (E) Percentages of CD107a⁺ on NK cells among BMMC (N=18) after 18 h of treatment. (F) Heatmap of the top 25 up- and down-regulated genes in the KEGG natural killer cell-mediated cytotoxicity pathway from bulk RNA-seq of JJN-3 cells treated with BoHV-1 (1 MOI) for 24 h or untreated (N=3 per group). Data were processed and visualized in R; the color scale represents Z-scores. (G) NK-92-mediated cytotoxicity of JJN-3 cells, untreated or pre-treated with BoHV-1 (1 MOI, 24 h), assessed after 4 h co-culture at increasing effector:target (E:T) ratios across three independent experiments. Data are shown as mean ± standard deviation. Each dot represents an individual patient. Bars and boxplots indicate medians with Interquartile Range; lines connect paired samples where applicable. Statistical analyses were performed using two-way ANOVA with Tukey's multiple comparisons test (G) and Friedman test with Dunn's multiple comparisons test (A-E). ***P*<0.01, ****P*<0.001, *****P*<0.0001.

cells by interrogating the KEGG natural killer cell-mediated cytotoxicity pathway within the BoHV-1 RNA-seq dataset from JJN-3 cells. This analysis revealed coordinated up-regulation of multiple core constituent genes, including NK-activating ligands (MICA, MICB, ULBP3), alongside down-regulation of classical HLA class I molecules (HLA-A, -B, -C), which normally suppress NK-cell response (Figure 5F). To functionally validate this transcriptional signature, BoHV-1-pretreated JJN-3 cells were co-cultured with NK-92 cells for 4 h at increasing effector-to-target (E:T) ratios. Compared to those untreated, virus-exposed targets exhibited significantly increased susceptibility to NK cell-mediated cytolysis (Figure 5G), confirming that BoHV-1 enhances NK recognition and cytotoxicity against MM cells.

BoHV-1 reshapes the myeloid bone marrow landscape and drives anti-tumor immune activation

Based on our observation that BoHV-1 treatment reduces myeloid cells (Figure 4D), we further examined the impact of the virus on specific myeloid subsets within the non-tumor BM compartment. First, $SSC^{high}CD11b^{+}$ cells were stratified into $CD14^{-}$ and $CD14^{dim/+}$ populations, corresponding to non-monocytic myeloid cells and mono/macrophages, respectively. At the later experimental time point, BoHV-1 treatment resulted in a significant reduction in $CD11b^{+}CD14^{-}$ cells, accompanied by a significant expansion of the $CD11b^{+}CD14^{dim/+}$ cells (Figure 6A). Then, within the $CD11b^{+}CD14^{-}$ subset, we considered the expression of CD16 to distinguish immature (CD16-) from more mature myeloid cells ($CD16^{+}$). A significant reduction in $CD16^{+}$ cells was observed, paralleled by an increase in $CD16^{-}$ cells (Figure 6B), indicating a virus-driven reshaping of the non-monocytic myeloid landscape with a predominant reduction in mature myeloid cells. Next, we investigated how BoHV-1 treatment impacts mono/macrophages. Specifically, exposure to BoHV-1 induced a significant and dose-dependent upregulation of both activation markers CD69 (Figure 6C) and CD38 (Figure 6D), detectable at 48 h and maintained through 96 h (*Online Supplementary Figure S5B*). Moreover, after 48 h of virus treatment, the percentage of $CD14^{+}CD16^{-}$ classical monocytes, typically associated with an M1-like anti-tumoral phenotype, increased significantly. In contrast, the rate of $CD14^{+}CD16^{+}$ non-classical monocytes, linked to an M2-like pro-tumoral phenotype, was significantly reduced (Figure 6E). This redistribution suggests that BoHV-1 actively drives mono/macrophage polarization toward an anti-tumor M1-like state.

