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学位論文

Oncolytic HSV-1 in combination with lenalidomide for plasma cell neoplasms

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Oncolytic HSV-1 in combination with lenalidomide for plasma cell neoplasms

Running short title: Oncolytic HSV-1 with lenalidomide for plasma cell neoplasms

Maki Oku,[1] Ryo Ishino,[1,2] Shumpei Uchida,[1] Osamu Imataki,[1] Naoshi Sugimoto,[3] Tomoki Todo,[4] Norimitsu Kadowaki[1]

[1] Department of Internal Medicine, Division of Hematology, Rheumatology and Respiratory Medicine, Faculty of Medicine, Kagawa University, Kagawa, Japan, [2] Department of Hematology and Oncology, Graduate School of Medicine, Kyoto University, Kyoto, Japan, [3] Department of Clinical Application, Center for iPS Cell Research and Application (CiRA), Kyoto University, Kyoto, Japan, and [4] Division of Innovative Cancer Therapy, Institute of Medical Science, the University of Tokyo, Tokyo, Japan.

Correspondence
Norimitsu Kadowaki, M.D., Ph.D.
Department of Internal Medicine, Division of Hematology, Rheumatology and Respiratory Medicine, Faculty of Medicine, Kagawa University, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan
Tel: +81-87-891-2143
Fax: +81-87-891-2147
E-mail: kado@med.kagawa-u.ac.jp

Summary

Oncolytic viruses exert an anti-tumor effect through two mechanisms: direct oncolytic and indirect immune-mediated mechanisms. Although oncolytic herpes simplex virus type 1 (HSV-1) has been approved for melanoma treatment and is being examined for its applicability to a broad spectrum of malignancies, it is not known whether it has an anti-myeloma effect. Here, we show that the third-generation oncolytic HSV-1, T-01, had a direct oncolytic effect on 5 of 6 human myeloma cell lines *in vitro*. The antitumor effect was enhanced in the presence of peripheral blood mononuclear cells (PBMCs) from healthy individuals and, to a lesser extent, from myeloma patients. The enhancing effect of PBMCs was abrogated by blocking type I interferons (IFNs) or by depleting plasmacytoid dendritic cells (pDCs) or natural killer (NK) cells, suggesting that pDC-derived type I IFNs and NK cells dominated the antitumor effect. Furthermore, the combination of T-01 and lenalidomide exhibited enhanced cytotoxicity, and the triple combination of T-01, lenalidomide, and IFN-α had a maximal effect. These data indicate that oncolytic HSV-1 represents a viable therapy for plasma cell neoplasms through direct oncolysis and immune activation governed by pDCs and NK cells. Lenalidomide is likely to augment the anti-myeloma effect of HSV-1.

Keywords

Introduction

Although an array of new therapeutics has been developed for plasma cell neoplasms, neoplastic cells eventually acquire drug resistance, and the prognosis is poor in most patients. Nevertheless, immunotherapy has been emerging as a promising therapy for multiple myeloma (Hoyos and Borrello 2016, Kumar and Anderson 2016). Of note, it has been recognized that the immunosuppressive tumor microenvironment promotes the progression of multiple myeloma (Kawano *et al*, 2015). Thus, modulation of such a microenvironment toward an immune-potentiating direction is likely to be a key factor in inducing anti-myeloma immune responses.

Oncolytic viruses represent a novel therapy for a variety of cancers (Bommareddy *et al*, 2018b), including hematological malignancies (Bais *et al*, 2012). Oncolytic viruses exert anti-tumor effects through two mechanisms: direct oncolysis and indirect immune-mediated mechanisms (Bommareddy *et al*, 2018b). Oncolysis and inflammation provoked in tumor tissues convert the immunosuppressive microenvironment into an immune-potentiating one, leading to the generation of so called the "Cancer–Immunity Cycle" (Chen and Mellman 2013).

Among oncolytic viruses, herpes simplex virus type 1 (HSV-1) has been leading the field (Bommareddy *et al*, 2018a). Mineta et al. generated a second-generation oncolytic HSV-1, G207 (Mineta *et al*, 1995), which had a deletion of the *γ34.5* gene and an inactivation of ICP6 with a *LacZ* gene insertion and could replicate only in cancer cells that can complement these mutations. The third-generation G47Δ has a further deletion of *α47*, enabling it to replicate more efficiently and increase the presentation of the MHC class I molecule by tumor cells (Todo *et al*, 2001). T-VEC (talimogene laherparepvec), an oncolytic HSV-1 that has deletions of the *γ34.5* and *α47* genes and has the gene encoding human granulocyte macrophage colony-stimulating factor, has been approved for advanced melanoma in the USA, Europe, and Australia (Bommareddy *et al*, 2018a). We have been conducting clinical trials using G47Δ for brain tumors (UMIN000015995, UMIN000011636) and prostate cancer (UMIN000010463).

Several oncolytic viruses have been applied to plasma cell neoplasms in preclinical as well as clinical studies (Meyers *et al*, 2017), indicating that oncolytic virus therapy has the potential for the treatment of plasma cell neoplasms. However, the application of oncolytic HSV-1 to plasma cell neoplasms has not been reported, because human hematopoietic cells are resistant to HSV-1 replication (Wu *et al*, 2001) and thus oncolytic HSV-1 has been considered unsuitable to treat hematological malignancies.

Immunomodulatory drugs (IMiDs) also exert an anti-tumor effect through two mechanisms: direct cytotoxicity (Hideshima *et al*, 2000) and indirect immune-mediated mechanisms, in which IMiDs activate natural killer (NK) cells by inducing T cells to produce IL-2 (Hayashi *et al*, 2005) and by lowering the activation thresholds of NK cells (Lagrue *et al*, 2015). Thus, a combination of oncolytic virus and IMiDs may increase direct cytotoxic and indirect immune-mediated effects of each

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other.

In this study, we used T-01, an HSV-1 containing modifications in the same genes as G47Δ, against plasma cell neoplasms. We first show our experience with a patient with relapsed and refractory multiple solitary plasmacytoma, whose primary tumor cells as well as a derived cell line were susceptible to T-01. We then examined whether combining T-01 with immune cells or a representative IMiD, lenalidomide, enhanced the anti-myeloma effect of T-01. We propose that the combination of oncolytic HSV-1 and IMiDs is a promising therapy for plasma cell neoplasms through both oncolytic and immune-mediated mechanisms.

Materials and methods

A case report

A 68-year-old male was referred due to left flank pain. Fluorine-18 fluorodeoxyglucose-positron emission tomography/computed tomography revealed multiple masses in the ribs, scapula, spine, iliac bone, and femur. Biopsy from the left flank mass showed a plasma cell neoplasm. Flow cytometric analysis of the biopsy sample indicated surface $CD138^+$, $CD38^+$, $CD19^-$, $CD20^-$, cytoplasmic λ^+, κ^- and negative for IgG, IgA, IgM, IgD, and IgE. Malignant plasma cells were not detected in bone marrow from the iliac bone. We made a diagnosis of multiple solitary plasmacytoma. The patient was treated with irradiation, bortezomib, carfilzomib, lenalidomide, dexamethasone, and combination chemotherapies for 8 months, with only transient effects. He developed left pleural effusion containing tumor cells, and died due to progressive disease.

The study was approved by Ethics Committees of Kagawa University Hospital and was conducted in accordance with the principles of the Helsinki Declaration. Pleural effusion was obtained with written informed consent. To examine whether the tumor cells were susceptible to T-01, mononuclear cells from the pleural effusion were cultured with the indicated multiplicity of infection (MOI) of T-01 for 72 h. After gating for $CD138^+$ cells, the percentages of propidium iodide-negative viable cells were measured by flow cytometry using the Accuri™ C6 Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Data were analyzed using FlowJo software (BD Biosciences).

