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Inhibition of Glycogen Synthase Kinase-3 Increases NKG2D Ligand MICA Expression and Sensitivity to NK Cell–Mediated Cytotoxicity in Multiple Myeloma Cells: Role of STAT3

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Engagement of NKG2D and DNAX accessory molecule-1 (DNAM-1) receptors on lymphocytes plays an important role for anticancer response and represents an interesting therapeutic target for pharmacological modulation. In this study, we investigated the effect of inhibitors targeting the glycogen synthase kinase-3 (GSK3) on the expression of NKG2D and DNAM-1 ligands in multiple myeloma (MM) cells. GSK3 is a pleiotropic serine/threonine kinase point of convergence of numerous cell-signaling pathways, able to regulate the proliferation and survival of cancer cells, including MM. We found that inhibition of GSK3 upregulates both MICA protein surface and mRNA expression in MM cells, with little or no effects on the basal expression of the MICB and DNAM-1 ligand poliovirus receptor/CD155. Moreover, exposure to GSK3 inhibitors renders myeloma cells more efficient to activate NK cell degranulation and to enhance the ability of myeloma cells to trigger NK cell–mediated cytotoxicity. We could exclude that increased expression of β-catenin or activation of the heat shock factor-1 (transcription factors inhibited by active GSK3) is involved in the upregulation of MICA expression, by using RNA interference or viral transduction of constitutive active forms. On the contrary, inhibition of GSK3 correlated with a downregulation of STAT3 activation, a negative regulator of MICA transcription. Both Tyr705 phosphorylation and binding of STAT3 on MICA promoter are reduced by GSK3 inhibitors; in addition, overexpression of a constitutively active form of STAT3 significantly inhibits MICA upregulation. Thus, we provide evidence that regulation of the NKG2D-ligand MICA expression may represent an additional immune-mediated mechanism supporting the antimyeloma activity of GSK3 inhibitors.

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In this regard, it has been shown that inhibition of GSK3 can induce growth arrest or apoptosis in MM cell lines, and enhance the anti-MM cytotoxic effect of bortezomib, by modulating critical signaling pathways in these cells (21–23).

Our laboratory has recently shown that low doses of different genotoxic drugs (e.g., melphalan, doxorubicin, and bortezomib) or inhibitors of the heat shock protein (HSP)-90 protein (e.g., 17-allylamino-17-demethoxy-geldanamycin, radicicol) can increase the expression of NKG2D and DNAM-1 ligands on these cells, via induction of the DNA damage response (DDR) or the activation of the heat shock factor-1 (HSF-1) transcription factor, respectively (19, 20). The upregulation of these ligands on MM tumor cells is associated with their ability to trigger increased NK cell degranulation.

In this study, we analyzed the possibility that treatment of MM cells with different inhibitors of GSK3 could regulate the expression of NK cell–activating ligands and, in turn, modify NK cell recognition and cytoxicity against these cancer cells.

Our results indicate that the GSK3 inhibitors can increase the surface expression levels of the NKG2D ligand MICA on MM cells, without significant effects on the expression of MICB or the DNAM-1 ligand PVR/CD155. Moreover, we found that MICA upregulation correlates with an increased gene promoter activity and a reduction of the Tyr \(^{705}\) phosphorylation and MICA promoter binding of the transcription factor STAT3, a negative regulator of MICA transcription (24).

In addition, inhibition of GSK3 could further enhance MICA expression levels in melphalan- or lenalidomide-treated cells, and significantly increase NK cell–mediated killing by promoting NKG2D recognition.

Overall, our data demonstrate that GSK3 inhibitors can regulate NKG2D-ligand expression, suggesting a new role of these agents in improving immune response to multiple myeloma.

Materials and Methods

Cell lines and clinical samples

The human MM cell lines SKO-007(J3), U266, and RPMI 8226 were provided by P. Trivedi (Sapienza University of Rome). The cell lines were maintained at 37°C and 5% CO\(_2\) in RPMI 1640 (Life Technologies, Gathersburg, MD) supplemented with 15% FCS. All cell lines were mycoplasma free (EZ-PCR Mycoplasma test kit; Biological Industries).

Bone marrow samples from patients with MM were managed at the Institute of Hematology (Sapienza University of Rome). Informed consent in accordance with the Declaration of Helsinki was obtained from all patients, and approval was obtained from the Ethics Committee of the Sapienza University of Rome. The bone marrow aspirates were processed, as described above (19). In some experiments, myeloma cells were selected using anti-CD138 magnetic beads (Miltenyi Biotec, Auburn, CA). More than 95% of the purified cells expressed CD138 and CD38.

