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Heat Shock Protein-90 Inhibitors Increase MHC Class I-Related Chain A and B Ligand Expression on Multiple Myeloma Cells and Their Ability to Trigger NK Cell Degranulation[1]

Cinzia Fionda,*† Alessandra Soriani,* Giulia Malgarini,*† Maria Luisa Iannitto,* Angela Santoni,[2,3]*† and Marco Cippitelli[2,3]*†

Modulation of the host immune system represents a promising therapeutic approach against cancer, including multiple myeloma. Recent findings indicate that the NK group 2D (NKG2D)- and DNAX accessory molecule-1 (DNAM-1)-activating receptors play a prominent role in tumor recognition and elimination by cytotoxic lymphocytes, suggesting that the levels of NKG2D and DNAM-1 ligand expression on tumor cells may be a critical factor to improve the immune response against cancer. In this study, we tested the effect of 17-allylaminogeldanamycin and radicicol, drugs targeting the heat shock protein-90 (HSP-90) chaperone protein and displaying antimyeloma activity, on the expression of NKG2D and DNAM-1 ligands in human myeloma cell lines. We demonstrate that HSP-90 inhibitors are able to up-regulate both MHC class I chain-related (MIC) A and MICB protein surface and mRNA expression in human myeloma cell lines, without any significant effect on the basal expression of the DNAM-1 ligand poliovirus receptor CD155, or induction of nectin-2 and UL16-binding proteins. Activation of the transcription factor heat shock factor-1 by HSP-90 inhibitors is essential for the up-regulation of MICA/MICB expression and knockdown of heat shock factor-1 using small hairpin RNA interference blocks this effect. Moreover, in vitro and in vivo binding of heat shock factor-1 to MICA and MICB promoters indicates that it may enhance NKG2D ligand expression at the transcriptional level. Finally, exposure to HSP-90 inhibitors renders myeloma cells more efficient to activate NK cell degranulation and a blocking Ab specific for NKG2D significantly reduces this effect. Thus, these results provide evidence that targeting NKG2D ligands expression may be an additional mechanism supporting the antimyeloma activity of HSP-90 inhibitors and suggest their possible immunotherapeutic value. ***The Journal of Immunology,*** **2009, 183: 4385–4394.**

Multiple myeloma (MM)[4] is a malignant plasma cell disorder characterized by neoplastic accumulation of clonal secretory plasma cells in the bone marrow, which accounts for ~10% of all hematological cancers and 2% of all cancer deaths (1). MM is often an incurable disease with a median survival of a few years, recently improved by the introduction of new antimyeloma chemotherapics such as bortezomib and thalidomide/lenalidomide and by the wider use of autotransplant procedures (1). In this regard, stem cell transplantation from allogeneic donors may be curative for 10–20% of patients with chemotherapy-resistant hematological malignancies, including MM, where much of curative potential of allografts is attributed to a graft-vs-tumor-effect mediated by donor T lymphocytes (2–5). However, emerging evidence also suggests that NK cells may have antimyeloma activity (2, 6–8) and, in the last years, in vitro studies demonstrated that allogeneic and autologous NK cells have the ability to kill CD138-purified primary MM cells (9–11). In contrast, $CD34^+$ hemopoietic stem cells, as well as PBMCs, are resistant to NK cell killing under similar conditions (12).

Several studies have shown that the engagement of the NK group 2D (NKG2D)- and DNAX accessory molecule-1 (DNAM-1)-activating receptors plays an important role in the recognition and killing of MM cells by NK cells (12–16). Moreover, the activity of a novel class of immunomodulatory drugs, i.e., thalidomide and lenalidomide, that are highly active agents in patients with MM also depends on their ability to stimulate NK cell cytotoxic functions (17, 18). In this regard, our laboratory has recently shown that low doses of different chemotherapeutic drugs (e.g., melphalan, Adriamycin, and bortezomib) can trigger the expression of NKG2D and DNAM-1 ligands on MM cells in an ataxia telangiectasia mutated/ataxia-telangiectasia and Rad3-related protein-dependent manner (10). The up-regulation of these ligands is associated with increased NK cell degranulation against the tumor cells, suggesting that pharmacological drugs can promote the recognition and NK cell cytotoxicity through up-regulation of the ligands of innate immune receptors.

Recently, the 90-kDa heat shock proteins (HSP-90) have emerged as attractive targets in cancer therapy (19). They are required for the stabilization of a large set of proteins termed HSP-90

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[4] Abbreviations used in this paper: MM, multiple myeloma; 17-AAG, 17-allylamino-17-demethoxygeldanamycin; DNAM-1, DNAX accessory molecule-1; HSF-1, heat shock factor-1; HSR, heat shock response; TM, tunicamycin; TG, thapsigargin; NEC-2, nectin-2; PVR, poliovirus receptor CD155; UPR, unfolded protein response; XBP-1, X-box-binding protein-1; MIC, MHC class I chain-related; HSE, HS response element; ChIP, chromatin immunoprecipitation assay; ER, endoplasmic reticulum; CHOP, C/EBP homologous protein; shRNA, small hairpin RNA; NKG2D, NK group 2D.

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clients, involved in intracellular signaling cascades that promote proliferation and/or survival. HSP-90s are often overexpressed in many cancers (including MM) (20), and it is presumed that they are required to sustain functional expression of oncoproteins and/or aberrant signaling, enabling the transformed cell to tolerate the imbalanced pathways this might create (19, 21). In this regard, a prominent characteristic of HSP-90 inhibition in MM cells is the down-regulation of cytokine-triggered signaling pathways, such as those leading to activation of STAT3, protein kinase B (PKB/Akt), MAPK, and NF-κB. Inhibition of HSP-90 with ansamycin-based compounds (e.g., 17-allylamino-17-demethoxygeldanamycin; 17-AAG) has been shown to induce myeloma cell death (20, 22, 23), and 17-AAG and IPI-504 (a novel highly soluble analog of the natural product geldanamycin) have been evaluated in clinical phase I/II trials with MM patients, with 17-AAG currently being evaluated in clinical phase II/III and phase III trials, together with different combinations of the proteasome inhibitor bortezomib (www.clinicaltrials.gov).

In this study, we analyzed the possibility that treatment of MM cell lines with different inhibitors of HSP-90 might regulate the expression of NK-activating ligands and, in turn, modify NK recognition and degranulation.

Our results demonstrate that HSP-90 inhibitors can enhance NKG2D ligand expression, suggesting a functional impact of higher MICA/MICB expression on promotion of NK recognition of target myeloma cells and propose a new role for these agents in improving immune response.

Materials and Methods

Cell lines and reagents

The human MM cell lines SKO-007(J3) and U266 were kindly provided by Prof. P. Trivedi (Sapienza University of Rome, Rome, Italy). The cell lines were maintained at 37°C and 5% CO_2 in RPMI 1640 (Life Technologies) supplemented with 15% FCS. All cell lines were *Mycoplasma* free (EZ-PCR *Mycoplasma* test kit; Biological Industries).

Radicicol, 17-AAG, tunicamycin (TM), thapsigargin (TG), and puromycin were purchased from Sigma-Aldrich.

The following unconjugated mAbs were used for immunostaining: anti-MICA (mAb 159227), anti-MICB (mAb 236511), anti-ULBP1 (mAb 170818), anti-ULBP2 (mAb 165903), and anti-ULBP3 (mAb 166510) from R&D Systems; anti-Nec-2 (R2.525) from Serotec; anti-poliovirus receptor (PVR; 46.31) kindly provided by Prof. M. Colonna (Washington University, St. Louis, MO); MHC class I (W6/32) from American Type Culture Collection. FITC-conjugated goat $F(ab')_2$ fragments directed against mouse IgG were purchased from Jackson ImmunoResearch Laboratories. Anti-CD107a/FITC (H4A3), CD56/FITC (NCAM16), and anti-CD16/PE (332779) were purchased from BD Biosciences. Anti-CD3/APC (HIT3a), anti-CD56/PE (HCD56), and anti-mouse IgG1-FITC, -PE, or -allophycocyanin (MOPC-21) were purchased from BioLegend.