Cell lines

Tumor cells in the pleural effusion were cultured in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (Corning, Corning, NY, USA), 100 U/ml penicillin G, and 100 μg/ml streptomycin (Sigma-Aldrich) in a 37°C incubator containing 5% CO_2. The cells were cloned by limiting dilution. The established cell line, named PC402, was $CD138^+$, $CD38^-$, $CD19^-$, cytoplastic λ^+, κ^-. Human myeloma-derived cell lines NCI-H929, MM.1S, and U266 were obtained from the American Type Culture Collection (ATCC). RPMI 8226 and KMS-12-BM were obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank.

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Virus

The details of the construction of T-01 were described previously (Fukuhara *et al*, 2005), with minor modifications. Viruses were titrated using Vero cells.

Cytotoxicity assay

For assays in the absence of peripheral blood mononuclear cells (PBMCs), myeloma cells were incubated with T-01 or mock (culture medium) in a volume of 100 μL per 1×10^5 myeloma cells at 37°C for 1 h. After adding culture medium, the infected cells were seeded at 2×10^4 cells/200 μL/well in flat-bottomed, 96-well plates and were incubated at 37°C for 72 h. Cell viability was measured using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) containing WST-8 as the detection dye, in accordance with the manufacturer's instructions.

For assays in the presence of PBMCs, the cells were obtained from healthy volunteers and myeloma patients under informed consent. Information about patients is shown in Table S1. After incubation with T-01 or mock for 1 h, 2×10^4 (PC402) or 5×10^4 (other cell lines) myeloma cells were co-cultured with PBMCs in flat-bottom, 96-well plates (200 μL/well) at the indicated ratios for 72 h. The cells were washed once with PBS, incubated with blocking reagent (5% human IgG) for 15 min on ice, and then labeled with FITC anti-human CD138 monoclonal antibody (mAb) (BioLegend, San Diego, CA, USA). Fifty microliters of CountBright™ Absolute Counting Beads (Molecular Probes, Eugene, OR, USA) was added to the final sample of 500 μL. The cells were analyzed by flow cytometry, and the absolute numbers of $CD138^+$ cells were calculated.

To block type I interferon (IFN) activity, human type I IFN neutralizing antibody mixture (mixture of antibodies directed against human type I IFN receptor and type I IFNs; PBL Assay Science, Piscataway, NJ, USA) was added to the co-culture of myeloma cells and PBMCs at 50-fold dilution. Lenalidomide dissolved in DMSO (Selleck Biotech, Tokyo, Japan) and IFN-α (PBL) were added at the indicated concentrations. Culture with lenalidomide was performed for 5 days instead of 72 h, because the kinetics of cell death induction with lenalidomide was relatively slow.

Real-time PCR for the glycoprotein B (gB) gene of HSV-1

DNA was isolated using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The samples were run on a ViiA7 Real-Time PCR System (Applied Biosystems, Waltham, MA). Amplification was performed using TaqMan Fast Advanced Master Mix (Thermo Fisher, Waltham, MA, USA) as follows: 95°C for 20 seconds followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. All the experiments were performed in triplicate. The TaqMan probe sequence for the *gB* gene was 5'-CACACCTGCGAAACGGTGACGTCTT-3'. Primer sequences used were: forward: 5'-GGCGCGGTCCTCAAAGAT-3', reverse: 5'-AGAACATCGCCCCGTACAAG-3'. DNA from T-01

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with a known plaque forming unit (pfu) was used as a control to quantify the amount of *gB* DNA of samples with pfu.

Detection of plasmacytoid dendritic cells (pDCs) and NK cells in blood

PBMCs were stained with FITC anti-human CD123 and APC anti-human CD303 mAbs (BioLegend, San Diego, CA, USA) or with FITC anti-human CD3 and APC anti-human CD56 mAbs (BioLegend). Monocytes were excluded based on the forward scatter/side scatter pattern on flow cytometry. $CD123^+CD303^+$ cells and $CD3^-CD56^+$ cells were detected as pDCs and NK cells, respectively.

Cell depletion from PBMCs

For depletion of NK cells, T cells, pDCs, and monocytes, PBMCs were stained with FITC anti-human CD16 plus APC anti-human CD56, APC anti-human CD3, APC anti-human CD303, or APC anti-human CD14 mAbs (BioLegend), respectively, and depleted using MoFlo Astrios cell sorter (Beckman Coulter, Tokyo, Japan). Residual target cells were consistently <1%.

ELISA

After PBMCs at 2×10^5 cells/200 µL/well in a 96-well flat bottom plate were stimulated by T-01 for 24 h, the supernatants were collected and concentrations of IFN-α (IFN-α pan ELISA development kit; Mabtech, Nacka Strand, Sweden) and IFN-β (rabbit anti-human IFN-β and biotinylated anti-human IFN-β; PeproTech, Rocky Hill, NJ, USA) were measured by ELISA. Detection limits were IFN-α 15.6 pg/mL and IFN-β 62.5 pg/mL.

Measurement of CD69 expression

PBMCs (2×10^5 cells/200 µL/well) from healthy volunteers were treated with T-01 for 24 h. The cells were washed, incubated with blocking reagent (5% human IgG) and then with APC anti-human CD56 and FITC anti-human CD69 mAbs (BioLegend), and were analyzed by flow cytometry.

Animal model

Five $\times 10^6$ MM.1S cells in 100 µL of PBS and 100 µL of Matrigel Matrix (Corning) were injected subcutaneously into the left flank of 6-week-old female SCID Beige mice (Charles River Laboratories Japan, Inc., Yokohama, Japan). When the tumors reached about 50 mm³, the mice were divided into four groups: control (culture supernatants of Vero cells), T-01, lenalidomide, and T-01 plus lenalidomide. T-01 (1×10^6 pfu) and lenalidomide (5 mg/kg/day, 5 days per week for 3 weeks) were administered intratumorally and intraperitoneally, respectively. As a control for lenalidomide, the same volume of PBS with 0.5% methyl cellulose and 0.25% Tween 80 was administered. Tumor

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diameters were measured twice a week, and the tumor volume was calculated using the formula $1/2 \times$ [long axis] $\times$ [short axis]^2. All mouse experimental procedures were carried out in accordance with guidelines for the Proper Conduct of Animal Experiments by Science Council of Japan and under approval of the Animal Care and Use Committee of Kagawa University.

Statistical analysis

Statistical analysis was conducted by a two-tailed unpaired t-test, one-way ANOVA followed by Tukey's test, or one-way ANOVA followed by Dunnett's test as described in Figure Legends, using Prism software version 6 (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

Results

T-01 kills primary plasmacytoma cells and human myeloma cell lines

We established a cell line PC402 from tumor cells in the pleural effusion of the patient with refractory multiple solitary plasmacytoma as described in the Materials and methods. The primary tumor cells were susceptible to T-01 and were almost totally killed by an MOI 1.0 of T-01 (submitted) (Fig. 1A). We also examined whether T-01 killed myeloma cell lines. Five out of six cell lines, including PC402, were strongly killed by T-01 at an MOI as low as 0.3 (Fig. 1B). All five cell lines had IC_{50} values between MOIs of 0.03 and 0.1. These data suggest that plasma cell neoplasms are susceptible to oncolytic HSV-1.