Reagents and Abs

The 4-hydroxytamoxifen, radicicol, and the GSK3 inhibitors (BI-201976, 9-Oxime (BIO), SB216763 (SB21), and LiCl) were purchased from Sigma-Aldrich (St. Louis, MO). STA-21, a selective inhibitor of STAT3, was purchased from BIOMOL/Enzo Life Sciences (Farmingdale, NY). Lenalidomide was purchased from BioVision (Milpitas, CA). The following mAbs were used for immunostaining or as blocking Abs: anti-MICA (MAB159227), anti-MICB (MAB236511), and anti-NKG2D (MAB149810) from R&D Systems (Minneapolis, MN); anti-PVR/CD155 (SKM14) provided by M. Colonna (Washington University, St. Louis, MO); anti-CD56 (C218) mAb provided by A. Moretta (University of Genoa, Genoa, Italy); anti-DNAM-1 (DX11) from Serotec (Oxford, U.K.); and anti-MHC class I (W6/32) from American Type Culture Collection (Manassas, VA). Allophycocyanin goat anti-mouse IgG (Poly4053), anti-CD3/allophycocyanin (HT13a), anti-CD56/PE (HC56), and anti-IgG1/FITC, IgG1/PE, or IgG1/allophycocyanin isotype control (MOPC-21) were purchased from BioLegend (San Diego, CA). Anti-CD107a/FITC (H1A3), anti-CD38, and anti-CD138 were purchased from BD Biosciences (San Jose, CA).

Immunofluorescence and flow cytometry

SKO-007(J3), U266, and RPMI 8226 cells were cultured in 6-well tissue culture plates for 48 h at a concentration of 3 \times 10^5 cells/ml in the presence of different concentrations of drugs. The expression of the NKG2D and DNAM-1 ligands on MM cells was analyzed by immunofluorescence staining using anti-MICA, anti-MICB, or anti-PVR/CD155-unconjugated mAb, followed by secondary goat anti-mouse allophycocyanin. In all experiments, cells were stained with propidium iodide (1 \mu g/ml) to assess cell viability. Nonspecific 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 flow cytometric data analysis software (Tree Star, Ashland, OR).

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

Total RNA was extracted using TRIzol (Life Technologies, Grand Island, NY), according to manufacturer’s instructions. The concentration and quality of the extracted total RNA were determined by measuring light absorbance at 260 nm (A\(_{260}\)) and the ratio of A\(_{260}/A_{280}\). Reverse transcription was carried out in a 25 \mu l reaction volume with 2 \mu g total RNA, according to the manufacturer’s protocol for Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). Real-time PCR was performed using the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). cDNAs were amplified in triplicate with primers for MICA (H\(_{400792195}\_m1) conjugated with fluorochrome FAM and \(\beta\)-actin (4326315E) conjugated with fluorochrome VIC (Applied Biosystems). The level of ligand expression was measured using threshold cycle (Ct). The Ct was obtained by subtracting the Ct value of the interest (MICA) from the housekeeping gene (\(\beta\)-actin) Ct value. In the current study, we used Ct of the untreated sample as the calibrator. The fold change was calculated according to equation \(2^{-\Delta\Delta Ct}\), where \(\Delta\Delta Ct\) was the difference between Ct of the sample and the Ct of the calibrator (according to the formula, the value of the calibrator in each run is 1). The analysis was performed using SDS version 2.2 software (Applied Biosystems).

Degranulation assay

NK cell–mediated cytotoxicity was evaluated using the lysosomal marker CD107a, as previously described (25). As source of effector cells, we used primary NK cells obtained from PBMCs isolated from healthy donors by Lymphoprep (Nycomed, Oslo, Norway) gradient centrifugation and then cocultured for 10 d with irradiated (30 Gy) EVB-transformed B cell line RPMI 8866 at 37°C in a humidified 5% CO\(_2\) atmosphere, as previously described (26). On day 10, the cell population was routinely >90% CD56\(^{+}\)CD16\(^{-}\)CD53\(^{-}\), as assessed by immunofluorescence and flow cytometry analysis. Drug-treated MM cell lines were incubated with activated NK cells at 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/FTTC (or cIgG/FTTC) for 45 min at 4°C. Cells were then stained with anti-CD3/allophycocyanin, 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. Fluorescence was analyzed using a FACSCalibur flow cytometer (BD Biosciences), and data were analyzed using FlowJo flow cytometric data analysis software (Tree Star).

