p38 MAPK differentially controls NK activating ligands at transcriptional and post-transcriptional level on multiple myeloma cells

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ABSTRACT
The mechanisms that regulate the expression of the NKG2D and DNAM-1 activating ligands are only partially known, but it is now widely established that their expression is finely regulated at transcriptional, post-transcriptional and post-translational level, and involve numerous stress pathways depending on the type of ligand, stressor, and cell context.

We show that treatment of Multiple Myeloma (MM) cells with sub-lethal doses of Vincristine (VCR), an anticancer drug that inhibits the assembly of microtubules, stimulates the expression of NKG2D and DNAM-1 activating ligands, rendering these cells more susceptible to NK cell-mediated killing. Herein, we focused our attention on the identification of the signaling pathways leading to de novo surface expression of ULBP-1, and to MICA and PVR upregulation on VCR-treated MM cells, both at protein and mRNA levels.

We found that p38MAPK differentially regulates drug-dependent ligand upregulation at transcriptional and post-transcriptional level. More specifically, we observed that ULBP-1 expression is attributable to both increased transcriptional activity mediated by ATM-dependent p53 activation, and enhanced mRNA stability; while the p38-activated E2F1 transcription factor regulates MICA and PVR mRNA expression. All together, our findings reveal a previously unrecognized activity of VCR as anticancer agent, and indicate that in addition to its established ability to arrest cell growth, VCR can also modulate the expression of NKG2D and DNAM-1 activating ligand on tumor cells and thus promoting NK cell-mediated immunosurveillance.

Introduction
The NKG2D and DNAM-1 activating receptors are major innate immunity recognition receptors for identification and elimination of transformed and infected cells, after engaging ligands that are inducibly expressed. The presence of NKG2D and DNAM-1 ligands on transformed, infected, or proliferating cells indicates that cells under different stressful-associated conditions activate specific intracellular signaling pathways leading to increased stress ligands that act as signals to alert the immune system (1,2). NKG2D is a C-type lectin-like molecule encoded on human chromosome 12 within the NK gene complex (3,4), and is expressed on NK cells, CD8+ T-cell receptor (TCR)-αβ T cells and TCR-γδ T cells. In humans, the NKG2D ligands include MICA and MICB (MHC class I chain-related proteins A and B), both encoded by genes in the MHC, and up to six different proteins belonging to the UL16-binding protein (ULBP) family ULBPs. DNAM-1 is an Ig-like family glycoprotein expressed on most human NK cells, monocytes, and T lymphocytes, including CD4+, CD8+, NKT, and TCR-γδ T cells (5-8). Two molecules belonging to the nectin protein family, PVR (Poliovirus Receptor; CD155, Necl5) and Nec-2 (Nectin-2; CD112, PRR2) have been so far identified as ligands of DNAM-1.

The mechanisms that control the expression of NKG2D and DNAM-1 ligands are only partially known and are largely dependent on the cell type and stressors including heat shock, UV and oxidative and proteoxic stress, DNA damaging, and chromatin remodeling agents (8-12). Regulation of NKG2D and DNAM-1 ligand expression mainly occurs at transcriptional level. Additional mechanisms operate at the post-
transcriptional level including inhibition of mRNA translation by cellular or viral microRNAs (miRNAs), ubiquitin-dependent proteasomal degradation, exosomal excretion, and proteolytic shedding from cell surface (2,13).

Relevant signaling pathways involved in the induction of the NKG2D and DNAM-1 ligand expression include stress pathways, such as DNA damage and heat shock response (8,14,15), proliferative pathways, generated mainly by oncogene activation (16,17), and tumor suppressor pathways, where the p53 protein is a key regulator. In particular, activation of p53 induces the expression of ULBP1/2 (18,19), although other NKG2D ligands have been found to be upregulated in response to DNA damage in a p53-independent manner. Whether and how the stress-activated MAPKs are involved in the regulation of NKG2D and DNAM-1 activating ligand expression is still unraveled.