Combined effects of T-01 and PBMCs

We examined whether the presence of immune cells augment the cytotoxic effect of T-01, by adding PBMCs from healthy individuals or myeloma patients to the myeloma cell lines. T-01 was added at a suboptimal MOI 0.05. Addition of PBMCs alone did not affect the viability of the cell lines (Fig. 2A, B). Addition of T-01 alone weakly reduced the viability. The combination of T-01 and PBMCs from healthy individuals exhibited significantly stronger cytotoxicity against all the cell lines than T-01 alone (Fig. 2A). Whereas addition of PBMCs to the cell lines at 1:1 was sufficient to significantly augment cytotoxicity for NCI-H929, MM.1S, and U266, addition at 5:1 was necessary for PC402. The combination of T-01 and PBMCs from myeloma patients exhibited significantly stronger cytotoxicity against MM.1S than T-01 alone, whereas the combined effects were not statistically significant against NCI-H929 or U266 (Fig. 2B). These data indicate that T-01 stimulates immune cells in PBMCs from healthy individuals to augment the direct oncolytic effect. Such augmenting effects were also observed using PBMCs from myeloma patients, although to a lesser extent.

pDC-derived type I IFNs and NK cells contribute to the combined effect of T-01 and PBMCs

Type I IFNs have a cytotoxic effect on myeloma (Chen *et al*, 2001) and also activate NK cells (Müller *et al*, 2017). pDCs are the predominant producer of type I IFNs in PBMCs (Kadowaki *et al*, 2000). Thus, we examined whether type I IFNs, pDCs, and NK cells play a part in the cytotoxic effect in Fig. 2. Frequencies of pDCs and NK cells in blood were lower in myeloma patients than in healthy individuals (Fig. 3A). Large amounts of IFN-α and IFN-β were secreted upon stimulation of PBMCs from healthy individuals with T-01 (Fig. 3Bi). Depletion of pDCs abrogated the secretion of IFN-α and IFN-β (Fig. 3Bi). PBMCs from myeloma patients secreted less IFN-α and IFN-β than those from healthy individuals (Fig. 3Bii).

IFN-α strongly reduced viability of NCI-H929, MM.1S, and U266 but not of PC402 (Fig. 4A). Neutralizing type I IFNs abrogated the combined effect of T-01 and PBMCs from healthy individuals on IFN-α-sensitive NCI-H929 and MM.1S strongly and on IFN-α-resistant PC402 marginally (Fig. 4Bi). Such abrogating effects of neutralizing type I IFNs were less prominent in NCI-H929 and MM.1S when PBMCs from myeloma patients were used (Fig. 4Bii).

Depletion of pDCs but not NK cells abrogated the combined effect of T-01 and PBMCs from healthy individuals on IFN-α-sensitive NCI-H929 and MM.1S (Fig. 5A). In contrast, depletion of NK cells but not pDCs did so on IFN-α-resistant PC402. Depletion of T cells (Fig. 5A) or monocytes (Fig. 5B) did not abrogate the combined effect of T-01 and PBMCs on any of the cell lines. Addition of T-01 to PBMCs induced upregulation of the activation marker CD69 on NK cells (Fig. 5C), consistent with the contribution of NK cells to the cytotoxicity against PC402 (Fig. 5A).

These data indicate that the combined antitumor effect of T-01 and PBMCs is attributed (i) to type I IFNs derived from T-01-stimulated pDCs if the tumor is sensitive to type I IFNs and (ii) to activated NK cells if the tumor is resistant to type I IFNs. Although activated NK cells may also contribute to the antitumor effect against the type I IFN-sensitive cell lines, such an effect may have been masked by the direct cytotoxic effect of type I IFNs. The lesser extent of the combined effects of T-01 and PBMCs (Fig. 2B) and of the abrogating effects of neutralizing type I IFNs (Fig. 4Bii) using PBMCs from myeloma patients may be due to lower frequencies of pDCs and NK cells (Fig. 3A) and lower levels of type I IFN production (Fig. 3Bii) in patients.

Combined effects of T-01 and lenalidomide

We next examined the combined effect of T-01 and a representative IMiD, lenalidomide. T-01 was added at suboptimal MOI 0.05. The concentrations of lenalidomide were adjusted to those expected to induce a partial cytotoxic effect on each cell line. Lenalidomide alone killed four (NCI-H929, MM.1S, U266, and PC402) out of six cell lines (Fig. 6A), which was consistent with previous reports (Hideshima *et al*, 2000, Lopez-Girona *et al*, 2011). The combination of T-01 and lenalidomide exhibited significantly stronger cytotoxicity against all lenalidomide-sensitive cell lines than each

alone (Fig. 6B). The addition of lenalidomide did not augment replication of T-01 in the cell lines as detected by quantification of the *gB* gene of HSV-1 (data not shown), suggesting that T-01 and lenalidomide worked independently rather than synergistically.

To examine the effect *in vivo*, we subcutaneously injected MM.1S to immunodeficient SCID Beige mice. Intratumor injection of T-01 and intraperitoneal injection of lenalidomide individually suppressed the growth of MM.1S (Fig. 6C). The combination of T-01 and lenalidomide further suppressed tumor growth. Thus, the combination may represent an effective therapy for plasma cell neoplasms accessible to intratumor injection of HSV-1.

Combined effects of T-01, lenalidomide, and IFN-α

We examined the combined effects of the three factors, T-01, lenalidomide, and IFN-α. T-01 and lenalidomide were added as in Fig. 6B, and IFN-α was added at concentrations close to the IC_{50} (500 U/mL) except for IFN-α-resistant PC402 at 5000 U/mL. The combination of T-01 with lenalidomide or with IFN-α was more effective than each alone (Fig. 7). The triple combination of T-01, lenalidomide, and IFN-α further enhanced the cytotoxic effects of the dual combinations for NCI-H929 and U266, or at least did not reduce them for MM.1S and PC402. Thus, the triple combination may cooperate in the antitumor effect on myeloma cells.

Discussion

In this study, we showed that oncolytic HSV-1 has potential for the treatment of plasma cell neoplasms through oncolytic and immune-mediated mechanisms. pDC-derived type I IFNs and NK cells play a key role in the immune-mediated effects. Furthermore, lenalidomide augmented the cytotoxic effect of HSV-1 apparently by an independent rather than synergistic mechanism. As lenalidomide is known to activate NK cells (Hayashi *et al*, 2005, Lagrue *et al*, 2015), oncolytic HSV-1 and lenalidomide may be a rational combination to treat plasma cell neoplasms.

Although plasmacytoma of the presented patient relapsed after multiple regimens and was highly aggressive, T-01 showed cytotoxicity to the primary tumor cells and the derived cell line. This suggests that the conventional therapies and oncolytic HSV-1 kill myeloma cells through different mechanisms and illustrates the potential of oncolytic HSV-1 as a novel therapy for plasma cell neoplasms resistant to conventional therapies.

HSV-1 stimulates pDCs to produce a large amount IFN-α/β (Kadowaki *et al*, 2000), which activate NK cells (Müller *et al*, 2017). HSV-1 also appears to directly activate NK cells (Dai and Caligiuri 2018). Furthermore, type I IFNs have direct antitumor activity against plasma cell neoplasms, in which type I IFNs induce TRAIL expression, cytochrome c release from mitochondria, and downregulation of Bcl-2 and Bcl-X_L, culminating in apoptosis (Chen *et al*, 2001). Thus, it is expected that oncolytic HSV-1 exerts anti-myeloma activity by activating pDCs and NK cells and that type I

IFNs are key mediators of the anti-myeloma activity. Accordingly, our data suggest that pDC-derived type I IFNs and activated NK cells are responsible for the immune-mediated anti-myeloma effect induced by HSV-1. The relative contributions of type I IFNs and NK cells to cytotoxicity appear to depend on the susceptibility of tumor cells to the direct cytotoxic effect of type I IFNs. Furthermore, the dual (T-01 + lenalidomide) and triple (T-01 + lenalidomide + IFN-α) combinations augmented the direct antitumor effect of T-01. Thus, the combination of oncolytic HSV-1 and lenalidomide may induce multi-pronged anti-myeloma activity through direct cytotoxicity and through IFN-α- and NK cell-mediated effects (Fig. 8). NK cells play an important role in antibody therapies with daratumumab and elotuzumab through antibody-dependent cellular cytotoxicity and activation of SLAMF7$^+$ NK cells with elotuzumab (Campbell *et al*, 2018, Pittari *et al*, 2017). Thus, multiple combinations including HSV-1, lenalidomide, and monoclonal antibodies to mobilize NK cells may represent intriguing strategies against plasma cell neoplasms. PBMCs from myeloma patients also exhibited combined effects with T-01, although to a lesser extent than PBMCs from healthy individuals. This may be due to lower frequencies of pDCs and NK cells and lower levels of type I IFN production in patients.