Cytotoxicity assay

A standard 4-h chromium-release assay was used, as previously described (27). SKO-007(J3) cells, stimulated as indicated in the text, were used as target cells and were labeled (100–200 \mu Ci \(^{3}Cr/10^6\) cells; Amersham BioSciences, Piscataway, NJ) for 90 min at 37°C and washed, and 5 \times 10^4 cells/well were plated. As source of effector cells, we used primary NK cells, as described above. The percentage of specific lysis was calculated by counting an aliquot of supernatant and using the following formula: 100 \times ([sample release – spontaneous release]/[total release – spontaneous release]). All determinations were made in triplicate, and E:T ratios ranged from 10:1 to 1:1, as indicated.

Plasmids

The pBabe-S33Y-ER-puro retroviral vector encoding a chimeric \(\beta\)-catenin/\(\beta\)-catenin receptor (ER) protein, in which the full-length S33Y/\(\beta\)-catenin (mutant \(\beta\)-catenin, codon 33 substitution of tyrosine for serine S33Y) is fused in-frame to a mutated ER ligand binding domain, was provided by E. Fearon (University of Michigan, Ann Arbor, MI). For knocking down \(\beta\)-catenin, we used a pLKO.1-sh-\(\beta\)-catenin lentivirus vector with puromycin resistance (TRCN0000003845, NM_001904.x-2279581c1, MISSION;
results of GSK3 inhibition and NKG2D ligand expression in multiple myeloma

GSK3 inhibitors upregulate MICA expression on human MM cells

In the last few years, our laboratory has investigated the expression and regulation of NKG2D and DNAM-1 ligands on human MM cells in response to different antimyeloma agents able to activate the DNA damage response (melphalan, doxorubicin) (19) or to inhibit the HSP-90 chaperone (20).

To characterize novel agents and pathways able to regulate the activity of these ligands in MM cells, we investigated the activity of different GSK3 inhibitors, a class of molecules recently studied for their ability to inhibit the proliferation of malignant lymphoid cells, including MM (21–23).

To analyze the effect of GSK3 inhibition on the expression of NKG2D and DNAM-1 ligands, we initially performed a flow cytometric analysis on three MM cell lines (SKO-007(J3), U266, and RPMI 8266), after 48-h treatment with three classical inhibitors of GSK3, LiCl, SB21, or BIO, a specific inhibitor of GSK3, and relative to the cyclin-dependent kinase inhibitor indirubin-3’-monooxime (23). As shown in Fig. 1A and 1B (and Supplemental Figs. 1A, 1B, 2A, 2B for U266 and RPMI 8266 cells), treatment of SKO-007(J3) cells was able to upregulate the basal expression of MICA, with little or no effects on MICB or PVR levels (Supplemental Figs. 3A and 4 for U266 and RPMI 8266 cells). These treatments did not affect the cell viability over the time chosen for these experiments (as assessed by propidium iodide staining) (Supplemental Figs. 1C, 2C, 3B for U266 and RPMI 8266 cells) and the expression of MHC1 on these cells (Supplemental Figs. 1D, 2D, 3C for U266 and RPMI 8266 cells). Previous observations have shown that these cell lines do not express detectable levels of UL16-binding proteins and nectin-2, and these ligands were not further analyzed in this study (20).

We then examined whether a possible mechanism underlying MICA upregulation on MM cells could be the consequence of an increased mRNA expression. To this aim, total RNA was isolated from SKO-007(J3), U266, and RPMI 8266 cells exposed to these inhibitors for 24 h and analyzed by real-time quantitative RT-PCR. As shown in Fig. 1C (and Supplemental Figs. 1E, 2E for U266 and RPMI 8266 cells), we found a significant increase of MICA mRNA levels in treated cells. Moreover, we confirmed the effect of GSK3 inhibitors on the cell surface expression of MICA in four patient MM cells; as shown in Fig. 2A, increased expression of MICA was observed in LiCl-treated MM cells, with no significant effects on MICB or PVR levels (Fig. 2B, e.g., patient 4). Accordingly, similar results were obtained for MICA mRNA levels, in LiCl-treated MM cells from three additional patients (Fig. 2C).

These results indicate that inhibition of GSK3 activity enhances the cell surface expression and the mRNA levels of the NKG2D ligand MICA, in human MM cells.