Mounting evidences suggest the use of drugs aimed at modulating and increasing the recognition of tumor cells by both innate and adaptive immune system (10,29,30). Several pharmacological drugs and other conventional treatments have been shown to induce expression of activating ligands on various solid and hematologic tumors (21-23). In particular, our group has demonstrated that genotoxic drugs including doxorubicin and melphalan by generating reactive oxygen species (ROS) induce a DNA Damage Response (DDR)-dependent increase of activating NK receptor ligand expression on senescent tumor cells (18). More recently, we have also proven that expression of the NKG2D and DNAM-1 activating ligands MICA and PVR, is enhanced by immunomodulatory drugs (IMiDs) (24).

It is well known that microtubules influence a variety of cell stress responses and act as coordinators of cell functions in response to stress (25). Recent work also suggests that induction of mitotic arrest by inhibitors of microtubule assembly can trigger DNA damage response pathways in mitotic-arrested cells (26-28). These observations raise the interesting question of whether perturbation of microtubule assembly, per se, might also evoke NK activating ligand expression.

A number of potent microtubule inhibitors, including Vin-cristine (VCR), belong to the Vinca alkaloids family and are widely used to treat a variety of solid and hematologic tumors. Their most common mechanism of action is to bind tubulin and inhibit its polymerization, thus preventing the assembly of the mitotic apparatus.

In the present study, we have investigated the signaling cascade initiated by VCR in microtubule targeting, and have asked whether it ultimately influenced NKG2D and DNAM-1 activating ligand expression on Multiple Myeloma (MM), an haematologic cancer characterized by clonal expansion of malignant plasma cells (PCs) and highly susceptible to recognition and killing by NK cells mainly through NKG2D and DNAM-1 receptors (10,29,30). VCR is widely used to treat haematologic cancers and solid tumors (21). Although a number of novel agents have been recently introduced into clinical practice of myeloma patients, including IMiDs, checkpoint inhibitors, proteasome inhibitors, and antibody-targeted therapy, the use of VCR remains an important therapeutic option, particularly in the treatment of relapsed and refractory MM patients (32). We provide evidence for a new role of tubulin polymerization inhibitors and p38 MAPK in the upregulation of NKG2D and DNAM-1 activating ligands. We found that activation of p38 is involved in the VCR-mediated MICA and PVR upregulation at both protein and mRNA levels, and in post-transcriptional control of NKG2D ligand ULBP-1 expression. In particular, we observed that the p38MAPK-dependent activation of E2F1 transcription factor contributes to MICA and PVR increased expression upon VCR treatment. Moreover, the observed ULBP-1 mRNA upregulation was associated with changes in the half-life of ULBP-1 transcripts upon VCR treatment and only partially depended on p53 activity. In addition, we provide data that extend the role of VCR in the regulation of the NK cell activating ligand to different cancer cells.

These results uncover a novel activity of the microtubule inhibitor VCR, of potential therapeutic value toward enhancing NKG2D- and DNAM-1-mediated immune responses in vivo.

Results

Vin-cristine-induced upregulation of NKG2D and DNAM-1 ligand expression on MM cells

To evaluate the effect of the microtubule polymerization inhibitor VCR on regulation of NKG2D and DNAM1 ligands, we first needed to identify the dose of VCR that would not affect cell viability in SKO-007(J3) MM cells. Cultures treated with low doses of VCR were analyzed by flow cytometry. After 48 h, VCR (0.05 ug/mL) induced some G2/M phase delay compare with untreated controls, yet did not induce complete G2/M arrest; consistent with this, Annexin-V/PI staining showed that the majority of cells (roughly 80%) remained viable after 48 h of treatment with low-dose VCR (Figs. S1A and B).