A recent study has shown that coxsackievirus also induces antitumor activity against myeloma through activating pDCs and NK cells (Müller *et al*, 2019). In addition, lenalidomide has been approved for other lymphoid malignancies such as mantle cell lymphoma, follicular lymphoma, marginal zone lymphoma, and adult T-cell leukemia/lymphoma. Thus, our study suggests that lenalidomide may enhance antitumor activities of different kinds of oncolytic viruses against various lymphoid malignancies.

Other oncolytic viruses have been applied to multiple myeloma via an intravenous route (Meyers *et al*, 2017). However, intravenously administered viruses are extensively diluted in the systemic circulation and also rapidly induce the production of neutralizing antiviral antibodies, which hinders repeated injection. Local intratumor injection induces a systemic immune-mediated effect, which is augmented by combining with immune checkpoint blockade (Ribas *et al*, 2017). Thus, HSV-1 is likely to be applicable to extramedullary tumor lesions of plasma cell neoplasms. In addition, as oncolytic HSV-1 is not toxic to human hematopoietic cells (Wu *et al*, 2001), HSV-1 may also be applicable to marrow myeloma lesions for intra-marrow injection.

We recently found that oncolytic HSV-1 kills various lineages of hematological tumor cells and that the expression of nectin-1, a receptor for glycoprotein D of HSV-1 (Geraghty *et al*, 1998), is a decisive factor for the cytotoxic effect (submitted). Together with this finding, the present study suggests that oncolytic HSV-1 is a viable therapy for nectin-1-expressing plasma cell neoplasms and that the combination with lenalidomide may represent a rational therapeutic option that exploits direct cytotoxic as well as indirect immune-mediated mechanisms.

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Acknowledgments

Authorship Contributions

M.O. performed the research, analyzed the data, and wrote the paper; R.I. and S.U. performed the research; O.I. collected the data; N.S. contributed vital experimental designs and techniques; T.T. contributed vital new reagents; and N.K. designed the research study, analyzed the data, and wrote the paper.

Conflict of interest

The authors declare no competing financial interests.

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Figure legends

Fig. 1. T-01 kills primary plasmacytoma cells and human myeloma cell lines

(A) Mononuclear cells obtained from the pleural effusion of a patient with multiple solitary plasmacytoma were treated with T-01 for 72 h. After gating for CD138$^+$ cells, the percentages of propidium iodide-negative viable cells were measured using flow cytometry. Left: dot plots of forward scatter and propidium iodide with gating of viable cells after treatment with mock or T-01 (MOI 1.0) for 3 days. Percentages of viable cells are indicated on each plot. Right: viability after treatment with mock or T-01 (MOI 0.01, 0.1, or 1.0) for 72 h, calculated as (viable cell percentage with T-01/viable cell percentage with mock) × 100 (%). (B) Myeloma cell lines were treated with the indicated MOI of T-01 for 72 h. Viability was measured using a WST-8 assay. The values are normalized to those of control cells. The data are shown as the mean ± SD of three independent experiments.

Fig. 2. Combined effects of T-01 and PBMCs

Cell lines were treated with T-01 at MOI 0.05 in the absence or presence of PBMCs from (A) healthy individuals or (B) myeloma patients for 72 h. The ratios of cell numbers (PBMCs : myeloma cells) were 1:1 for NCI-H929, MM.1S, U266 (5 × 10^4/200 µL) and 5:1 for PC402 (2 × 10^4/200 µL). The starting cell numbers of each cell line were adjusted by their growth rates to avoid overgrowth. The absolute numbers of CD138$^+$ myeloma cells were calculated by adding CountBright^{TM} Absolute Counting Beads and by analyzing with flow cytometry. The values are normalized to those of control cells. The data are shown as the mean ± SD of (A) six or (B) five independent experiments. Statistical analysis was conducted by one-way ANOVA followed by Tukey's test. **$P < 0.01$ compared with T-01 alone.

Fig. 3. Frequencies of pDCs and NK cells and type I IFN production in PBMCs

(A) Frequencies (%) of pDCs and NK cells among PBMCs (other than monocytes) from 7 healthy individuals and 5 myeloma patients. Monocytes were excluded based on the forward scatter/side scatter pattern. The horizontal bars indicate mean values. (B) Type I IFN production. After total PBMCs from (i) 3 healthy individuals and (ii) 5 myeloma patients or PBMCs depleted of pDCs were stimulated by the indicated MOI of T-01 for 24 h, the supernatants were collected and concentrations of IFN-α and IFN-β were measured by ELISA.

Fig. 4. Type I IFNs contribute to the combined effect of T-01 and PBMCs

(A) Cell lines were treated with IFN-α (5000 U/mL) for 72 h. Viability was measured using a WST-8 assay. The values are normalized to those of control cells. The data are shown as the mean ± SD of three independent experiments. Statistical analysis was conducted by a two-tailed unpaired t-test. ***$P < 0.001$. (B) Cell lines were treated with T-01 at MOI 0.05 in the absence or presence of PBMCs from

(i) healthy individuals or (ii) myeloma patients for 72 h. Human type I IFN neutralizing antibody mixture was added to the last three conditions of each cell line. The ratios of cell numbers (PBMCs : myeloma cells) were 1:1 for NCI-H929, MM.1S ($5 \times 10^4/200$ μL) and 5:1 for PC402 ($2 \times 10^4/200$ μL). The absolute numbers of $CD138^+$ myeloma cells were calculated by adding CountBright^{TM} absolute counting beads and by analyzing using flow cytometry. The values are normalized to those of control cells. The data of (i) three and (ii) five independent experiments are shown. The horizontal bars indicate mean values.

Fig. 5. pDCs and NK cells contribute to the combined effect of T-01 and PBMCs

(A) Cell lines were treated with T-01 at MOI 0.05 for 72 h in the absence or presence of total PBMCs from healthy individuals or PBMCs depleted of NK cells, T cells, or pDCs. The ratios of cell numbers (PBMCs : myeloma cells) were 1:1 for NCI-H929, MM.1S ($5 \times 10^4/200$ μL) and 5:1 for PC402 ($2 \times 10^4/200$ μL). The absolute numbers of $CD138^+$ myeloma cells were calculated by adding CountBright^{TM} absolute counting beads and by analyzing using flow cytometry. The values are normalized to those of control cells. The data are shown as the mean ± SD of six independent experiments. Statistical analysis was conducted by one-way ANOVA followed by Dunnett's test. ***$P < 0.001$ compared with T-01 plus PBMCs. (B) Experiments were performed as in (A) with or without monocyte depletion. The data are shown as the mean ± SD of three independent experiments. (C) PBMCs from three healthy volunteers were treated with T-01 for 24 h. The expression levels of CD69 on $CD56^+$ cells are shown.