To confirm the contribution of $CD14^{+}$ monocytes to the anti-MM activity of BoHV-1, we compared virus-treated total BMMC with their CD14-depleted counterparts. Depletion of monocytes led to a significant increase in the viability of malignant PC (Figure 6F), indicating that $CD14^{+}$ cells actively contribute to the indirect oncolytic effects of the virus. Finally, we investigated the BM cytokine milieu in relation to BoHV-1 treatment. ELISA analysis revealed a significant increase in IFN-α, TNF-α, and IFN-γ levels in the supernatants of BoHV-1-treated compared to untreated BMMC at 48 h post infection (Figure 6G). At 96 h, IL-6 and IL-1β levels were also significantly up-regulated (*Online Supplementary Figure S5C*), consistent with a sustained inflammatory environment accompanying BoHV-1-mediated PC cytotoxicity.

BoHV-1 enhances the efficacy of clinically relevant anti-multiple myeloma therapies

The limited efficacy of OV as monotherapies, together with the reliance on combination regimens in MM, prompted us to test whether BoHV-1 could enhance the efficacy of clinically relevant anti-MM treatments.

Building on our transcriptomic analyses, which showed that BoHV-1 suppresses multiple proteostasis-related pathways, including the Hallmark UPR gene set (Figure 2D) and KEGG proteasome and ribosome pathways (*Online Supplementary Figure S3B*), we tested its combination with BTZ, a proteasome inhibitor that induces proteotoxic stress. In preliminary experiments using the JJN-3 cell line, co-treatment with BoHV-1 (1 MOI) and BTZ (2, 3, 5 nM) significantly reduced cell viability compared to either agent alone (*Online Supplementary Figure S6A*). In MM patient-derived BMMC (N=11), BoHV-1 (1 and 2 MOI) combined with BTZ (2 nM) significantly decreased PC viability (Figure 7A).

We further evaluated BoHV-1 in combination with the immunomodulatory drug LENA, chosen for its ability to enhance immune effector functions. In JJN-3 cells, co-treatment with BoHV-1 (1 MOI) and LENA (1, 2, 10 μM) reduced cell viability compared to either agent alone (*Online Supplementary Figure S6B*). A similar effect was observed in BMMC from MM patients (N=9), where BoHV-1 combined with LENA (10 μM) significantly reduced PC viability (Figure 7B). We then focused on the effects of BoHV-1 on CD38 expression, which was significantly up-regulated not only on NK cells (Figure 5D) and mono/macrophages (Figure 6D), but also on malignant PC (Figure 7C). CD38 is a clinically relevant target of anti-MM monoclonal antibodies, including DARA. Accordingly, DARA (10 μg/mL) was administered 48 h post infection (Figure 7D), corresponding to the time point at which CD38 upregulation was consistently observed in BM PC. In patient-derived BMMC (N=12), co-treatment with BoHV-1 and DARA significantly reduced PC viability (Figure 7E).

Finally, to explore BoHV-1 interactions with other immune-engaging agents, we assessed its combination with the bispecific antibody ELRA (BCMA×CD3). In BMMC from MM patients (N=8), BoHV-1 (1 and 2 MOI) combined with a suboptimal dose of ELRA (0.01 nM), administered 24 h post infection (*Online Supplementary Figure S6C*), significantly reduced PC viability (*Online Supplementary Figure S6D*).