We then evaluated whether the sub-lethal dose of VCR identified, could modulate the expression of NK cell receptor activating ligands by immunofluorescence and FACS analysis, and we observed that 48 h treatment differentially modulated NKG2D and DNAM-1 ligands on the SKO-007(J3) cells (Fig. 1A). It is very important to note that VCR treatment led to a de-novo induction of ULBP-1 ligand, the basal level of which was barely detectable. Ligand surface expression on treated MM cells was accompanied by a corresponding increase in mRNA levels as shown by real-time PCR (Fig. 1B ). As far the NKG2D ligands, VCR upregulated MICA mRNA already expressed at basal levels, and more strongly ULBP-1 transcript levels that were not or weakly detectable (Ct = 35). Like MICA, the DNAM1 ligand PVR transcript was already present in the untreated MM cells and its expression increased in response to drug treatment. To evaluate whether VCR could affect NKG2D and DNAM-1 ligand expression in other tumors, either at protein or mRNA levels, we extended our analysis to HeLa cells, derived from HPV-originated cervical cancer, and to the metastatic melanoma cell line MeC (Fig. S2 and data not shown). VCR treatment significantly increased the surface expression of the activating ligands on both tumor cell lines, except for MICB and ULBP3 on melanoma cells where the treatment did not influence the expression.

To determine whether VCR-induced activating ligand upregulation occurred at transcriptional level, we transiently transfected MICA, ULBP-1, and PVR gene promoters in SKO-007(J3) cells. The activity of the luciferase reporter ULBP-1 gene construct was highly enhanced by drug treatment, whereas MICA promoter
activity was only slightly, but significantly increased upon VCR treatment; a similar promoter induction was observed for PVR gene (Fig. 1C).

Collectively, these data suggest that ULBP1, MICA, and PVR drug-induced surface expression on MM cells by the VCR is due to transcriptional events.

We also extended our analysis to MM patients’ malignant PCs at different states of the disease obtained from patients prior to any therapeutical protocol (Table 1). At variance with the results obtained in the MM cell line, we found a marked increase of all NKG2D and DNAM-1 ligand surface expression on VCR-treated PCs, although it varied among different patients and was not related to the state of disease (Fig. 1D).

VCR stimulated p38 MAPK activation is involved in MICA, ULBP-1, and PVR ligand upregulation on drug-treated MM cells

The p38 MAPkinase is preferentially activated by many stress stimuli, including perturbation of microtubule assembly, via

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**Table 1.** Patient characteristics. Patients were classified according to the state of the disease.

| Patient no. | Clinical stage | Monoclonal Ig | % PCs in BM |
|-------------|----------------|---------------|-------------|
| 1           | Relapse        | IgA-k         | 28          |
| 2           | Smoldering     | IgG-L         | 22          |
| 3           | Onset          | IgG-k         | 8           |
| 4           | Relapse        | IgG-L         | 45          |
| 5           | Smoldering     | IgG-L         | 13          |
| 6           | Smoldering     | IgG-L         | 3           |
| 7           | Relapse        | IgA-L         | 9           |
| 8           | MGUS           | IgG-k         | 7           |
| 9           | Relapse        | IgG-k         | 52          |
| 10          | Onset          | IgG-k         | 40          |
| 11          | Smoldering     | IgG-k         | 30          |
| 12          | Onset          | IgA-k         | 97          |
| 13          | Onset          | IgG-L         | 67          |
| 14          | Relapse        | IgG-k         | 32          |
| 15          | Onset          | IgG-k         | 24          |
| 16          | Onset          | IgG-L         | 34          |
| 17          | Relapse        | IgA-L         | 30          |
| 18          | Relapse        | IgG-L         | 28          |
the MEKK1-MKK3 cascade (33-35). We first analyzed the ability of VCR to trigger p38 MAPK activation by employing a phospho-specific antibody that recognizes the active form of the kinase when dually phosphorylated at T180/Y182. VCR treatment promoted p38 MAPK phosphorylation evident at 3 h, peaking at 10 h, and returning at basal level after 24 h (Fig. 2A). Of note, the other members of the MAPK family ERK and JNK were not activated under the same experimental conditions.

Next, we focused our attention on the role of this MAPK in the control of ligand expression on MM cells. To this purpose, SKO-007(J3) and patients’ malignant PCs were treated with SB203580, a specific p38 MAPK inhibitor, prior to administering VCR, and MICA, ULBP-1, and PVR surface expression was evaluated. Drug-induced ligand upregulation was almost completely abolished by the p38 MAPK inhibitor (Figs. 2B and C).