Immunofluorescence and flow cytometry

SKO-007(J3) and U266 cells were cultured in six-well tissue culture plates respectively for 24 or 48 h at a concentration of 3×10^5 cells/ml with different concentrations of drugs (17-AAG, radicicol, etc.). The expression of the NKG2D and DNAM-1 ligands on MM cells was analyzed by immunofluorescence staining using anti-MICA; anti-MICB; anti-ULBP-1, -2, and -3; anti-PVR; and anti-nectin-2 (NEC-2) unconjugated mAbs, followed by secondary goat anti-mouse FITC Ab. In all experiments, cells were stained with propidium iodide (1 μg/μl) to assess cell viability. Aspecific fluorescence was assessed by using an isotype-matched irrelevant mAb (R&D Systems) followed by the same secondary Ab. Fluorescence was analyzed using a FACSCalibur flow cytometer (BD Biosciences), and data were analyzed using FlowJo (version 7.2.5) flow cytometric data analysis software (Tree Star).

RNA isolation, RT-PCR, and real-time PCR

Total RNA from MM cell lines was extracted using TRIZOL (Life Technologies), after 6 and 18 h of treatment with HSP-90 inhibitors according to the manufacturer's instructions. The concentration and quality of the extracted total RNA were determined by measuring OD_{260} and the OD_{260}:OD_{280} ratio. Reverse transcription was conducted in a 25-μl reaction volume with 2 μg of total RNA according to the manufacturer's protocol for Moloney MLV reverse transcriptase (Promega).

Real-time PCR was performed using the ABI Prism 7900 sequence detection system (Applied Biosystems). cDNAs were amplified in triplicate with primers for MICA (Hs00792952_m1) and MICB (Hs00792952_m1), all conjugated with fluorochrome FAM, and β-actin (4326315E) conjugated with fluorochrome VIC (Applied Biosystems). The average of the threshold cycles was used to interpolate standard curves and to calculate the transcript amount in samples using SDS version 1.7a software (Applied Biosystems). Relative mRNA amount, normalized with β-actin, was expressed as arbitrary units and referred to untreated cells considered as calibrator.

To analyze splicing of X-box-binding protein-1 (XBP-1), cDNA samples were used in PCR reactions using the following primer sets: human XBP-1 sense, 5′-ccttgtagttgagaaccagg-3′ and human XBP-1 antisense, 5′-ggggcttggtatatatgtgg-3′; human β-actin sense 5′-gtggggcgccccaggcacca-3′ and β-actin antisense, 5′-ctccttaatgtcacgcacgatttc-3′. Semiquantitative PCR conditions were optimized to obtain reproducible and reliable amplification within the logarithmic phase of the reaction.

Degranulation assay

NK cell-mediated cytotoxicity was evaluated using the lysosomal marker CD107a as previously described (24). As a source of effector cells, we used PBMCs isolated from healthy donors by Lymphoprep (Nycomed) gradient centrifugation and then cocultured for 10 days with irradiated (30 Gy) EBV-transformed B cell line RPMI 8866 at 37°C in a humidified 5% CO_2 atmosphere, as previously described (25). On day 10, the cell population was routinely >90% $CD56^+CD16^+CD3^-$, as assessed by immunofluorescence and flow cytometry analysis. NK cells were activated overnight with 200 U/ml human rIL-2 (R&D Systems).

Drug-treated MM cell lines were incubated with activated NK cells at an E:T ratio of 2.5:1, in a U-bottom 96-well tissue culture plate in complete medium at 37°C and 5% CO_2 for 2 h. Thereafter, cells were washed with PBS and incubated with anti-CD107a/FITC (or cIgG FITC) for 45 min at 4°C. Cells were then stained with anti-CD3 allophycocyanin or anti-CD56 PE to gate the $CD3^-CD56^+$ NK cell population. In some experiments, cells were pretreated for 20 min at room temperature with anti-NKG2D- or anti-DNAM-1-neutralizing mAbs. The anti-NKG2D (IgG1, clone 149810) was purchased from R&D Systems, and the anti-DNAM-1 (IgG1, clone DX11) was from Serotec. Fluorescence was analyzed using a FACSCalibur flow cytometer (BD Biosciences), and data were analyzed using FlowJo (version 7.2.5) flow cytometric data analysis software (Tree Star).

Plasmids

For knocking down heat shock factor-1 (HSF-1), we used RNAi-Ready pSIREN-RetroQ vector with puromycin resistance (BD Biosciences). Target sequence for HSF-1 small interfering RNA was 5′-TATGGACTCCAACCTGGATAA-3′. The vectors were kindly provided by Dr. M. Y. Sherman (University Medical School, Boston, MA).

Virus production and in vitro transduction

Phoenix retrovirus packaging cell lines were cultured in DMEM plus 10% FCS. Phoenix cells were transfected with viral DNA (5 μg of pSIREN-RetroQ or pSIREN-RetroQ/HSF-1 small hairpin RNA; shRNA) at 50% confluence with Lipofectamine Plus (Invitrogen). After transfection, the cells were placed in fresh medium. After a further 24-h culture, virus-containing supernatants were harvested, filtered, and either stored at −80°C or used immediately for infections. Infections were performed on 0.5×10^6 U266 and SKO-007(J3) cells in 3 ml of complete medium with polybrene (8 μg/ml; hexadimethrine bromide; Sigma-Aldrich) for 2 h. After infection, cells were allowed to expand for 24 h and were then selected for puromycin resistance. The amount of puromycin used during selection was 1 μg/ml.

EMSA

Nuclear proteins were prepared as described in Ref. 26. Protein concentration of extracts was determined by the BCA method (Pierce). The nuclear proteins (10 μg) were incubated with radiolabeled DNA probes in a 20-μl reaction mixture containing 20 mM Tris (pH 7.5), 60 mM KCl, 2 mM EDTA, 0.5 mM DTT, 1–2 μg of polydeoxyinosinic-polydeoxycytidylic acid, and 4% Ficoll. Where indicated, a molar excess of double-strand oligomer was added as a cold competitor, and the mixture was incubated at room temperature for 10 min before adding the DNA probe. Nucleoprotein complexes were resolved as described in