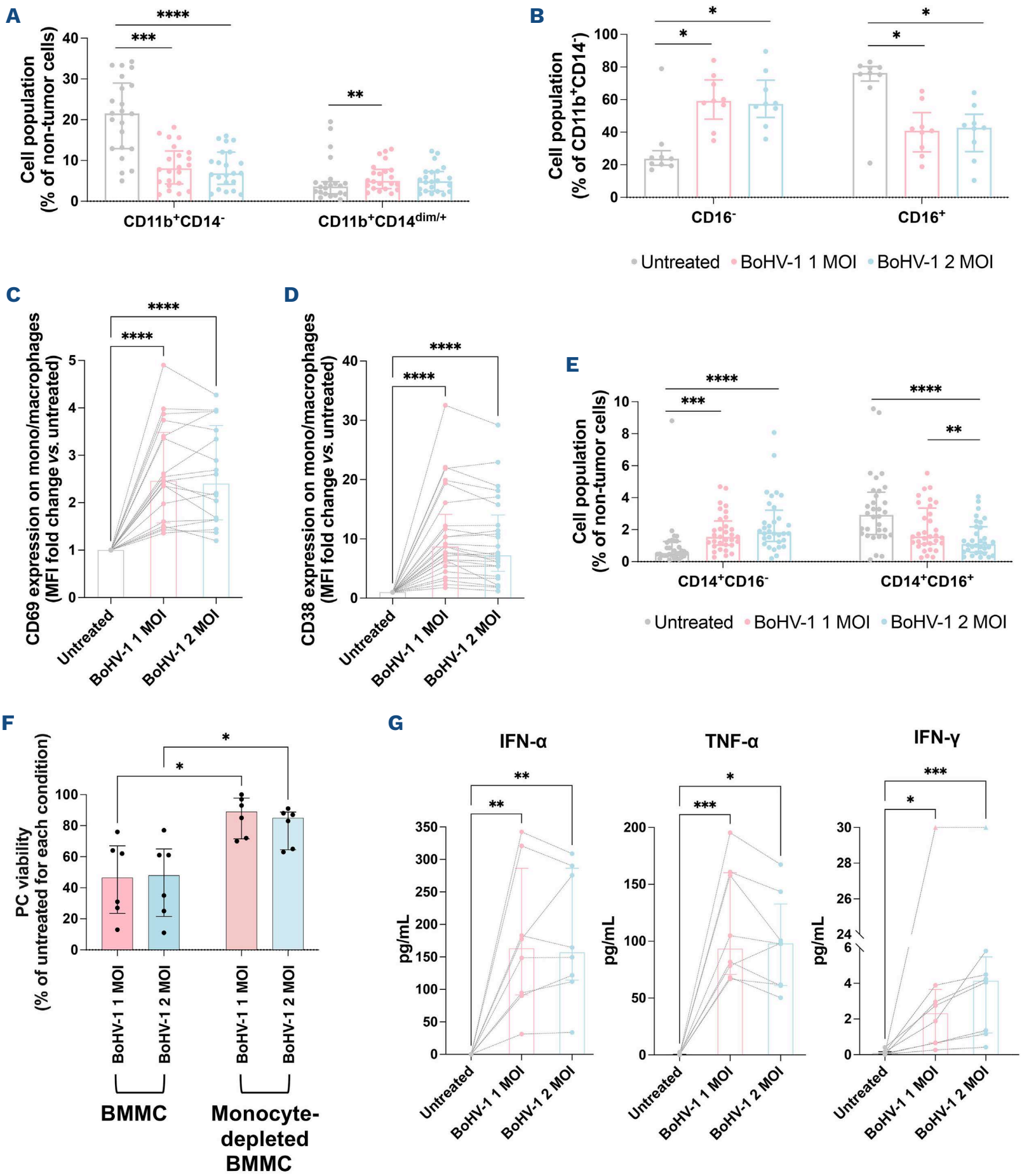


Figure 6. BoHV-1 reduces suppressive myeloid subsets, activates mono/macrophages, and induces a pro-inflammatory cytokine milieu. (A) Percentages of CD11b+CD14- and CD11b^{+}CD14$^{dim/+}$ myeloid subsets among non-tumor cells from multiple myeloma (MM) patients' bone marrow mononuclear cells (BMMC) (N=22) after 96 hours (h) of BoHV-1 treatment. (B) Relative frequencies of CD16^{+} and CD16^{-} cells within the CD11b^{+}CD14^{-} compartment from MM patients' BMMC (N=9) after 96 h of BoHV-1 treatment. (C) Relative CD69 expression on CD11b^{+}CD14$^{dim/+}$ mono/macrophages among BMMC at 18 h and (D) relative CD38 expression on the same population at 48 h following BoHV-1 treatment (1 and 2 MOI), compared to untreated samples (results pooled from 18 patients for CD69 and 26 patients for CD38). (E) Percentages of the CD14^{+}CD16- and CD14^{+}CD16^{+} subsets among non-tumor cells from MM patients' BMMC (N=32), untreated or treated with BoHV-1 (1 and 2 MOI) for 48 h. (F) Relative cell viability of plasma cells (PC) in total and monocyte-depleted BMMC from MM patients (N=6) following BoHV-1 treatment at 1 and 2 MOI for 96 h. (G) IFN-α, TNF-α, and IFN-γ levels in the cell-free supernatants of MM patients' BMMC (N=8) treated with or without BoHV-1 (1 and 2 MOI for 48 h). Triangles indicate values exceeding the assay's detection limit; these were assigned the maximum detectable concentration and are, therefore, underestimated. Each dot represents an individual patient. Bars and boxplots indicate medians with Interquartile Range; 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.