We then analyzed whether p38 activation was involved in the regulation of activating ligand transcripts and assessed their mRNA levels by real-time PCR with or without pretreatment with SB203580 inhibitor before MM cell exposure to VCR. We found that SB induced a partial but significant reduction of MICA and PVR levels; surprisingly, ULBP-1 mRNA expression was instead not affected by this treatment, suggesting that p38 kinase regulates at different levels the NKG2D ligands MICA and ULBP1 (Fig. 2D). Moreover, in accordance with the failure of p38 inhibitor to reduce ULBP1 mRNA expression, we observed by confocal microscopy high levels of ULBP-1 in the cytoplasm of VCR-treated cells (Fig. 3).

All together, these findings show that p38 MAPK controls MICA and PVR ligand upregulation on drug-treated MM cells through activation of E2F1 transcription factor.

E2F1 transcription factor is responsible for the transcriptional regulation of NKG2D and DNAM-1 ligand in cells undergoing...
proliferation or genotoxic stress (14,36). This transcription factor ULBP-1 protein is still evident in the cytoplasm of VCR-treated cells after p38 inhibition. SKO-007(J3) cells were left untreated or treated with VCR alone or VCR-SB203580, stained with anti-ULBP1 mAb (green) and Hoechst (blue), and analyzed by Confocal Microscopy. (A) Representative images are shown as a single optical section acquired with a 60X/1.35NA oil immersion objective. Scale bar: 5 μm. (B) Green fluorescence intensity was measured with Fiji/ImageJ software in 20 cells randomly acquired from two independent experiments. Bars represent the mean ± SD. *p ≤ 0.005.

Vincristine treatment of MM cells increases their susceptibility to NK cell-mediated lysis

We investigated whether increased NKG2D and DNAM1 ligand surface expression could result in higher MM cell susceptibility to lysis mediated by NK cells. The expression of the lysosomal marker CD107a that correlates with NK cell cytotoxicity (39), was evaluated by immunofluorescence and FACS analysis by gating on NK cells upon interaction with drug-treated or untreated SKO-007(J3) MM cells used as targets. The upregulation of NKG2D and DNAM-1 ligands was verified before the degranulation assay. We observed increased degranulation of NK cells contacting VCR-treated MM as compared to untreated cells and in accordance with our previous findings, this increase was abrogated when target cells were pretreated with the p38 inhibitor before VCR treatment (Fig. 7A).
The average of the CD107a % of at least five independent experiments is shown in Fig. 7B. Conversely, MM cell pretreatment with caffeine before VCR treatment has no effect on NK cell degranulation (Fig. 7A).

Based on these findings, we evaluated the role of NKG2D and DNAM-1 in MM cell killing by performing the degranulation assay in the presence of anti-NKG2D and/or anti-DNAM-1 blocking mAbs. Drug-treated SKO-007(J3) myeloma cells were killed in an NKG2D-dependent manner, whereas treatment with a DNAM-1 neutralizing mAb has only a marginal albeit significant effect on the CD107a expression (Fig. 7C). In addition, ULBP-1 blocking antibody partially controls NK cell degranulation, leading to the conclusion that this ligand also has a minimal role in the NK cell-mediated killing of MM cells.

These functional data indicate that treatment of tumor cells with VCR by upregulating surface expression of ligands for activating NK cell receptors, promotes NK cell recognition and killing mainly through the engagement of the NKG2D activating receptor.

**Discussion**

Much evidence indicate the ability of DNA-damaging agents and genotoxic drugs to potentiate antitumor NK cell effector functions by upregulating the expression of activating ligands on tumor cells and thus enhancing their recognition and elimination by NK cells.

Regulation of activating ligand expression is a rather complex phenomenon and can occur at different levels. Indeed, NKG2D ligand expression is finely regulated by transcriptional, post-transcriptional and post-translational mechanisms, and involves numerous stress pathways (2); whereas the mechanisms regulating DNAM-1 ligand expression are less characterized.

Herein, we first provide evidence that also alterations of microtubule dynamics by means of a microtubule inhibitor such as VCR results in stimulation of NKG2D and DNAM-1 ligand expression on MM cells by triggering signaling events, namely activation of the stress-induced p38MAPK and ATM, the upstream kinase of DDR, with different contribution depending on the activating ligand. In particular, ATM-p53 pathway controls drug-induced NKG2D ligand ULBP-1 expression at transcriptional level, whereas the p38 kinase regulates through the activation of E2F1 transcription factor MICA and PVR expression, and ULBP1 expression at post-translational level.