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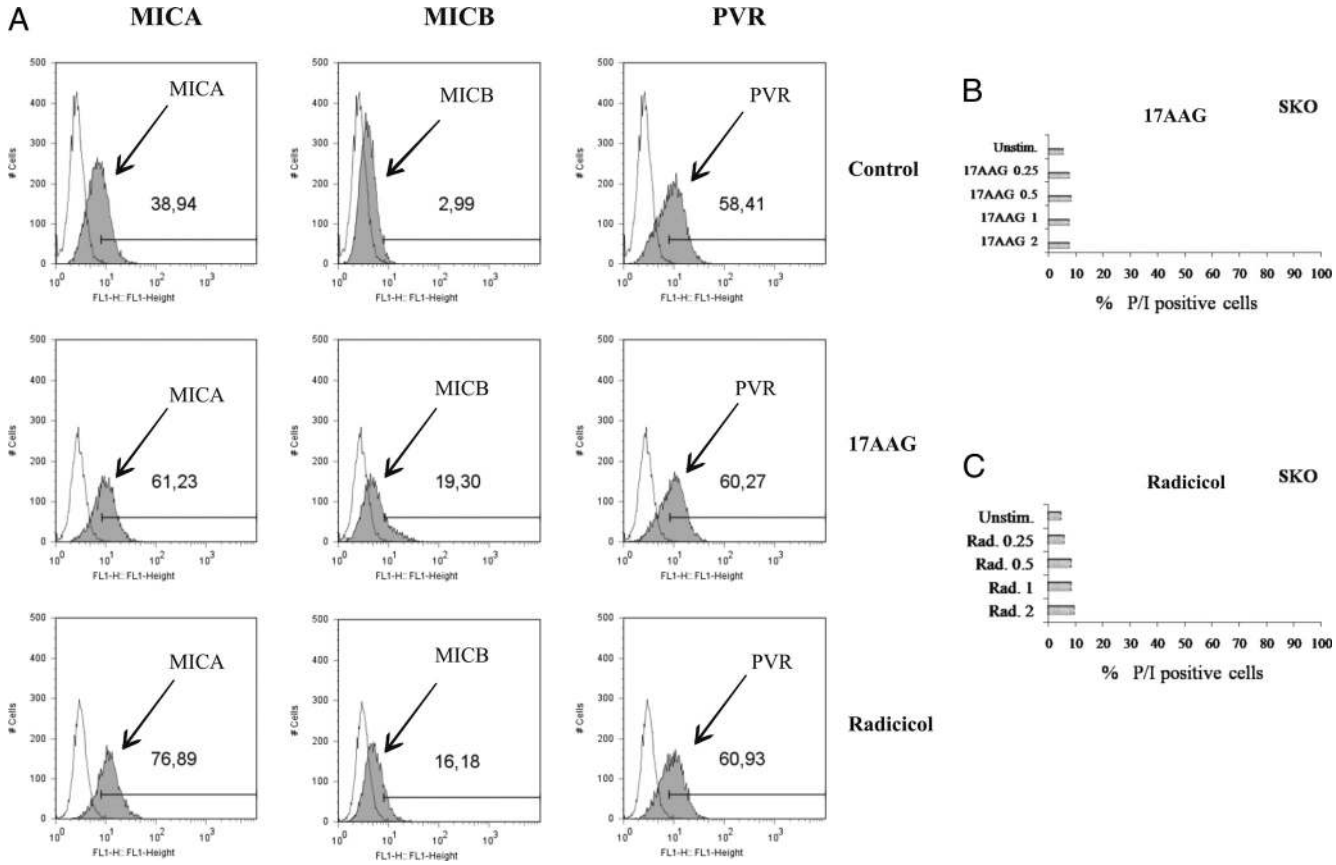

FIGURE 1. Modulation of MICA, MICB, and PVR expression on the SKO-007(J3) cell line following treatment with HSP-90 inhibitors. *A,* MICA, MICB, and PVR surface expression was analyzed by flow cytometry on SKO-007(J3) cells treated with 17-AAG (1 μM) or radicicol (Rad.; 2 μM) for 48 h. Data are representative of one of three independent experiments. The percent of positive cells with respect to the isotype control Ab is shown. Gray histograms represent the specific ligand. *B* and *C,* Cell viability after drug treatment. The percent of propidium iodide (P/I)-positive cells in the presence of different concentrations (micromolar) of 17-AAG or radicicol is shown. FL, Fluorescence; -H, -height; Unstim., unstimulated.

(26). Oligonucleotides were purchased by Eurofins MWG Operon. Complementary strands were annealed and end-labeled as described in Ref. 26. Approximately 3×10^4 cpm of labeled DNA were used in a standard EMSA reaction. In supershift analysis, the specific Ab was added to the binding reaction, and the mixture was incubated for 30 min at room temperature before adding the labeled DNA probe. The Ab against HSF-1 and Oct-1 were purchased from Santa Cruz Biotechnology.

The following double-stranded oligomers were used as specific labeled probes or cold competitors (sense strand): HSE-MICA, 5′-ccagcccactg gaattttctcttccaagcg-3′; HSE-MICB, 5′-ccaggccgctagaattttctcttctgaacg-3′; octamer-(h-histone H2b), 5′-agctcttcaccttatttgcataagcgat-3′.

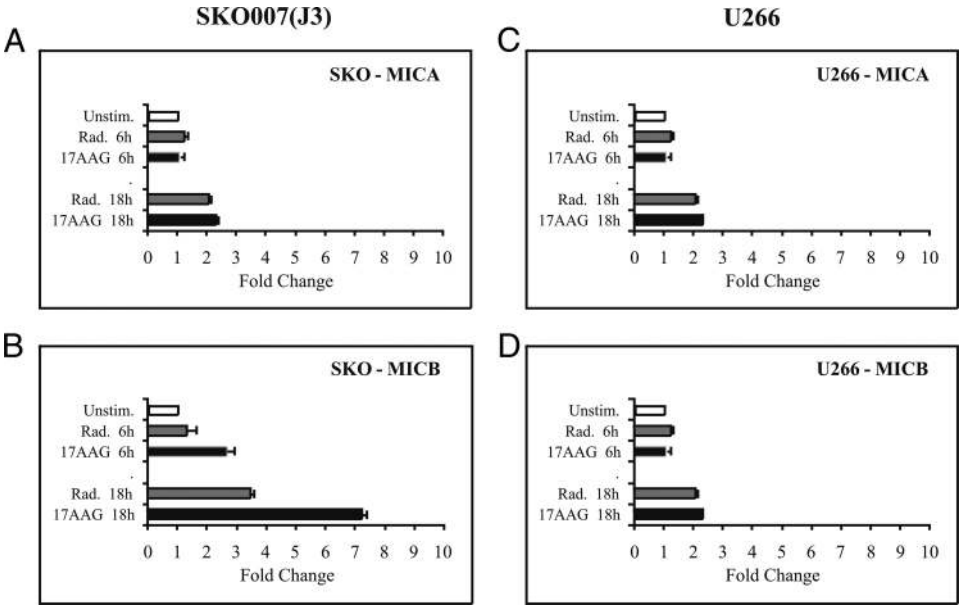

FIGURE 2. HSP-90 inhibitors increase MICA and MICB mRNA levels in MM cell lines. *A–D,* Real-time PCR analysis of total mRNA obtained from SKO-007(J3) or U266 cells, untreated (−) or treated with 1 μM 17-AAG or 2 μM radicicol (Rad.) for the indicated time. Real-time PCRs are representative of various independent experiments all displaying similar results. Data, expressed as arbitrary units, were normalized with β-actin and referred to untreated cells considered as calibrator. Unstim., Unstimulated.

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Western blot analysis

For Western blot analysis, SKO-007(J3) and U266 cells were pelleted, washed once with cold PBS, resuspended in lysis buffer (1% Nonidet P-40 (v/v), 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM PMSF, COMPLETE protease inhibitor mixture (Roche) in PBS), and subsequently incubated for 30 min on ice. The lysate was centrifuged at 14,000 × *g* for 15 min at 4°C, and the supernatant was collected as whole-cell extract. Nuclear proteins were prepared as described before. Protein concentration of nuclear and whole cell extracts was determined by the BCA method (Pierce). From 30 to 50 μg of nuclear extract or whole-cell extract were run on 12% denaturing SDS-polyacrylamide gels. Proteins were then electroblotted onto nitrocellulose membranes (Schleicher & Schuell) and blocked in 3% milk in TBST buffer. Immunoreactive bands were visualized on the nitrocellulose membranes, using HRP-coupled goat anti-rabbit or goat anti-mouse Igs and the ECL detection system (Amersham), following the manufacturer's instructions. Abs against HSF-1, Oct-1, C/EBP homologous protein (CHOP), and HSP-70 were purchased from Santa Cruz Biotechnology.