Fig. 6. Combined effects of T-01 and lenalidomide

(A) Cell lines were treated with the indicated concentrations of lenalidomide for 5 days. Viability was measured using a WST-8 assay. The values were normalized to those of control cells. The data are shown as the mean ± SD of three independent experiments. (B) Cell lines were treated with T-01 at MOI 0.05 in the absence or presence of lenalidomide for 5 days. Concentrations of lenalidomide were 0.5 μM for MM.1S, 3 μM for NCI-H929 and PC402, and 5 μM for U266. Viability was measured using a WST-8 assay. The values were normalized to those of control cells. The data are shown as the mean ± SD of six independent experiments. Statistical analysis was conducted by one-way ANOVA followed by Tukey's test. *$P < 0.05$, ***$P < 0.001$. (C) MM.1S was injected subcutaneously into the left flank of SCID Beige mice (n=6). Mock or T-01 was injected into the established tumor on day 0 and 3 intratumorally. Lenalidomide was administered intraperitoneally 5 days per week for 3 weeks from day 0. The tumor size was measured every 3 or 4 days. The data are shown as the mean ± SD.

Fig. 7. Triple combination effects of T-01, lenalidomide, and IFN-α

Cell lines were treated with T-01 (MOI 0.05), lenalidomide, or IFN-α for 5 days. Concentrations of

lenalidomide were 0.5 µM for MM.1S, 3 µM for NCI-H929 and PC402, and 5 µM for U266. Those of IFN-α were 500 U/mL for NCI-H929, MM.1S, and U266, and 5000 U/mL for PC402. Viability was measured using a WST-8 assay. The values are normalized to those of control cells. The data of three independent experiments are shown. The horizontal bars indicate mean values.

Fig. 8. Multi-pronged effects of HSV-1 and lenalidomide

HSV-1 directly kills myeloma cells, and directly stimulates pDCs (through Toll-like receptor 9) and reportedly NK cells. pDC-derived IFN-α directly kills IFN-α-susceptible myeloma cells and stimulates NK cells. Activated NK cells kill myeloma cells. Lenalidomide kills myeloma cells and activates NK cells. Such multi-pronged effects likely cooperate to kill myeloma cells efficiently.
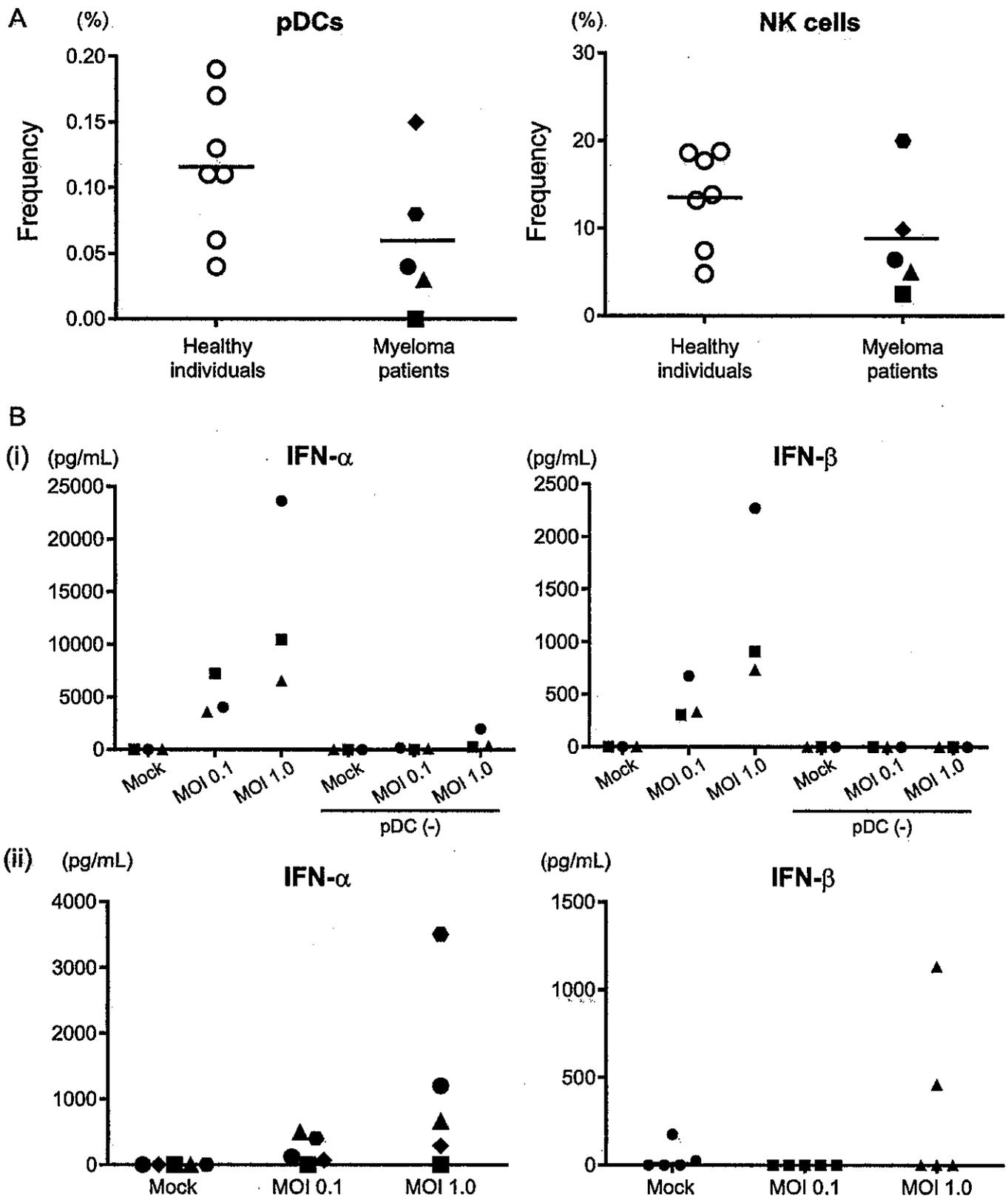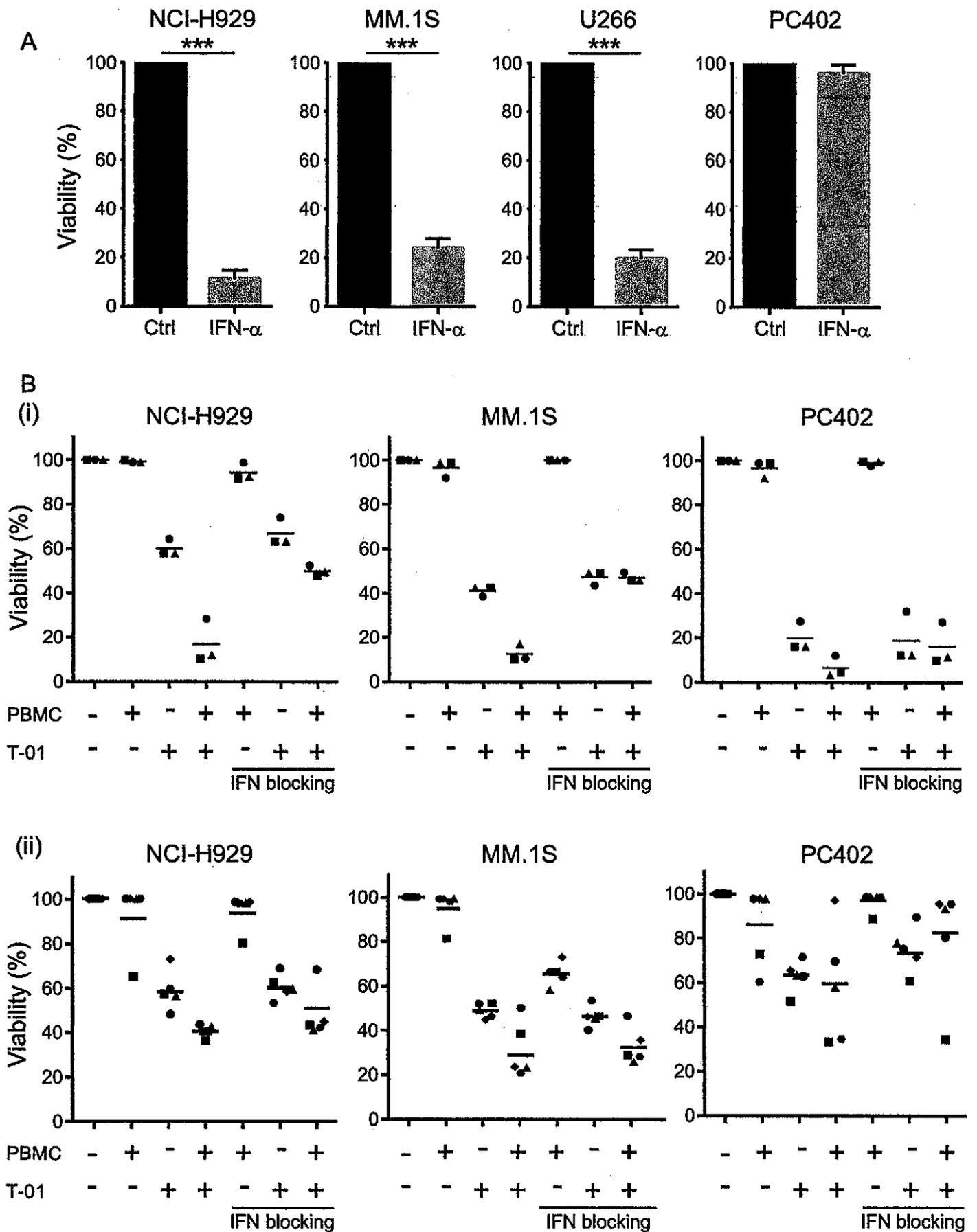
Figure 1