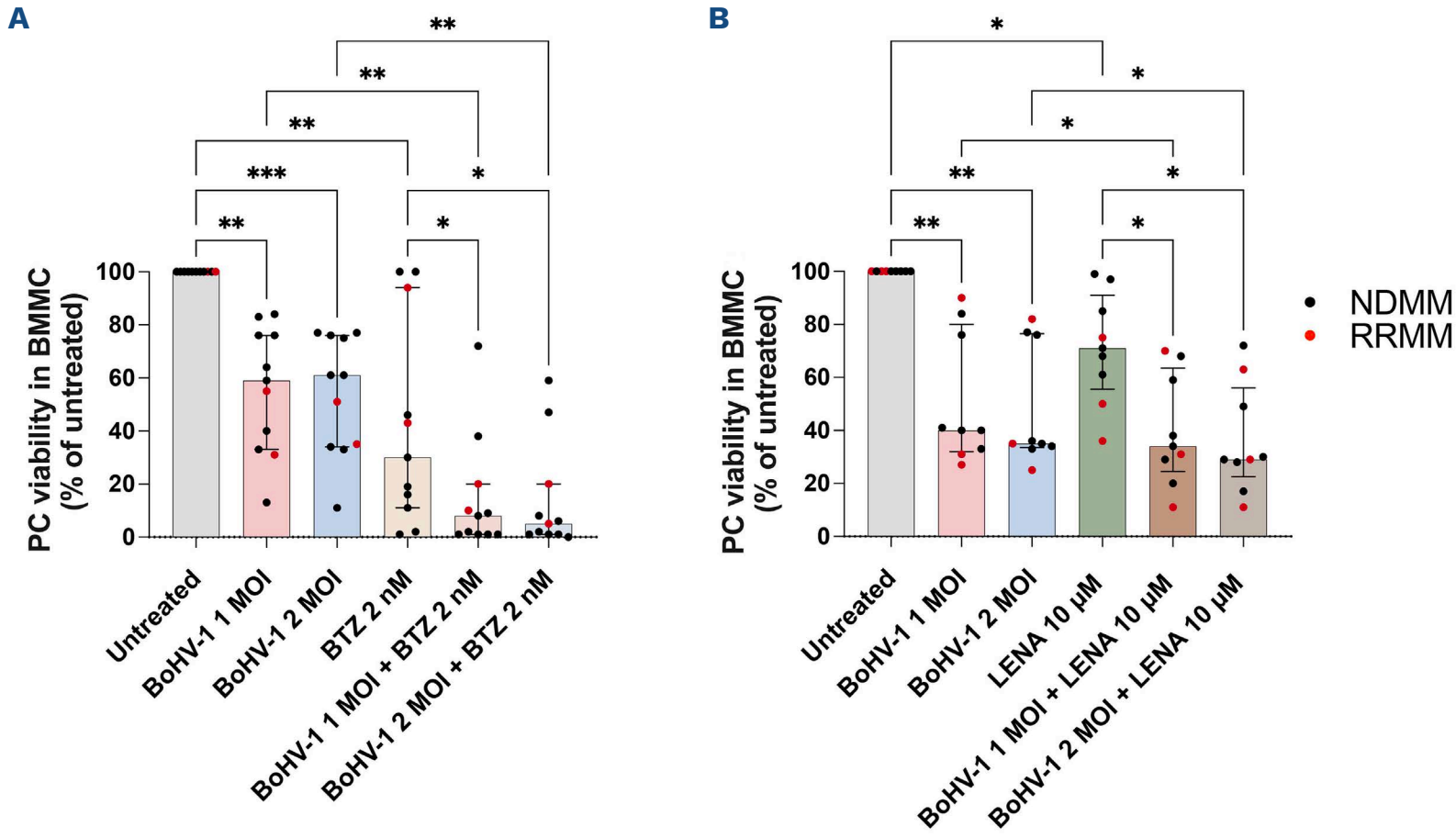


Figure 7. Combination treatments with BoHV-1 and standard anti-multiple myeloma agents reduce tumor cell viability in patient-derived bone marrow mononuclear cells. (A) Relative viability of plasma cells (PC) from multiple myeloma (MM) patients' bone marrow mononuclear cells (BMMC) (N=11) 96 hours (h) after treatment with BoHV-1 (1 and 2 MOI), bortezomib (BTZ) (2 nM), alone or in combination. (B) Relative viability of PC from MM patients' BMMC (N=9) 96 h after treatment with BoHV-1 (1 and 2 MOI), lenalidomide (LENA) (10 µM), alone or in combination. (C) Relative CD38 expression on PC from MM patients' BMMC (N=34) after 48 h of treatment. (D) Experimental design for BoHV-1 and daratumumab (DARA) combination. (E) Relative viability of PC from MM patients' BMMC (N=12) 96 h after treatment with BoHV-1 (1 and 2 MOI), DARA (10 µg/mL), alone or in combination. All treatments were compared to untreated controls. Data from patient-derived BMMC are presented as median with Interquartile Range from paired experiments. Black dots represent patients with newly diagnosed MM (NDMM), while red dots represent those with relapsed/refractory MM (RRMM). Statistical analysis was performed using the Friedman test with Dunn's correction. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

Discussion

The clinical efficacy of OV in MM has remained modest, largely due to inefficient tropism for malignant PC or pre-existing antiviral immunity that limits viral propagation.[13,14,16,17] To overcome these barriers, non-human OV represent a promising alternative. We previously showed that BVDV exerts direct cytotoxic effects on MM cells in both *ex vivo* and *in vivo* models.[31] Here, we extend this approach by investigating BoHV-1 as a novel oncolytic agent in MM, providing the first evidence that a bovine virus can reprogram the BM microenvironment from a 'cold' immunosuppressive state into a 'hot' pro-inflammatory and immunostimulatory niche.