Chromatin immunoprecipitation assay (ChIP)

SKO-007(J3) and U266 cells untreated or treated for 1 h with HSP-90 inhibitors (1 μM 17-AAG and 2 μM radicicol) were processed for ChIP assays, following the kit protocol from Upstate Biotechnology. Chromatin samples were immunoprecipitated overnight with a polyclonal rabbit anti-HSF-1 Ab (2 μg/ml; Santa Cruz Biotechnology). Immunocomplexes were collected with protein A-salmon sperm DNA-agarose beads and sequentially washed, as detailed in the Upstate Biotechnology protocol. Chromatin was eluted with 1% SDS, 0.1 M $NaHCO_3$, digested with proteinase K, and cross-linking was reversed overnight in the presence of 200 mM NaCl at 65°C. DNA was recovered by phenol-chloroform extraction and ethanol precipitation and dissolved in 25 μl of diethylpyrocarbonate-water. Fractions of precleared chromatin were processed similar to immunoprecipitated chromatin for input DNA controls. The forward and reverse primers for PCR amplification (all 5′-3′) were: GGGCCCTGGCCGTGCTTAT GAAGTTGG (-317/-291) and CGCCACCCTCTCAGCGGCTCAAGC (-12/-35) for MICA; GCGACAGGGTCCAGGTCGTGCTC (-337/-315) and CCCTACGTCGCCACCTTCTCAGCT (-4/-27) for MICB; CT GAGAGGCAGAGGTGGCTGAGA (-541/-518) and GCGGGAGCG CAGCCTAAAGCAGGG (-239/-267) for MICA (ΔHSE).

Results

HSP-90 inhibitors up-regulate MICA and MICB expression on human MM cell lines

Our laboratory has recently shown that the expression of NKG2D and DNAM-1 ligands on human MM cells can be up-regulated by chemotherapy agents through the activation of the DNA damage response (10).

In this report, we wanted to investigate whether different therapeutic agents with antimyeloma activity, the HSP-90 inhibitors, could modulate the expression of these ligands on MM cell lines.

To investigate the effect of these drugs on the surface expression of NKG2D and DNAM-1 ligands on MM cells, we initially performed a flow cytometric analysis on SKO-007(J3) and U266 MM cells after treatment with two classical inhibitors of HSP-90, 17-AAG, and radicicol. As shown in Fig. 1*A,* treatment of SKO-007(J3) and U266 cells (shown in supplemental Fig. 1*A*)[5] with these inhibitors was able to up-regulate the basal expression of MICA and MICB, without any significant effect on PVR levels. As shown in Fig. 1, *B* and *C* (and supplemental Fig. 1, *B* and *C*), these treatments did not affect cell viability over the time chosen for these experiments, as assessed by propidium iodide staining.

FACS analysis revealed also that SKO-007(J3) and U266 cells did not express significant levels of ULBP1, ULBP2, ULBP3, and NEC-2, and these ligands were not induced by the two HSP-90 inhibitors (supplemental Figs. 2 and 3, for SKO and U266, respectively).

[5] The online version of this article contains supplemental material.

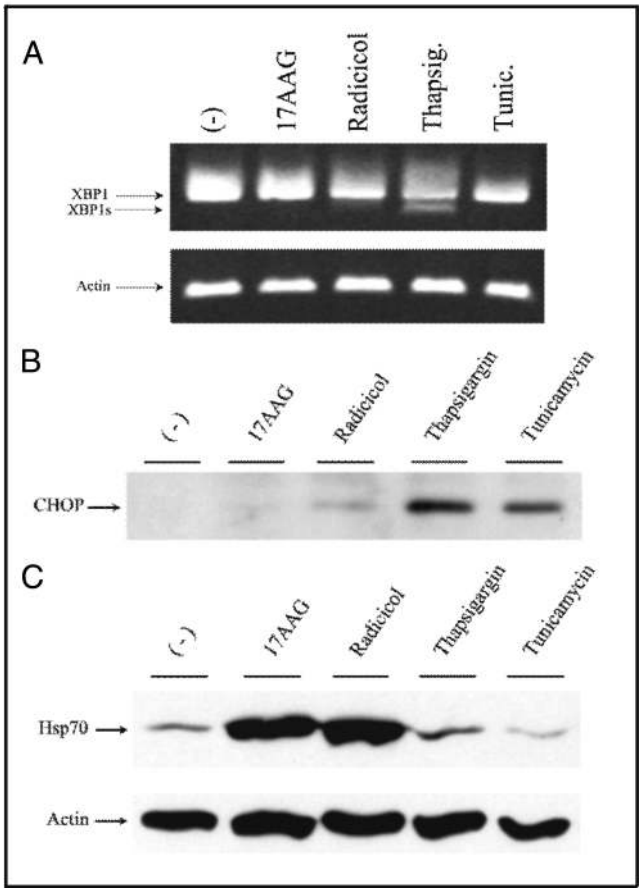

FIGURE 3. Activation of the ER/UPR and HSPR on the SKO-007(J3) cell line following treatment with HSP-90 inhibitors. SKO-007(J3) cells were treated with the ER/UPR activators TG (Thapsig.; 3 μM) and TM (Tunic.; 2 μM). In the same experimental setting, cells were also treated with 17-AAG (3 μM) and radicicol (5 μM). *A,* Induction of *XBP-1* slicing after treatment for 2 h, as determined by RT-PCR. *B* and *C,* Induction of CHOP and HSP-70 after treatment for 18 h as indicated above. Western blotting of nuclear extracts (for CHOP) and cytosolic fractions (for HSP-70) are shown. The proteins transferred to nitrocellulose membranes were stained with Ponceau to verify that similar amounts of protein had been loaded in each lane. Data are representative of one of two independent experiments.

Thus, in SKO-007(J3) and U266 MM cells, 17-AAG and radicicol enhance the surface expression of the two NKG2D ligands, MICA and MICB.

We then examined whether this effect could be the consequence of an up-regulation of their mRNA expression. To this aim, total RNA was isolated from SKO-007(J3) and U266 cells exposed to HSP-90 inhibitors for 6 and 18 h and analyzed by real-time qRT-PCR. We found that 17-AAG and radicicol increased MICA and MICB mRNA levels in a time-dependent manner (Fig. 2).

These results showed that HSP-90 inhibitors are able to enhance MICA and MICB expression on MM cell lines both at level of mRNAs and proteins.

Activation of the UPR is not sufficient to up-regulate MICA-MICB expression in MM cell lines

An important key feature that characterizes myeloma plasma cells is the large quantity of monoclonal paraprotein that they synthesize and secrete. The high level of Ig secretion requires a secretory

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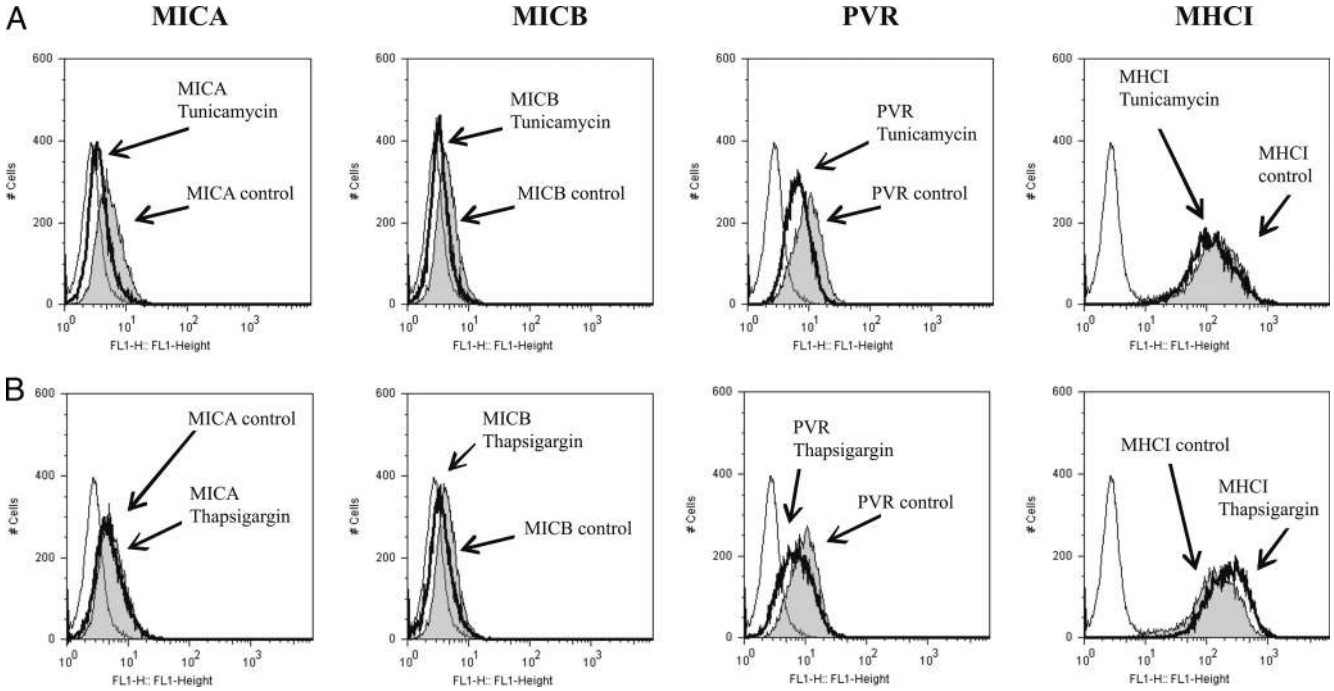