At present, the role of p38 MAPK in the control of activating ligand expression refers only to the NKG2D MICA/B and ULBP2 ligand expression with no evidence on the molecular mechanisms involved (40-43).

Our findings indicate that activation of p38 by the microtubule inhibitor controls the NKG2D ligand MICA and the DNAM1 ligand PVR expression at transcriptional level through activation of E2F1 transcription factor by phosphorylation at Ser364. They are consistent with previous evidence showing that E2F1 transcription factor is responsible for the transcriptional regulation of MICA and PVR in a manner dependent on ROS-induced DDR signaling and p53 independent, and of the mouse NKG2D ligand
Rae-1 in response to proliferative signals (14, 44). As far as ULBP1, this kinase affects the protein cellular distribution, but not gene transcription. At present, the mechanisms underlying this regulation are still unclear but we can envisage a role for p38 in the protein trafficking to the plasma membrane or in the protein internalization/recycling from the cell surface.

In regard to ATM/ATR initiated DDR cascade, accumulating evidence indicates that the upregulation of NKG2D ligands under different physiological and pathological conditions, including mitosis, viral infections, and cancer, involves DNA damage response activation, with a variable involvement of the downstream ATM effector p53.

As far as DNAM-1 ligands, very little information are available on the mechanisms underlying their regulation and we recently reported that the upregulation of PVR and Nec2 in response of genotoxic stress requires DDR signaling, but not p53 activation. In addition, a putative site for the oxidative stress-induced transcription factor Nrf1 in PVR promoter region has been found (45).

Moreover, the transcription factor AP-1 was found to regulate mouse PVR expression in response to growth factor-induced stimulation of the RAS-MAPK pathway (46), but no AP-1-binding sites are present in the core promoter of human PVR.

Besides the involvement of transcriptional activity, the presence of ARE (AU-Rich Elements) regions in the 3’UTR of all human NKG2D ligands (43,47) suggests that mRNA stability could play an important role among the various levels of regulation. Our data demonstrate that VCR affects the stability of ULBP-1 but not MICA and PVR mRNA and are consistent with the evidence showing that the microtubule disruption can increase the binding to AU sequences of ELAV proteins involved in the stability of the mRNA (48).

Our work also shows that as a consequence of the VCR ability at sub-lethal doses to promote NK activating ligand expression on tumor cells, NK cells display an increased propensity to

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**Figure 5.** ATM controls ULBP-1 ligand upregulation on drug-treated MM cells. (A) SKO-007(J3) cells were left untreated (NT) or stimulated with VCR, for the indicated time at 37°C. Cell lysates were immunoblotted with anti-phospho-ATM, or anti-ATM. Data shown are representative of 1 out of 3 independent experiments. Numbers represent densitometric analysis of pATM normalized to ATM relative to the control of untreated cells. (B, C) Lysates of SKO-007(J3) cells left untreated or treated for 10 h with VCR were probed with antibodies to phospho-p53-Ser15 or total p53, and β-actin. Numbers represent densitometric analysis of pp53 and β-actin respectively, relative to the control untreated cells. (D) Phosphorylation of H2AX was evaluated by immunofluorescence and FACS analysis by staining with anti-γH2AX or anti-cIgG. (E) Cells were pre-incubated for 1 h with the ATM/ATR inhibitor caffeine, then treated with VCR for further 48 h. NKG2D and DNAM1 surface expression was analyzed by immunofluorescence and flow cytometry. Data shown are representative of 1 out of 4 independent experiments. The light gray histogram represents the isotype control antibody, dark gray histogram represents the basal expression of specific ligands, while full and dashed lines represents VCR-treated cells in absence or in presence of the caffeine inhibitor, respectively. (F) SKO-007(J3) cells were pre-incubated for 1 h with Caffeine, then treated with VCR for further 24 h. The effect of the inhibitor has been tested by real-time PCR. Data represent the mean of at least four independent experiments. *p ≤ 0.05.
degranulate upon drug-treated target cell interaction. Increased degranulation was not observed when ligand upregulation was reverted by inhibition of p38 MAPK activation, whereas no effects were obtained in the presence of ATM/ATR inhibitor caffeine. In addition, our experiments performed in the presence of anti-NKG2D or anti-DNAM-1 blocking mAbs reveal that the lysis of MM cells was mainly dependent on NKG2D, while the DNAM-1 receptor has a comparatively marginal, albeit significant, role (Fig. 7C).