Exposure to GSK3 inhibitors renders myeloma cells more susceptible to NK cell–mediated cytotoxicity

Because changes in the expression of NKG2D ligands (such as MICA) on tumor cells can modify the recognition and the acti-
vation of NK cells via NKG2D, we tested whether treatment of myeloma cells with GSK3 inhibitors could also lead to increased activation and NK cell–mediated killing.

We analyzed the degranulation of NK cells isolated from healthy donors against SKO-007(J3) cells, by evaluating the expression of the CD107a [a surrogate marker for granule mobilization (25)], by immunofluorescence and FACS analysis.

As shown in Fig. 3A and 3B, basal expression of CD107a on NK cells contacting SKO-007(J3) cells was enhanced following LiCl treatment. This increase of degranulation was significantly dependent on NKG2D activation, because considerably reduced in the presence of a blocking anti-NKG2D mAb; little changes of degranulation were observed upon treatment with an anti–DNAM-1 mAb or with a control mAb, this in accordance with our ob-

FIGURE 1. Upregulation of MICA expression on MM cells following treatment with GSK3 inhibitors. (A) MICA cell surface expression was analyzed by flow cytometry on SKO-007(J3) cells treated with the GSK3 inhibitors, LiCl (10 mM), SB21 (5 μM), or BIO (1.5 μM) for 48 h. The gray-colored histograms represent basal expression of MICA, whereas thick black-colored histograms represent the expression of MICA after treatment with the indicated inhibitor. Data are representative of one of four independent experiments. (B) The mean fluorescence intensity (MFI) of MICA was calculated based on at least four independent experiments and evaluated by paired Student t test (*p < 0.05). For each treatment, histograms represent the MFI with specific mAb-MFI with isotype control. (C) Real-time PCR analysis of total mRNA obtained from SKO-007(J3) cells, unstimulated or treated with the indicated GSK3 inhibitor for 24 h, as described above. Data, expressed as fold change units, were normalized with β-actin and referred to the untreated cells considered as calibrator and represent the mean of three experiments (*p < 0.05).

FIGURE 2. Upregulation of MICA expression on patient MM cells following treatment with GSK3 inhibitors. (A) Cell surface expression of MICA was analyzed by flow cytometry on patient MM cells treated with LiCl (10 mM) for 48 h. The gray-colored histograms represent basal expression of MICA, whereas thick black-colored histograms represent the expression of MICA after treatment with LiCl. (B) MICA, MICB, and PVR/CD155 cell surface expression (arrows), representative of patient (P4), was analyzed by flow cytometry, as described above. (C) Real-time PCR analysis of total mRNA obtained from patient MM cells, unstimulated or treated with LiCl for 24 h, as described above. Data, expressed as fold change units, were normalized with β-actin and referred to the untreated cells considered as calibrator.
GSK3 inhibition and NKG2D ligand expression in multiple myeloma

Figure 3. Inhibition of GSK3 enhances NK cell–mediated cytotoxicity against myeloma cells. (A) NK cells prepared from PBMCs of healthy donors were incubated with SKO-007(J3) cells, untreated or treated with LiCl (10 mM) for 48 h, and used as target cells in a degranulation assay. The assay was performed at the 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. (B) The mean fluorescence intensity of CD107 was calculated based on at least three independent experiments and evaluated by paired Student t test (*p < 0.05). For each treatment, histograms represent the mean fluorescence intensity with specific mAb–mean fluorescence intensity with isotype control. (C) NK cells isolated from PBMCs of healthy donors were incubated with SKO-007(J3) cells, untreated or treated with LiCl for 48 h, as described above, and used as target cells in a standard 4-h chromium-release assay. The percentage of specific lysis was calculated by counting an aliquot of supernatant and using the following formula: 100 × ([sample release − spontaneous release]/[total release − spontaneous release]). All determinations were made in triplicate, and E:T ratios ranged from 10:1 to 1:1, as indicated. Data represent the mean (n = 4 experiments, *p < 0.05).

Analysis of possible molecular mechanisms involved in MICA upregulation by GSK3 inhibitors

To investigate whether one of the mechanisms involved in GSK3 inhibition-mediated increase of MICA expression could be a direct effect on the transcriptional activity of its promoter, transient transfection assays were performed in SKO-007(J3) cells. As shown in Fig. 4A, the activity of a luciferase reporter gene construct, containing 1 kb of the mica gene promoter region, was enhanced by LiCl. Thus, MICA mRNA expression and promoter activity are enhanced by GSK3 inhibition in MM cells.