FIGURE 4. Activation of the ER/UPR is not sufficient to up-regulate MICA/MICB expression in MM cell lines. MICA, MICB, and PVR surface expression was analyzed by flow cytometry on SKO-007(J3) cells treated with ER/UPR activators TG (3 μM) and TM (2 μM) for 48 h. In the same experimental setting, cells were also treated with 17-AAG (3 μM) and radicicol (5 μM). As control, the MHC class I (MHCI) surface expression has been analyzed in the same samples. Gray histograms represent the specific ligand; thick histograms represent the expression of specific ligand after treatment. Data are representative of one of three independent experiments. FL, Fluorescence; -H, -height.

machinery and the production of chaperone proteins to allow proper protein translation and folding in the endoplasmic reticulum (ER). One of the cellular responses induced by HSP-90 inhibitors is the accumulation of misfolded proteins and the induction of the ER stress, leading to the activation of complex signaling and transcriptional pathways, called the unfolded protein response (UPR), that maintain the equilibrium between the rate of protein production and the capacity for nascent protein folding (27, 28). In this regard, recent experimental observations have provided contradictory data on the actions of the HSP-90 inhibitors. In fact, activatory and inhibitory actions on the basal level of UPR in MM cells have been described in response to HSP-90 inhibitors. Inhibition of HSP-90 is expected to induce more ER stress and lead to a higher UPR response (28). Alternatively, several of the ER stress sensors have been shown to be HSP-90 clients, and therefore an HSP-90 inhibitor could block the induction of the UPR (29).

To investigate the possible role of the UPR on the expression of NKG2D and DNAM-1 Ligands on MM cells, we initially analyzed the induction of two main arms of it, activation of XBP-1s (alternative splicing of *XBP-1* mRNA to form *XBP-1s*) and induction of the transcription factor CHOP, by two classical ER stress inducers, TG and TM. In parallel, we also analyzed the induction of the HSP-70, as a classical readout for the heat shock response (HSR) activation. As shown in Fig. 3, *A* and *B,* treatment of SKO-007(J3) and U266 cells (supplemental Fig. 4, *A* and *B*) with TG was able to induce *XBP-1s* splicing and to up-regulate CHOP, whereas TM only induced CHOP. Under the same experimental conditions, 17-AAG and radicicol (even at higher doses) were not able to induce *XBP-1s* mRNA splicing and only weakly (in the case of radicicol) CHOP. On the contrary, only 17-AAG and radicicol strongly induced HSP-70 in the same context (Fig. 3*C* and supplemental Fig. 4*C*).

We then evaluated the effect of these UPR activators on MICA, MICB, and PVR expression, and as shown in Fig. 4 and supplemental Fig. 5, *A* and *B*), treatment with these agents failed to enhance MICA and MICB expression in SKO-007(J3) and U266 MM cells. These observations are in agreement with previous data from another laboratory indicating that TM or TG are not able to increase NKG2D ligands in Jurkat T cell leukemia cells (30). Thus, in our MM cell lines, UPR activation is weakly induced by 17-AAG and radicicol at the doses used in our experiments; moreover, as in the case of TG, UPR activation per se is not sufficient to enhance NKG2D ligands or PVR expression, suggesting that it is not involved in the regulation of their expression by HSP-90 inhibitors.

Activation of HSF-1 by HSP-90 inhibitors plays a central role in MICA-MICB induction

HSP-90 has emerged as a key factor in the regulation of HSF-1, a transcription factor involved in the induction of the HSR, a cellular response characterized by increased synthesis of HSPs to prevent denaturation of cellular proteins under stress. In particular, HSP-90 together with the action of different components of the HSP-90 chaperone machine, regulates key steps in the HSF-1 activation-deactivation process and normally represses its activity under nonstress conditions. In this regard, pharmacological inhibition of the HSP-90 can disrupt the HSP-90-inhibitory complex and thus strongly activate the transcriptionally active form of HSF-1 (31, 32). HSF-1 may act as a positive or negative regulator of many different genes, including cytokines such as IL-1, TNF, and NKG2D ligands (e.g., MICA and MICB; Refs. 33–36). In this regard, a conserved canonical HSE consensus (HS response element; HSE) located -280/-260 bp 5′ from the transcription start site, has been identified in *MICA* and *MICB* promoters, and HSF-1 has been shown to mediate their activation by stress conditions (e.g., heat shock; Ref. 36).

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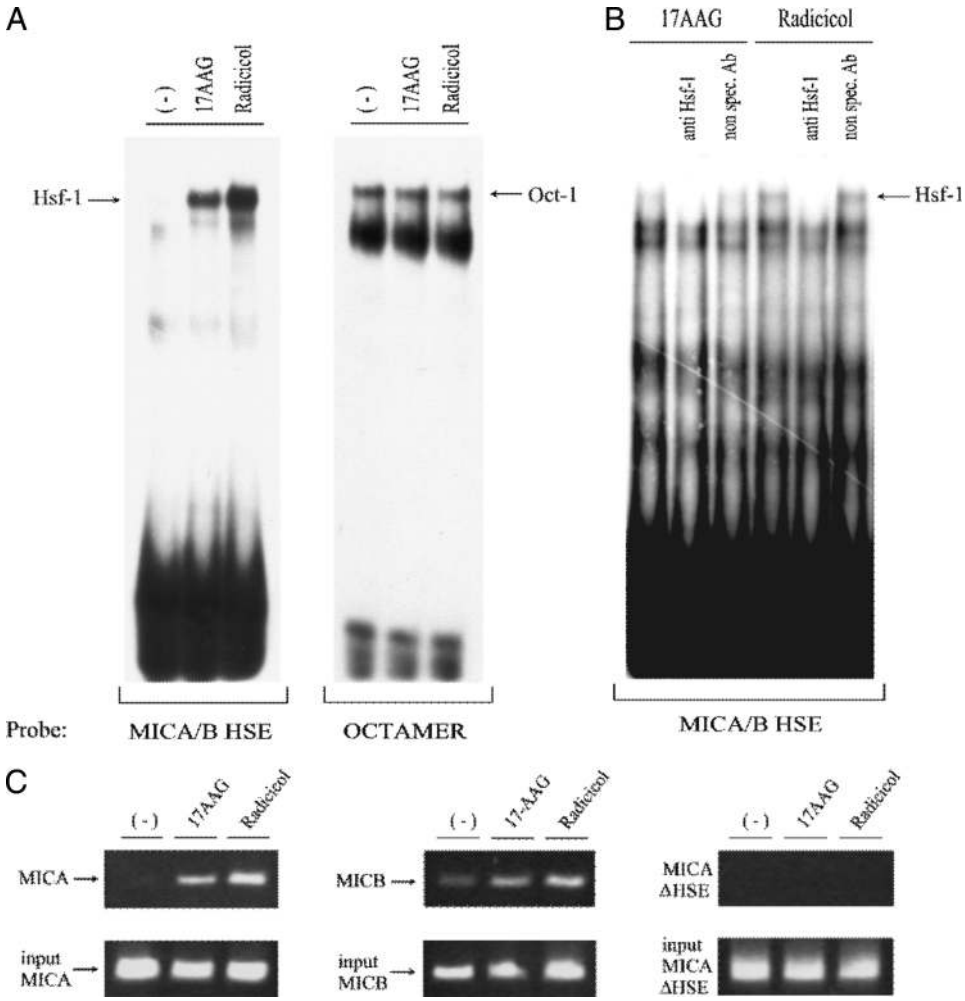