Across MM cell lines, putative BoHV-1 attachment and entry receptors were consistently expressed at high levels, supporting the concept of a receptor landscape compatible with viral entry. Functionally, BoHV-1 infection induced marked cytopathic effects in all tested HMCL, including the KRAS wild-type OPM-2 cells, accompanied by activation of the intrinsic apoptotic cascade.

Antibody-mediated blockade implicated CD138 in viral tropism, reinforcing the rationale for BoHV-1 targeting within the MM niche, although incomplete protection suggests involvement of additional host factors. Transcriptomic profiling further showed that BoHV-1 reprograms MM cells by inducing apoptotic, p53, and inflammatory pathways while suppressing MYC, oxidative phosphorylation, and UPR programs, thereby exploiting their dependence on proteostasis and mitochondrial metabolism.

In patient-derived BM samples, BoHV-1 attachment and entry receptors were highly expressed on PC, monocytes, and non-monocyte myeloid cells, supporting the capacity to infect multiple cellular populations within the BM niche. Consistent with this distribution, BoHV-1 reduced PC viability and led to a decrease in myeloid cells with a relative increase in lymphoid subsets; this shift is more likely attributable to a selective decrease of myeloid cells rather than active expansion of lymphoid subsets. Importantly, HSPC remained unaffected by BoHV-1 treatment, underscoring the selective cytotoxicity of the virus and its ability to preserve hematopoietic integrity.

Beyond its direct oncolytic activity, BoHV-1 infection markedly reshapes the immune microenvironment. $CD8^+$ T cells showed early functional priming upon viral exposure, whereas NK cells underwent a more pronounced activation, consistent with their intrinsic capacity for rapid innate responses. Infected MM cells up-regulated NK-activating ligands while concomitantly down-regulating classical HLA class I molecules, generating a surface phenotype that aligns with the observed increase in NK cell-mediated cytotoxicity and innate immune recognition.