A

B
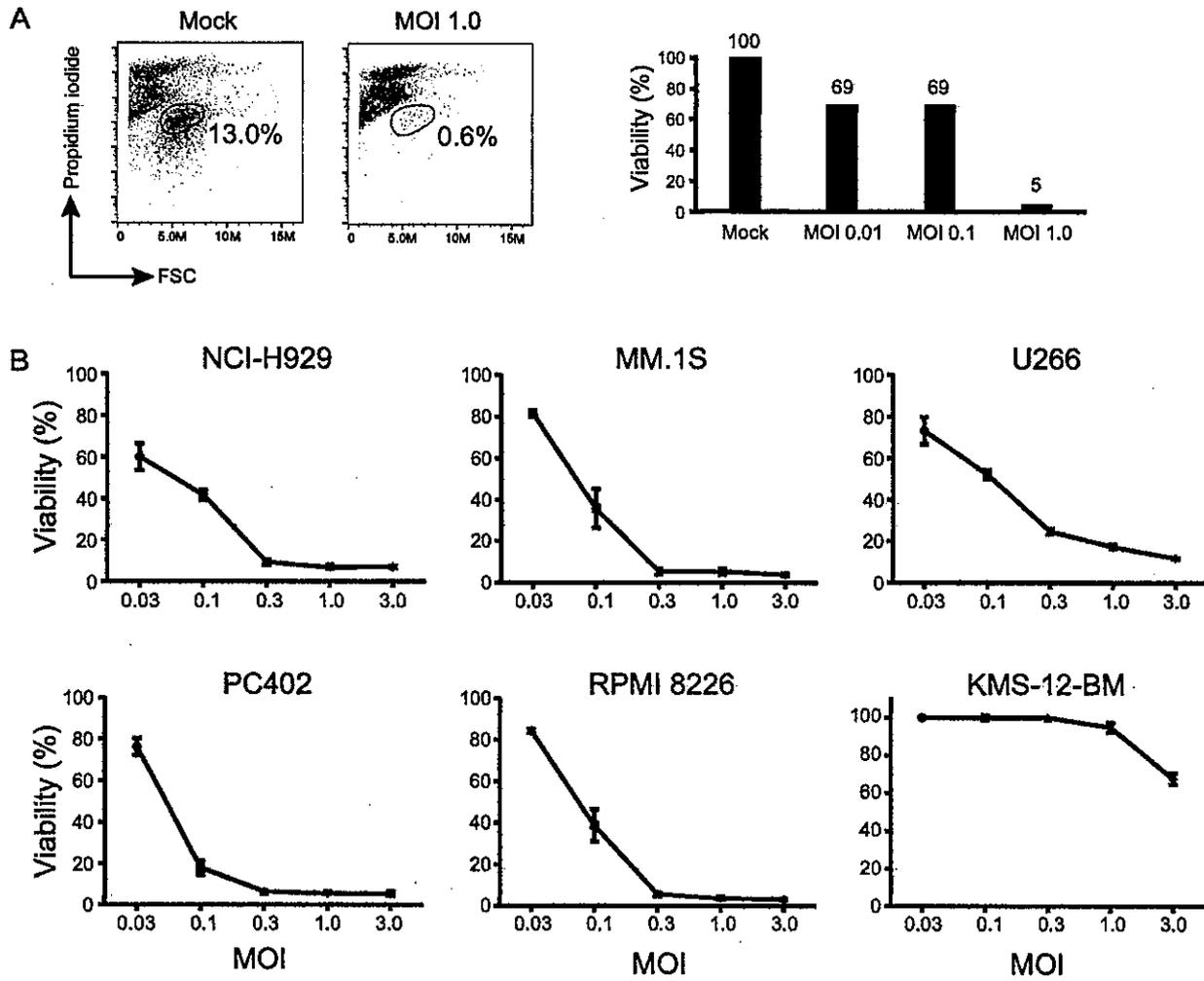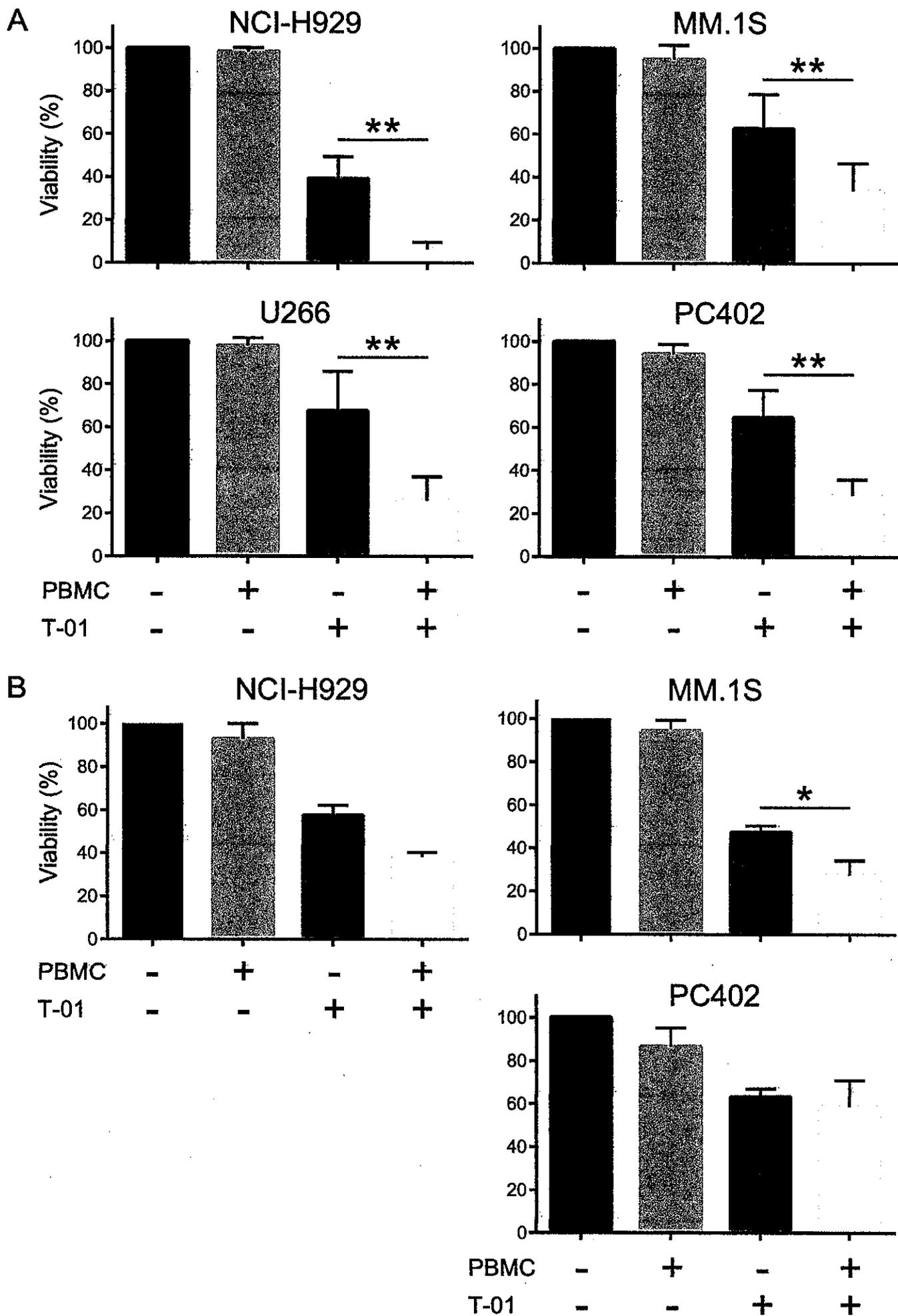
Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