FIGURE 5. Recruitment of HSF-1 on MICA-MICB promoters by HSP-90 inhibitors. *A,* EMSA was performed using a ^{32}P-labeled MICA/B HSE oligonucleotide or a canonical octamer-binding element as a probe, in the presence of nuclear extracts (10 μg) from SKO-007(J3) cells untreated (−) or treated for 1 h with 1 μM 17-AAG or 2 μM radicicol. Arrows, DNA binding of HSF-1- or Oct-1-specific (spec.) complexes. *B,* Supershift analysis of HSF-1 complexes bound to the MICA HSE oligonucleotide; where indicated, purified anti-HSF-1 was added to the reaction mixture. The same amount of a nonspecific Ab (anti Octamer-1) did not supershift or inhibit HSF-1-bound complexes. *C,* In vivo binding of HSF-1 to MICA and MICB promoters was examined using ChIP analysis. Samples were immunoprecipitated from the sonicated lysates of SKO-007(J3) cells unstimulated (−) or treated for 1 h with 1 μM 17-AAG or 2 μM radicicol, using an Ab against human HSF-1 protein. After reversing the cross-linking, DNA was precipitated and PCR was performed using primers to amplify the MICA and MICB promoter regions encompassing the conserved HSE element. The specificity of this interaction was demonstrated by the absence of PCR amplification, using primers to amplify an upstream region of MICA promoter lacking of the HSE element (MICA; ΔHSE). As a positive control of equal loading and quality of chromatin samples, 1% input DNA was used as template with the same primers.

As described above, we observed higher levels of HSP-70 in SKO-007(J3) and U266 cells treated with HSP-90 inhibitors, suggesting the activation of HSF-1 in our system.

To determine whether one of the mechanisms involved in 17-AAG/radicicol-mediated up-regulation of MICA and MICB could be a direct transcriptional action of HSF-1, we analyzed its activation and capability to bind to *MICA/MICB* HSE sequences in SKO-007(J3) and U266 cells treated with these drugs. EMSA assays, performed with synthetic double-stranded oligonucleotides spanning this promoter element and nuclear extracts from drug-treated cells, showed a shift in the electrophoretic mobility due to a specific binding of HSF-1. The specificity of this interaction was confirmed by the addition of a molar excess of unlabeled MICA-MICB HSE probe (data not shown) and by a specific anti-HSF-1 Ab, that inhibited the induction of DNA binding complexes (Fig. 5, *A* and *B,* and supplemental Fig. 6, *A* and *B*).

In vivo binding of HSF-1 to the promoters of MICA and MICB was investigated by ChIP assay on chromatin prepared from HSP-90 inhibitor-treated or -untreated MM cells. The cross-linked chromatin was immunoprecipitated with an anti-HSF-1 Ab. After immunoprecipitation and cross-linking reversal, enrichment of MICA and MICB promoters was monitored by PCR amplification with specific primers for the region between nucleotides (-317/-12)-MICA and (-337/-4)-MICB encompassing the HSE element. As shown in Fig. 5*C* (and supplemental Fig. 6*C*), both 17-AAG and radicicol induced recruitment of HSF-1 to this region of MICA and MICB promoters; the specificity of this interaction was demonstrated by the absence of PCR amplification when an upstream region of MICA promoter, lacking the HSE element, was amplified (MICA; ΔHSE). As a positive control of equal loading and quality of chromatin samples, 1% input DNA was used as template with the same primers.

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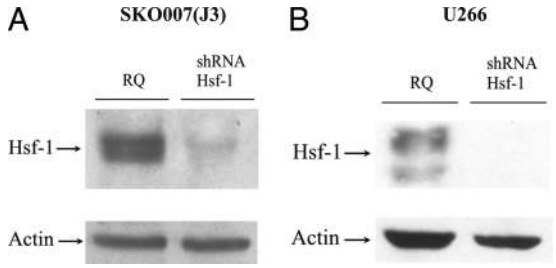

FIGURE 6. Western blot was performed using whole-cell extracts from pSIREN-RetroQ (RQ) or pSIREN-RetroQ/HSF-1 (shRNA HSF-1) retrovirus-infected SKO-007(J3) or U266 cells. The proteins transferred to nitrocellulose membranes were stained with Ponceau to verify that similar amounts of protein had been loaded in each lane.

Taken together, these data indicate that MICA and MICB promoters are bound by HSF-1 in myeloma cells exposed to HSP-90 inhibitors.

To confirm the involvement of HSF-1 in MICA-MICB induction by 17-AAG and radicicol, we inhibited HSF-1 expression in SKO-007(J3) and U266 cells using RNA interference. We infected the cell lines with a retrovirus encoding a shRNA specific for HSF-1 (RQ-shRNA HSF-1) or with a control retrovirus (RQ). After drug selection, we obtained resistant cells where HSF-1 was specifically inhibited, as confirmed by Western blot (Fig. 6).

We then conducted flow cytometric assays to analyze the effect of HSP-90 inhibitors on NKG2D ligands expression in pRQ and pRQ-shRNA-HSF-1-infected cell lines.

As previously reported, we observed that the absence of HSF-1 makes cells more susceptible to apoptotic signaling (data not shown); thus, in these experiments we used lower doses of radicicol and 17-AAG to avoid cell death. As shown in Fig. 7 (and supplemental Fig. 7), up-regulation of MICA and MICB expression persists in control RQ-SKO-007(J3) and RQ-U266 cells even at lower doses of drugs, and it is significantly blocked by HSF-1 interference.

Thus, the overall data indicate that induction of HSF-1 plays a central role in the regulation of MICA and MICB expression by 17-AAG and radicicol in MM cell lines.

HSP-90 inhibitors enhance NK cell degranulation against myeloma cells

Because changes in the expression of NKG2D ligands, such as MICA and MICB, on tumor cells can modify the recognition and activation of NK cells via NKG2D, we tested whether treatment of myeloma cells with HSP-90 inhibitors could lead to increased NK cell degranulation.

We analyzed the degranulation of NK cells against SKO-007(J3) and U266 cells, by evaluating the expression of the lysosomal marker CD107a on the effector cells by immunofluorescence and FACS analysis, that correlates with NK cell cytotoxicity (24).