It has been well recognized that successful anti-MM immunotherapeutic strategies should be capable of overcoming immunosuppressive barriers and redressing the balance between immunogenic and tolerogenic inflammation in the BM microenvironment.[32,33] Analyzing the reduction in myeloid cells, we found that BoHV-1 infection led to a profound quantitative and qualitative remodeling of the myeloid compartment. The activation of mono/macrophages indicates their engagement in response to viral pattern recognition, while the significant skewing toward the $CD14^{dim/+}CD16^-$ subset reveals the virus-driven polarization from an M2-like pro-tumoral to a pro-inflammatory and anti-tumor M1-like phenotype. Interestingly, a previous study has reported a significantly higher enrichment of M2 tolerogenic macrophages in rapid progressors, supporting the concept that virus-induced macrophage reprogramming may contribute to counteracting the immunosuppressive microenvironment associated with disease progression.[34]

As expected, the result that the depletion of $CD14^+$ monocytes markedly reduced the ability of BoHV-1 to diminish PC viability confirms that these cells actively contribute to the anti-tumor effects of the virus. At later experimental time points, among BoHV-1-treated BMMC, we observed that the decrease in myeloid cells was associated with a change in their relative subset composition. Indeed, BoHV-1 treatment resulted in a significant reduction in the non-monocyte myeloid cells, particularly those with a $CD16^+$ phenotype, counteracted by the increase in mono/macrophage frequency. We are unable to accurately identify myeloid-derived suppressor cells (MDSC), but there is evidence in the literature to suggest that cells with suppressive activity may be included among the more mature depleted $CD11b^+CD14^-CD16^+$ subset.[35,36] Of note, since viability analyses are based on relative frequencies within total BMMC, the reduction in myeloid populations may partially mask the extent of PC loss and even underestimate the anti-tumor effect of BoHV-1.

Consistent with a virus-driven shift toward an immunostimulatory BM environment, BoHV-1 exposure induced early increases in IFN-α, TNF-α, and IFN-γ that coincided with the onset of PC cytotoxicity. The increased levels of IL-6 and IL-1β observed at later time points may indicate that this inflammatory milieu persists beyond the initial cytotoxic phase. Although the absence of comprehensive kinetic profiling precludes formal causal inference, these data support a model in which BoHV-1 triggers a temporally evolving inflammatory program that accompanies, and may contribute to, its anti-MM activity. Overall, these findings support a model in which direct BoHV-1-mediated oncolysis and concomitant immune remodeling within the BM microenvironment co-evolve and reinforce the anti-MM response, rather than acting as independent or strictly sequential processes.

Current MM treatment relies on multidrug regimens, typically including dexamethasone, immunomodulatory agents, proteasome inhibitors, and monoclonal antibodies targeting PC-associated antigens. Building on this rationale and the multifaceted activity of BoHV-1, we investigated its potential in combination with established anti-MM agents.

Transcriptomic analyses revealed downregulation of UPR and proteasome pathways, two critical stress-adaptation programs in MM, prompting the hypothesis that BoHV-1 might potentiate the effects of proteasome inhibition. Indeed, co-treatment with BTZ led to markedly enhanced cytotoxicity in a representative MM cell line and, most importantly, in primary patient samples. Beyond proteostasis collapse, BTZ is a well-characterized inducer of apoptosis through activation of caspase-8 and caspase-9 cascades converging on caspase-3,[37] whereas our functional studies demonstrated that BoHV-1 infection directly promotes mitochondrial apoptosis. Thus, the combination likely amplifies caspase-3-dependent cell death through complementary mechanisms. This effect may also extend to immunogenic cell death (ICD): proteasome inhibitors are recognized inducers of ICD, and a recent study has demonstrated that BoHV-1 alone can elicit bona fide ICD in immunocompetent tumor models.[38] Although we did not directly assess ICD in this setting, the observed immune activation and transcriptional remodeling are consistent with engagement of ICD pathways in MM.