A

NCI-H929

MM.1S

U266

PC402

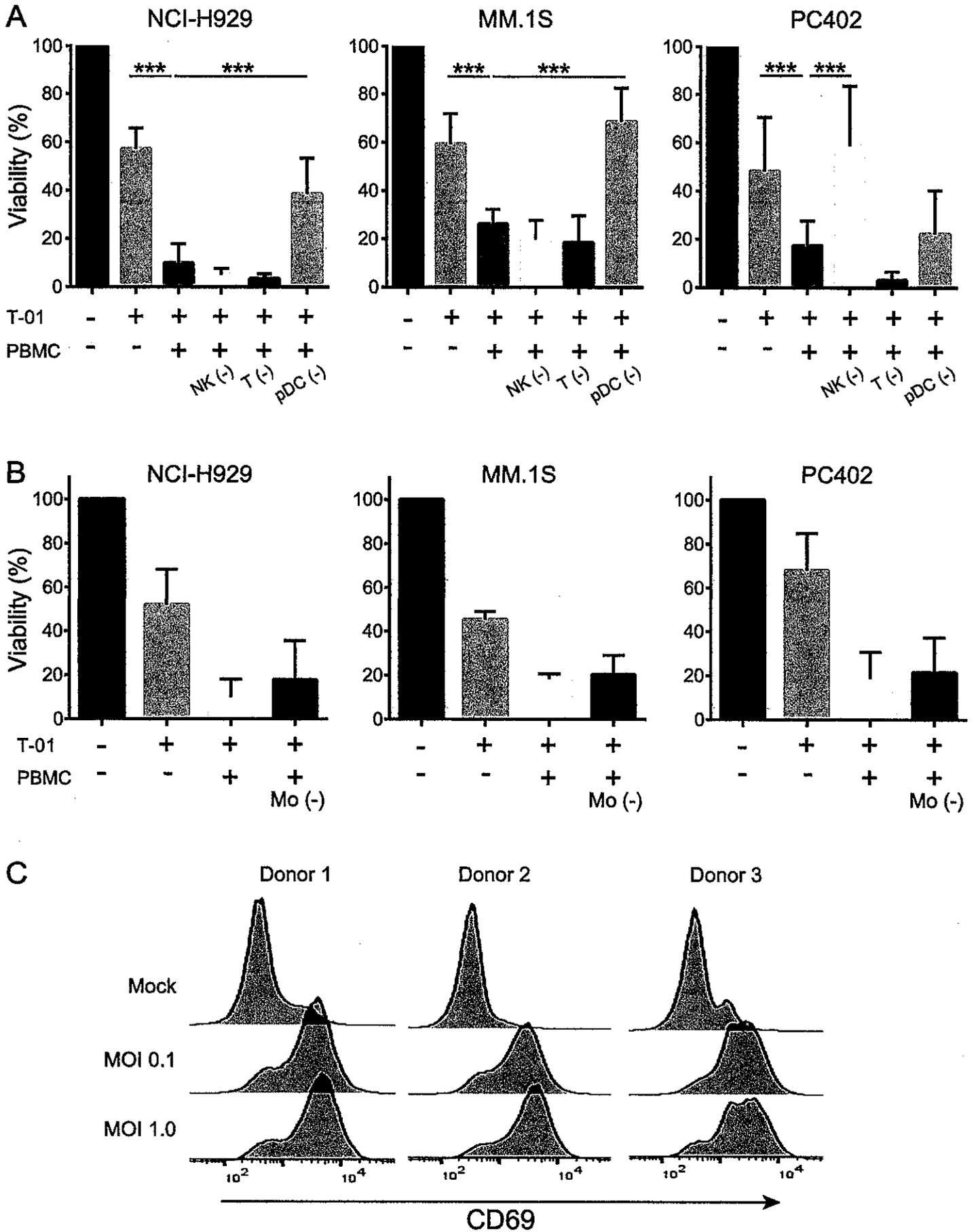RPMI 8226

KMS-12-BM

B

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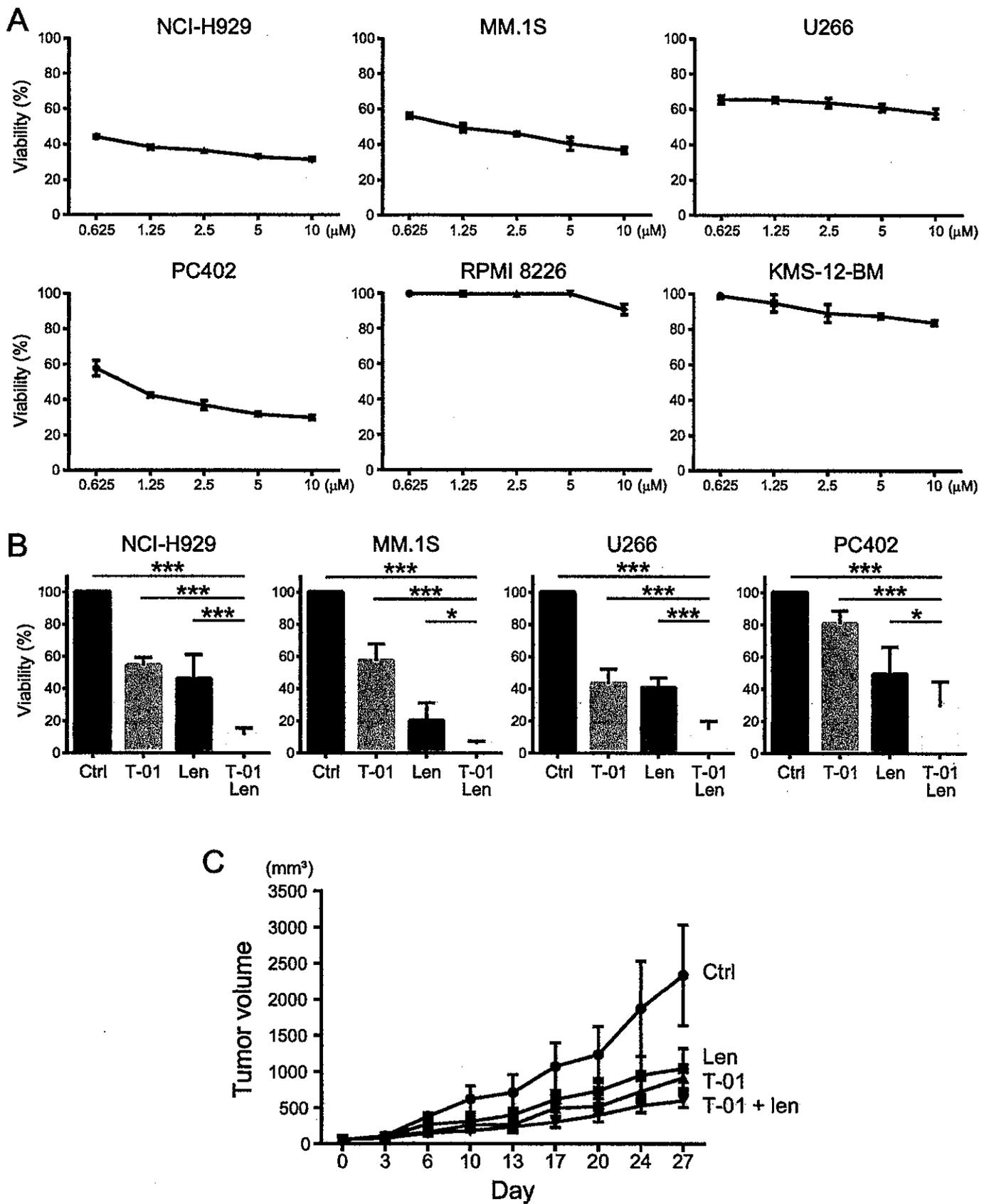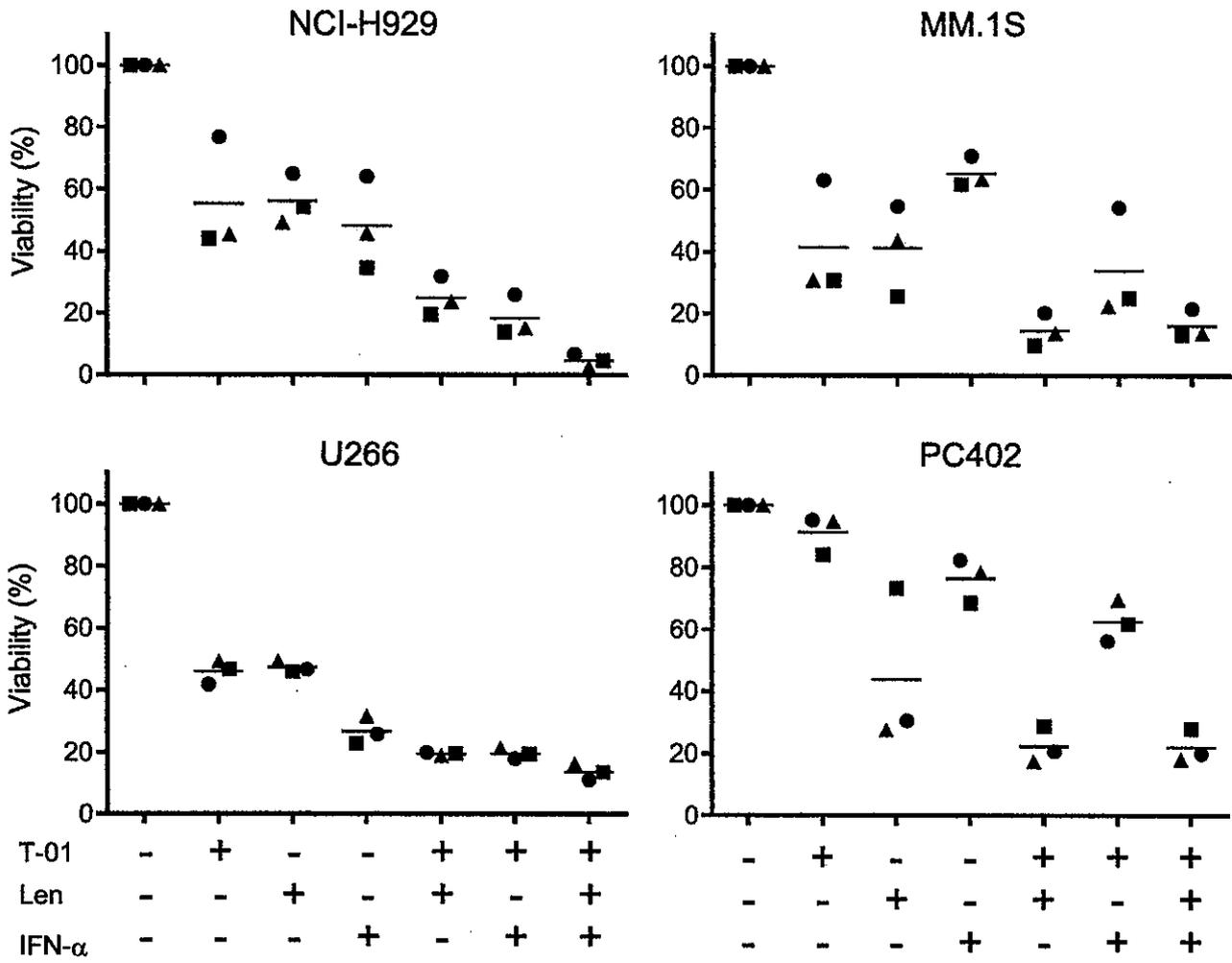
Figure 7

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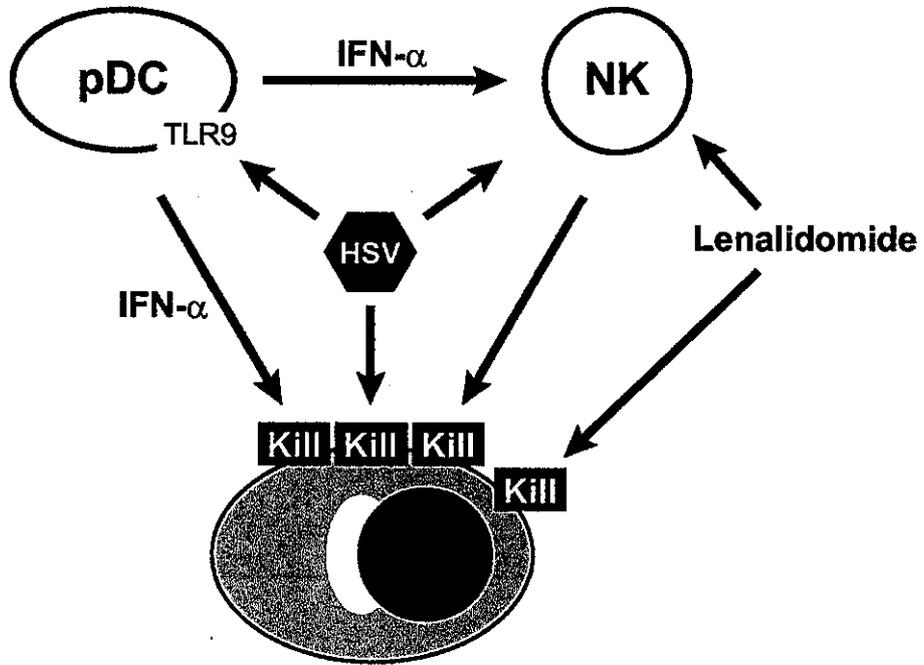
Table S1. A list of myeloma patients

Patient #	Age	Sex	Therapy
1	79	F	Kd (5th line)
2	77	F	Rd (1st line)
3	58	F	Untreated
4	58	M	EPd (4th line)
5	78	M	Therapy-free (after 5th line)

Kd: carfilzomib + dexamethasone

Rd: lenalidomide + dexamethasone

EPd: elotuzumab + pomalidomide + dexamethasone