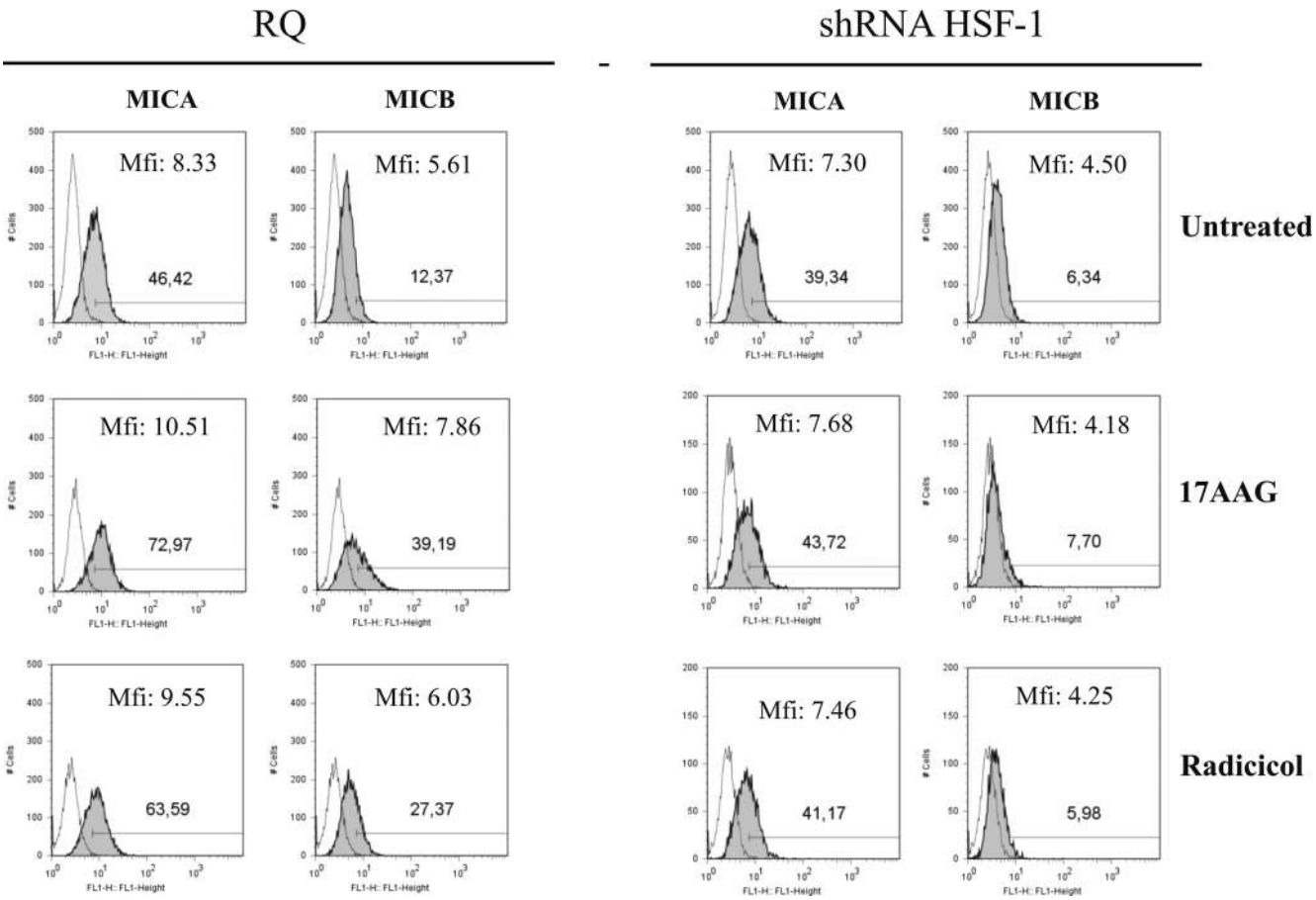

FIGURE 7. Activation of HSF-1 by HSP-90 inhibitors plays a central role in MICA-MICB induction. MICA and MICB surface expression was analyzed by flow cytometry on RQ or shRNA HSF-1 retrovirus-infected SKO-007(J3) cells, treated with 17-AAG (0.75 μM) or radicicol (1 μM) for 48 h. Data are representative of one of three independent experiments. The percent of positive cells with respect to the isotype control Ab and the mean fluorescence intensity (MFI) are shown. Gray histograms represent the specific ligand. Data are representative of one of three independent experiments.

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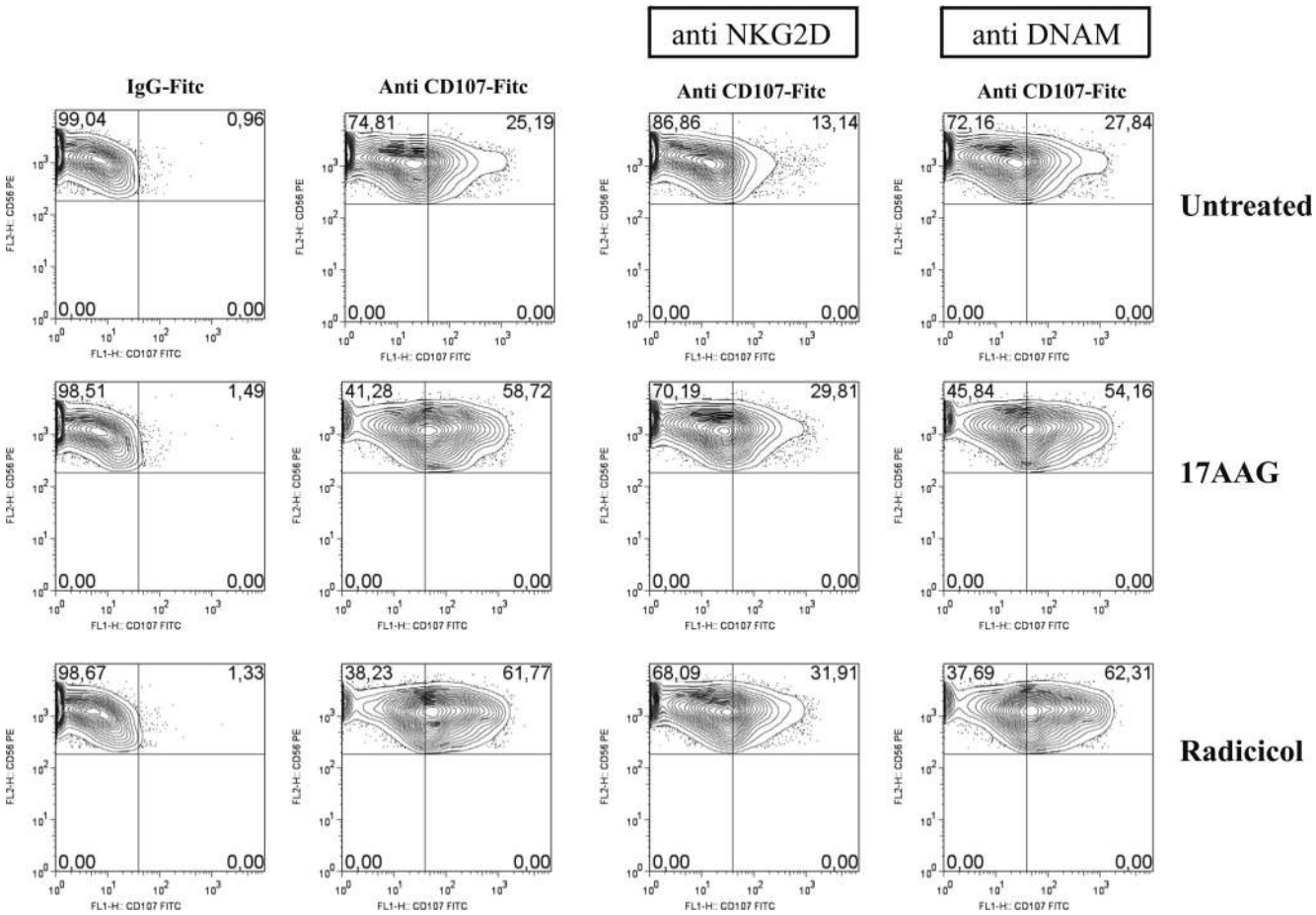

FIGURE 8. Effect of HSP-90 inhibitors on NK cell degranulation against myeloma cells. NK cells prepared from PBMCs of healthy donors, preactivated with 200 U/ml IL-2 for 12 h, were incubated with SKO-007(J3) cells, untreated or treated with 17-AAG (1 μM) or radicicol (1 μM) for 48 h, and used as target cells in a degranulation assay. The assay was performed at an E:T ratio of 2.5:1. After 3 h at 37°C, cells were stained with anti-CD56, anti-CD3, and anti-CD107a mAbs. Cell surface expression of CD107a was analyzed on $CD56^+CD3^-$ cells. To evaluate the role of NKG2D and DNAM-1, the assay was performed in parallel treating NK cells with blocking anti-DNAM-1 or anti-NKG2D Abs. Results are representative of one of three independent experiments. FL, Fluorescence; -H, -height.