GSK3 has been demonstrated to be a central integrator of many signaling pathways involved in a multitude of cellular physiological and pathological processes (1–3). In particular, GSK3 has been shown to posttranslationally modify degradation, nuclear export, and the activity of different transcription factors, via phosphorylation of consensus site-specific serine or threonine residues (30–32).

To determine whether one of the mechanisms involved in GSK3 inhibition-mediated enhancement of mica gene expression could be related to its ability to regulate the function of specific transcription factors, we analyzed the role of β-catenin and HSF-1, two classical targets of this kinase and involved in regulating MICA expression in stressed cells (20, 33).

Inhibition of GSK3 decreases phosphorylation of β-catenin, thus preventing its degradation by the ubiquitin-mediated pathway; the stabilized β-catenin acts in the nucleus by activating the T cell factor/lymphoid enhancer factor–mediated transcription of different target genes (34). As shown in Fig. 4B, treatment of SKO-007(J3) cells with LiCl, SB21, or BIO increased β-catenin protein levels, confirming GSK-3 inhibition in our experimental conditions. To investigate the possible link between the activity of this transcription factor and the induction of MICA expression, we used SKO-007(J3) cells stably expressing an inducible chimeric β-catenin/ER fusion protein (SKO/S33Y-ER), in which a full-length constitutive active (S33Y) β-catenin had been cloned upstream of a mutated ER ligand binding domain, capable of binding 4-hydroxytamoxifen (4-OHT), but not estrogen (35) (Fig. 4C). As shown in Fig. 4D, following 48-h treatment of the SKO/S33Y-ER cells with 4-OHT, no change in MICA expression was observed.
was detected in these cells, indicating that accumulation and activation of β-catenin are not sufficient to induce MICA expression. In addition, increased expression of MICA in the presence of LiCl was not affected in SKO-007(J3) cells in which β-catenin expression was blocked by shRNA interference (Fig. 4E, 4F).
To investigate the involvement of HSF-1 in MICA upregulation by GSK3 inhibitors, we used SKO-007(J3) cells in which HSF-1 expression was blocked by shRNA interference, as already described (20). As previously reported, we observed that upregulation of MICA mediated by radicicol (a HSP-90 inhibitor able to activate HSF-1) was significantly blocked by HSF-1 interference; on the contrary, increased expression of MICA in the presence of LiCl was not affected in the same experimental setting (Fig. 4G).

Inhibition of GSK3 enhances MICA expression: role of STAT3

Recently, a novel role for the transcription factor STAT3 in the regulation of NKG2D ligands has been described; STAT3 inhibition is able to specifically upregulate MICA transcription and protein surface expression in a colorectal tumor cell line. As a result, this upregulation can enhance NKG2D-mediated NK cell activation and functions (24).

Different pathways converge in regulating the activity of STAT3; in fact, in addition to being downstream of cytokine receptors, STAT3 is also activated by growth factor receptor and nonreceptor tyrosine kinases that phosphorylate and induce functional dimers able to translocate in the nucleus and to activate transcription [reviewed in (36)].

In this regard, GSK3 activity has been shown to positively influence the Tyr705 phosphorylation and DNA-binding activity of STAT3 in response to different cytokines, and inhibition of this kinase can significantly modulate the expression of STAT3-regulated genes (37).

Thus, we investigated the possible correlation among GSK3 inhibition, enhancement of MICA expression, and modulation of the activity of STAT3 in MM cells.

Fig. 5A shows that treatment of SKO-007(J3) cells with STA-21, a molecule able to inhibit STAT3 DNA binding, dimerization, and transcriptional activity (38), can increase the expression of MICA, thus confirming the repressive action of STAT3 on this gene in our experimental system. Similar results were obtained using the JAK2-specific inhibitor AG490, which predominantly represses STAT3 activity (Fig. 5B).

Interestingly, as already described in different models (5, 37, 39, 40), LiCl, SB21, and BIO could significantly reduce Tyr705 phosphorylation of STAT3 in SKO-007(J3) cells (Fig. 5C, 5D), suggesting that the constitutive Tyr705 phosphorylation of STAT3 in these cells was dependent on the GSK3 activity.