The understanding of the signaling events and the molecular mechanisms that underlying the regulation of NKG2D and DNAM-1 activating ligand expression might open the scenario for the development of new therapeutic anticancer approaches aimed at boosting the tumoricidal activity of NK cells.

Materials and methods

Cell lines and clinical samples

The MM cell line SKO-007(J3), kindly provided by Prof. Trivedi (Sapienza University of Rome, Italy), was maintained in RPMI-1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% Fetal Calf Serum (FCS), 2% Glutamine and 2% Penicillin/Streptomycin at 37°C and 5% CO2. SKO-007(J3) cells were mycoplasma-free (EZ-PCR Mycoplasma Test Kit; Biological Industries, Haemek, Israel). MelC metastatic melanoma cell line was kindly provided by Dr. Licia Rivoltini, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy). HeLa cells were obtained from the American Type Culture Collection (ATCC).

Bone marrow samples from patients with untreated MM were managed at the Department of Cellular Biotechnologies and Hematology – Institute of Hematology (Sapienza University of Rome, Italy). Informed consent was obtained from all patients, and approval was achieved from the Ethics Committee of the “Sapienza” University of Rome (Rif. 3373).

The bone marrow aspirates were lysed to obtain Bone Marrow Mononuclear Cells (BMMCs) using a buffer composed of 1.5 M NH4Cl, 100 mM NaHCO3, and 10 mM EDTA. BMMCs were maintained at 37°C and 5% CO2 in complete medium supplemented with 20 ng/mL human recombinant IL-3 and 10 mM EDTA. BMMCs were maintained at 37°C and 5% CO2 in complete medium supplemented with 20 ng/mL human recombinant IL-3 and 2 ng/mL human recombinant IL-6 (PeproTech, Rocky Hill, NJ). NK cells were purified using human NK cell isolation kit (Miltenyi Biotec, San Diego, CA) from healthy donor PBMCs, isolated through lymphoprep (Stemcell technologies, Vancouver, Canada).

Antibodies and reagents

Unconjugated monoclonal antibodies (mAbs) used for immunostaining were the following: anti-MICA (MAB159227), anti-MICB (MAB236511), anti-ULBP1 (MAB170818), anti-ULBP2 (MAB165903) and anti-ULBP3 (MAB166510) from R&D Systems (Minneapolis, MN); anti-Nec-2 (R2.525) from BD PharMingen (San Diego, CA); anti-p85 (10H11.E12) was purchased from Cell Signaling (Cambridge, UK), anti-p53Ser15 and anti-p38, anti-phospho-p38, anti-p38, were purchased from Cell Signaling.

Figure 6. Upregulation of ULBP1 by Vincristine treatment depends both by p53 activity and enhanced mRNA stability. (A) MM cells were infected with a retrovirus encoding shRNA specific for p53 (shRNA p53) or with a control retrovirus (shRNA GFP). The upregulation of ULBP-1 expression upon VCR treatment was evaluated by immuno-fluorescence and flow cytometry. The light gray histogram represents the isotype control antibody, dark gray histogram represents the basal expression of specific ligands, while full line represents VCR-treated cells. (B) MM cells were infected with a retrovirus encoding shRNA specific for p53 (shRNA p53) or with a control retrovirus (shRNA GFP). ULBP-1 mRNA expression was evaluated upon VCR treatment by real-time PCR. Data represent the mean of five independent experiments. *p < 0.05. (C) Cells were either, left untreated or treated for the indicated hours with VCR, in presence or absence of 5 μg/mL actinomycin D, and ULBP1, MICA, and PVR mRNA expression was monitored by quantitative real-time PCR. Relative mRNA expression compared with the expression before the addition of ActD is shown. Expression of ligand mRNA was normalized with 18S. Data represent the mean of at least two independent experiments. *p < 0.05.
Technology (Danvers, MA); anti-p53 (DO-1) and anti-E2F1 (C20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-β-actin (AC-15) and anti-mouse IgG1 (MOPC-21) were purchased from Sigma-Aldrich (St. Louis, MO). Allophycocyanin (APC)-conjugated GAM was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Anti-γH2AX/FITC (JBW301) was purchased from Millipore. PE-conjugated anti-CD38, anti-CD138/FITC, anti-CD107a/APC, anti-CD56/PE (NCAM16.2) antibodies were purchased from Becton Dickinson. Anti-mouse IgG1/FITC, IgG1/PE, or IgG1/APC (MOPC-21) were purchased from BioLegend (San Diego, CA). Propidium Iodide (PtdIns), actinomycin-D, SB203580, and caffeine were purchased from Sigma-Aldrich.