As shown in Fig. 8, basal expression of CD107a on NK cells contacting SKO-007(J3) or U266 target cells (supplemental Fig. 8) was enhanced following drug treatment. The assay was performed at an E:T ratio of 2.5:1, and similar results were obtained using different E:T ratios (data not shown).

This increase of degranulation was dependent on NKG2D activation, because it was considerably reduced in the presence of a blocking anti-NKG2D mAb, whereas no significant change of degranulation was observed upon treatment with an anti-DNAM-1 mAb or with a control mAb (data not shown), this in according with our observation of the unmodified PVR surface expression in drug-treated cells. NKG2D (and to a lesser extent DNAM-1 in U266 cells)-blocking Abs partially affected basal degranulation, indicating that constitutive NK cell degranulation also involves these activating receptors.

Our results, therefore, demonstrate that augmented expression of MICA and MICB on SKO-007(J3) and U266 cells treated with 17-AAG and radicicol enhances NK cell degranulation by promoting NKG2D recognition.

Discussion

In the last few years, increasing evidence has shown that anticancer immune responses may contribute to the control of tumors after conventional chemotherapy. A number of different observations have indicated that some chemotherapeutic agents, in particular anthracyclines or radiotherapy, can induce specific immune responses that result in immunogenic cancer cell death or immunostimulatory side effects (37–39). In this regard, tumor cells can be stressed by a variety of stimuli (intrinsic or extrinsic) that may induce membrane expression or release of specific signals such as danger signals, eat-me signals, or killing signals that may facilitate immune recognition/activation and enhance the eradication of stressed/treated tumor cells.

A particular example of this functional connection between chemotherapy and therapeutic immunomodulation is the finding that several chemotherapics, such as genotoxic agents, histone deacetylase inhibitors, or proteasome inhibitors (e.g., bortezomib), can increase the expression of NKG2D ligands, thus facilitating the activation of NKG2D-expressing lymphocytes (including NK cells, NKT cells, and CTLs) and tumor cell lysis. Moreover, activation of the DNA damage response by chemotherapy or radiotherapy can also increase the expression of death receptors in several tumors (e.g., CD95/FAS and TRAIL receptors), facilitating the cytotoxic action of FAS ligand- and TRAIL-positive immune effectors on tumor cells (37–39).

In this study, we described the effect of the HSP-90 inhibitors, 17-AAG and radicicol, on the expression of NKG2D and DNAM-1 ligands in human MM cell lines.

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We found that treatment of two MM cell lines, SKO-007(J3) and U266, with HSP-90 inhibitors is able to increase the expression of the MICA and MICB NKG2D ligands, but not the DNAM-1 ligand PVR, rendering these cells more efficient to activate NK cell degranulation.

NK cells are effector cells of the innate immune system and their functions are regulated by a number of killer cell-inhibitory and -activating receptors. Depending on their intracellularly located ITIM or activatory motifs, these receptors can mediate NK cell activation or tolerance toward self-Ags and/or tumor cells (40, 41). In this regard, evidence is accumulating that, apart from classical missing-self control or specific genotoxic-mediated activation of NKG2D ligands, different additional activating signals originating from a stressed tumor cell can contribute to NK cell recognition and cytotoxic action.

Indeed, a number of inducible proteins can be recognized by NK cells on the surface of stressed cells. These stress-inducible proteins include the NKG2D ligands (42), DNAM-1 ligands (10), together with the membrane expression and release of the HSP-70, that can trigger and redirect cytolytic and migratory capacity of NK cells toward tumors (43). Similarly, the NF HLA-B-associated transcript 3 can be relocalized and released from tumor cells in response to stress signals (e.g., heat shock) and engage the activating receptor NKp30 (44), thus further supporting also in the case of NK cell activity, the general model for a damage-induced recognition by factors expressed and/or released from tumor cells (37, 39, 45).

The pharmacological inhibition of the HSP-90 chaperone can be considered an interesting target relevant for cancer therapy, given that tumor cells, as compared with their normal counterparts, can exhibit a greater stressed phenotype, with an enhanced dependency on the cytoprotective actions mediated by this protein. Indeed, HSP-90 is often overexpressed in many cancers, including MM (20) and accounts for the maturation and functional stability of a plethora of client proteins required to maintain aberrant signaling pathways in tumors (19, 21). Moreover, in malignant cells, HSP-90 is present in an activated superchaperone complex that is hypersensitive to HSP-90 inhibition, whereas in normal cells HSP-90 is mainly uncomplexed and less responsive to pharmacological inhibitors (46).

A general characteristic feature of HSP-90 inhibitors is that they can induce the heat shock response, a complex cascade pathway activated mainly by the transcription factor HSF-1 and the specific induction of a number of antiapoptotic HSPs such as HSP-70, HSP-27, and HSP-90 itself (31), that may prevent denaturation of cellular proteins under stress, and also activate a protective mechanism against HSP-90 inhibition and/or the action of other cytotoxic chemotherapics (19). On the other hand, membrane-bound and extracellular-located HSP-70 is known to activate cancer immunity and thus could ameliorate clinical outcome (43).

In this work, we have shown that treatment of MM cells with HSP-90 inhibitors can up-regulate both MICA and MICB protein surface and mRNA expression and that activation of HSF-1 is involved in this pathway. On the contrary, basal expression of PVR was unaffected after drug treatment. Moreover, SKO-007(J3) and U266 cells did not express significant levels of ULBP1, ULBP2, ULBP3, and NEC-2, and these ligands were not induced by the two HSP-90 inhibitors.

In this regard, the regulation of expression of NKG2D ligands is not well understood yet, and a wide variety of signals in the context of the cell stress can cooperate at different levels. The activation of the HSF-1 has been shown to regulate MICA/B expression (36) and potential heat shock elements can be predicted from the *ULBP1* and *ULBP2* promoter sequence (47, 48). Therfore, it is possible that activators of HSF-1 could induce these ligands via a HSR. On the other hand, the expression of specific NKG2D and DNAM-1 ligand genes and the action of HSF-1 could be inhibited or silenced by DNA methylation and/or specific repressive histone proteins in the cell lines used in this work. Additional experiments will be needed to clarify these hypotheses.

Our data show that NK cell recognition and activation in response to SKO-007(J3) and U266 MM cells was enhanced following HSP-90 inhibition, using either 17-AAG or radicicol. The increase of degranulation was significantly dependent on NKG2D activation, because considerably reduced in the presence of a blocking anti-NKG2D mAb, whereas no change of activation was observed upon treatment with an anti-DNAM-1 mAb or a control mAb. However, the presence of a blocking anti-NKG2D mAb did not completely abrogate this effect, suggesting that other parallel activating pathways might be involved.

Additional experiments will be needed to clarify these hypotheses and to verify the action of HSP-90 inhibitors also in the context of ex vivo primary plasma cells derived from MM patients.

In conclusion, this study provides novel information about the therapeutic potential of HSP-90 inhibition in MM; promotion of tumor immunosurveillance, via enhancement of NKG2D ligand expression, could be considered an additional mechanism supporting the antimyeloma activity of HSP-90 inhibitors and suggests their possible immunotherapeutic value.

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Disclosures

The authors have no financial conflict of interest.

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