Similarly, BoHV-1 treatment with LENA increased anti-tumor cytotoxicity across our experiments. This result may be due to the combination of direct virus and drug antiproliferative effects on MM cells, as well as to shared pleiotropic effects on immune cells.[39] LENA promotes NK cell-mediated killing by inducing IKZF1/3 degradation and up-regulating activating ligands.[40] BoHV-1 infection triggered comparable changes and increased NK activation, suggesting that the combination improves NK-cell recognition and elimination of infected MM cells. In addition, LENA has been shown to repolarize MM-associated macrophages from an M2 to M1 phenotype and to enhance phagocytic activity in mouse models,[41,42] consistent with the immunostimulatory effects observed following BoHV-1 treatment alone.

To assess the translational relevance of BoHV-1 in contemporary MM immunotherapy, we examined its compatibility with antibody-based therapies. The virus-induced upregulation of CD38 on MM cells, NK cells, and mono/macrophages provides a strong biological rationale for combining with DARA. Increased CD38 expression may potentiate DARA-mediated cytotoxicity by enhancing target antigen density on tumor cells and facilitating Fc-dependent engagement of immune effector cells.[43] Consistent with this rationale, the results of a greater reduction in MM cells viability with co-treatment with DARA compared to either agent alone support the capacity of BoHV-1 to sensitize both tumor and immune effector compartments to anti-CD38 antibody-based therapies. In a more exploratory setting, administration of the BCMA×CD3 bispecific antibody ELRA after viral infection was associated with improved anti-MM activity, consistent with the hypothesis that BoHV-1-mediated immune remodeling may also favor T cell-redirecting approaches.[35,44]

A limitation of this study is the absence of *in vivo* preclinical validation. However, BoHV-1 does not efficiently bind to or enter murine cells, precluding the use of conventional mouse models and preventing faithful reproduction of virus-tumor-immune interactions.[23,45] Although pseudotyping with heterologous glycoproteins can enhance entry into murine cells, viral gene transcription remains inefficient due to intrinsic host defenses, and such approaches rely on engineered viruses not intended for translation into a clinical setting. Alternative species, such as cotton rats, have been used in anti-BoHV-1 vaccination studies and selected tumor models, but these systems are not established for MM.[46] In this context, patient-derived *ex vivo* BM cultures provide a biologically relevant human platform to assess BoHV-1 activity and combinatorial strategies within a complex and immunologically intact microenvironment. Future work may extend these findings using advanced human-based systems, including 3-dimensional BM organoids or other *ex vivo* platforms that more faithfully recapitulate tissue architecture and long-term immune-tumor dynamics.

Ultimately, BoHV-1 lacks pre-existing human immunity, exerts potent therapeutic activity at low MOI, requires no engineering, allows straightforward genetic manipulation,[47] and can be produced at scale on established platforms, making it a practical and versatile oncolytic candidate.

In conclusion, this study establishes BoHV-1 as a novel oncolytic agent capable of inducing direct cytotoxicity and deep immunologic remodeling in MM. These findings provide a strong rationale for further preclinical development of BoHV-1-based immunovirotherapy, potentially in combination with standard-of-care agents, to improve outcomes for MM patients.

Disclosures

NG received research funding and honoraria from Amgen, Bristol-Myers Squibb, Celgene, Pfizer, Takeda, Millennium Pharmaceutical, and Janssen Pharmaceutical. All the other authors have no conflicts of interest to disclose.

Contributions

VR performed all in vitro experiments, with support from RV, PS, VF, GP, CS, NTI, DT, MD, SM and OL. VF, SM and GD supplied the oncolytic virus. RV and VR conducted the flow cytometry analysis. BDP and MS provided the clinical samples. SR was responsible for patient enrollment and clinical data management. VR and FM performed the bioinformatic analysis. VR, RV, GD and NG analyzed the data and wrote the manuscript. PS, PM, GD and NG contributed to the interpretation of the data. PM, GD and NG provided critical revision. All authors read and approved the final version of the manuscript for publication.

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Data-sharing statement

All data generated or analyzed during this study are included in this published article and its Online Supplementary Appendix.

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