**Drug treatment**

Tumor cells were cultured for 24–48 h in absence or presence of the microtubule targeting agent VCR (0.05 μM). In some experiments, cells were pre-treated for 1 h with the pharmacological inhibitors caffeine (1 mM) and SB203580 (20 μM). SKO-007(J3) cells and BM-derived plasma cells (B MPCs) were plated at a density of 3 × 10^5/mL and 1 × 10^6/mL, respectively.

**Immunofluorescence and flow cytometry**

NKG2D and DNAM-1 ligand surface expression was evaluated by immunofluorescence staining in two steps: a first incubation with unconjugated primary mAbs followed by secondary GAM-APC. Samples were analyzed by using a FACS Canto II (BD Biosciences, San Jose, CA) and flow cytometric analysis was performed by using the FlowJo software version 8.8.7 (TreeStar, Ashland, OR).

**RT- and real-time PCR**

MICA, MICB, ULBP1, ULBP2, ULBP3, PVR, and Nec-2 mRNA expression was analyzed by real-time PCR. Total RNA from SKO-007(J3) cells was extracted using Trizol (Invitrogen). Total RNA (1 μg) was used for cDNA first-strand synthesis using oligo-dT (Promega, Madison, WI) in a 25 μL reaction volume. To analyze ligand mRNA expression, the cDNA was amplified in triplicate with the following primers: Hs00792952_m1 for MICA, Hs00792952_m1 for MICB, Hs00 197846_m1 for PVR, Hs00607609_m1 for ULBP2, Hs002 25909_m1 for ULBP3, Hs01071562_m1 for Nec2, Hs0300 3631_g1 for 18S, and Hs99999903_m1 for β-actin, all conjugated with fluorochrome FAM (Life Technologies). The level of expression was calculated using the 2^(-ΔΔCt) method.
of ligand expression was measured using the threshold cycle value (Ct). The ΔCt was obtained by subtracting the Ct value of the gene of interest from the housekeeping gene Ct value. We used ΔCt of the NT sample as the calibrator. The fold change was calculated according to the formula $2^{-\Delta \Delta Ct}$, where $\Delta \Delta Ct$ was the difference between ΔCt of the sample and that of the calibrator (according to the formula, the value of the calibrator in each run is 1). For inhibition of transcription, SKO-007(J3) cell line was treated with 5 μg/mL actinomycin D or the respective amount of dimethyl sulfoxide (DMSO; both Sigma-Aldrich) 6 h after VCR treatment.

**Virus production and in vitro transduction**

pLKO.1-puro lentiviral vectors expressing short hairpin RNAs (shRNAs) targeting E2F1 (shE2F1_1: NM_005225.1–502s1c1 and shE2F1_2:NM_005225.1–948s1c1) were kindly provided by Dr Tschan (University of Bern, Bern, Switzerland), and the non-targeting control shRNA vector was purchased from Sigma-Aldrich; pRETRO Super GFP-p53shRNA was kindly provided by Dr Soddu (Regina Elena National Cancer Institute, Rome, Italy). Virus production and infection of SKO-007(J3) cells were performed as previously described (49).

For lentivirus production of the different ligand gene promoters, 293T packaging cell line at 70% confluence were transfected with commercially available pEZX-LvPG04 vectors for ligand promoters or a control vector (5 μg) (GeneCopoeiaTM, Rockville, MD) together with the packaging vectors pVSVG and psPAX2 with Lipofectamine Plus. After an additional 48 h, virus-containing supernatants were harvested, filtered, and used immediately for infections. Infections were performed on 0.5 × 10^6 SKO-007(J3) cells in 2 mL complete medium with Polybrene (8 μg/mL) resistance.

**SDS-PAGE and western blot**

Drug-treated SKO-007(J3) cells were lysed for 20 min at 4°C in ice-cold lysis buffer containing 0.2% Triton X-100, 0.3% NP40, 50 mM Tris HCl pH 7.6, 1 mM EDTA, 150 mM NaCl, 10 μg/mL leupeptin, 1mM PMSF, 10 μg/mL aprotinin, 10 mM NaF and 1 mM Na2VO4 to detect phospho-ATM and p85 or in ice-cold lysis buffer containing 1% Triton X-100, 0.5% DOC, 0.1% SDS, 50 mM Tris HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 50 mM Na3PO4, 150 mM NaCl, 5 mM MgCl2, 10 μg/mL leupeptin, 1mM PMSF, 10 μg/mL aprotinin, 100 mM NaF, and 1 mM Na2VO4 to detect phospho-p38, p38, phospho-p53, p53, and β-actin. To detect ATM phosphorylation or E2F1 expression, cells were maintained in RPMI-1640 with 2% FCS for 24 h at 37°C before drug treatment. The Bio-Rad Protein Assay (Bio-Rad Laboratories; Hercules, CA) was used to measure protein concentration. Fifty μg of total lysates were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore) or nitrocellulose membranes (Whatman GmbH; Dassel, Germany). After blocking with BSA, membranes were probed with specific antibodies. A Horseradish Peroxidase (HRP)-conjugated secondary antibody and an enhanced chemiluminescence kit (Amersham, GE Healthcare; Buckinghamshire, UK) were used to reveal immunoreactivity.

**Immunofluorescence and confocal microscopy**

Cells were plated on poly-L-lysine-coated multichamber glass plates, fixed and permeabilized as previously described (24), and stained with anti-ULBP1 mAb (R&D Systems) followed by an AlexaFluor 488-conjugated goat anti-mouse antibody (Invitrogen), both diluted in blocking buffer (0.01% Triton X-100, 1% FCS). After extensive washing, cells were counterstained with Hoechst and coverslips were mounted with SlowFade Gold reagent (Life Technologies). High-resolution images (800 × 800 pixel, 8 μs/pixel) were acquired with a FV1200 laser-scanning confocal microscope using a 60 ×/1.35 NA UPlanSAPO oil immersion objective (all from Olympus). Sequential acquisition was used to avoid cross-talk between different fluorophores. Images were acquired with FluoView 4.2 software, and processed with ImageJ software.

**Degranulation assay**

NK cell-mediated cytotoxicity was evaluated using the degranulation lysosomal marker CD107a as previously described (14). As a source of effector cells, we used human peripheral blood mononuclear cells (PBMCs) isolated from healthy donors by Lymphoprep (Nycomed, Oslo, Norway) gradient centrifugation and then co-cultured for 10 d with an irradiated (30 GY) Epstein-Barr virus (EBV)-transformed B-cell line. Cells were grown in RPMI 8866 at 37°C in a humidified 5% CO2 atmosphere. On day 10, the cell population was routinely more than 90% CD56+CD16+CD3−, as assessed by IF and flow cytometric analysis. After a 48 h treatment with VCR, SKO-007(J3) cells were incubated with activated NK cells at effector:target (E:T) ratios of 2.5:1 in complete medium. The plates were incubated at 37°C in a 5% CO2 atmosphere for 2 h. Thereafter, the cells were washed with PBS and incubated with anti-CD107a/APC (or clgG/APC) for 45 min at 4°C.

**Statistics**

Error bars represent Standard Deviation (SD) or Standard Error of the Mean (SEM). Data were evaluated by paired Student’s t test. For the experiments with patient, PCs Wilcoxon and Friedman test were used.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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