To determine whether one of the mechanisms involved in the upregulation of MICA gene expression could be a direct regulatory action mediated by STAT3 on its promoter activity, quantitative ChIP assays were done on the promoter region encompassing the repressor element MICA/STAT3-RE (24). As shown in Fig. 5E, we observed that GSK3 inhibition can decrease the interaction of STAT3 on the MICA promoter; in addition, overexpression of a constitutively active mutant form of STAT3 [dimerized by cysteine–cysteine residues instead of pY-SH2 interactions (28)] significantly inhibited MICA up-regulation (Fig. 6), indicating that specific repression mediated by active STAT3 on this gene can be released by GSK3 inhibition.

**FIGURE 5.** Inhibition of GSK3 enhances MICA expression: role of STAT3. (A and B) MICA cell surface expression was analyzed by flow cytometry on SKO-007(J3) cells treated with the STAT3 inhibitor STA-21 (30 μM) or the JAK2 inhibitor AG490 (40 μM) for 48 h. The gray-colored histogram represents basal expression of MICA, whereas thick black-colored histogram represents the expression of MICA after treatment with STA-21 or AG490. Data are representative of one of three independent experiments. (C) SKO-007(J3) cells were treated with the indicated GSK3 inhibitors for 18 h, as described above, and cell lysates were immunoblotted for phospho-Tyr705-STAT3, total STAT3, and β-actin. The proteins transferred to nitrocellulose membranes were stained with Ponceau to verify that similar amounts of protein had been loaded in each lane. (D) The ratio of phospho-Tyr705-STAT3 to total STAT3 was calculated by densitometric analysis of the immunoblots. Data represent the mean \( n = 3 \) experiments, *\( p < 0.05 \). (E) In vivo binding of STAT3 to the MICA promoter was examined using ChIP analysis. Samples were immunoprecipitated from sonicated lysates of SKO-007(J3) cells, unstimulated or treated for 18 h with the indicated inhibitor, as described above, using an Ab against human STAT3 protein or isotype control. After reversing the cross-linking, DNA was precipitated and real-time PCR was performed using primers to amplify the STAT3 promoter region encompassing the STAT3 responsive element (the MICA STAT3-RE is indicated in the box). Results are expressed as relative enrichment as compared with the input. Data represent the mean \( n = 3 \) experiments, *\( p < 0.05 \).
As previously described, treatment of SKO-007(J3) cells with melphalan increases the expression of MICA (19); interestingly, this upregulation was further enhanced in the presence of LiCl, both at the protein and mRNA expression (Fig. 7A, 7C, 7E), suggesting that GSK3 inhibition can cooperate with the DDR induced by genotoxic drugs to increase MICA expression by a mechanism that can involve removal of STAT3-mediated repression. Moreover, similar results were obtained using lenalidomide (Fig. 7B, 7D, 7F), an antmyeloma chemotherapeutic agent (derived using structural modifications of the chemical structure of thalidomide) endowed with antiangiogenic effects in the bone marrow and able to induce NK cell anti-MM immune responses (9, 13, 42).

These results therefore indicate that GSK3 inhibitors can further enhance the expression of MICA in MM cells induced by standard chemotherapy drugs.

Discussion

In the last few years, increasing evidence has suggested that anticancer immune responses may contribute to the control of tumors after conventional chemotherapy; indeed, different observations have indicated that chemotherapeutic agents (e.g., genotoxic drugs) or radiotherapy can induce specific immune responses that result in immunogenic cancer cell death or immunostimulatory side effects (43, 44). An example of this functional connection between chemotherapy and therapeutic immunomodulation is the finding that several genotoxic agents or drugs, such as inhibitors of histone deacetylases or of the HSP-90 molecular chaperone, can increase the expression of NKG2D ligands, thus facilitating the activation of,NK cells (including NKT cells, macrophages or inhibit the constitutive activity or the polarization of Th17 cells in different models of autoimmune disease (5, 47). Moreover, GSK3 has been shown to modulate specific functions of NK cells, given that inhibition of its activity can increase cytokine secretion and cytotoxicity, possibly due to nuclear translocation of functional β-catenin (48).

In this study, we described the effect of different GSK3 inhibitors on the expression of the NKG2D and DNAM-1 ligands in human MM cells.

We found that treatment of different MM cell lines with GSK3 inhibitors is able to increase the expression of the NKG2D ligand MICA, rendering these cells more susceptible to NK cell–mediated killing (Fig. 3). In this regard, our observations provide the additional information that pharmacological modulation of this kinase could potentiate the activity of NK cells against the tumor, via mechanisms that can involve a parallel action on NK cells and tumor cells, increasing the expression of ligands that promote recognition and enhancing the cytolytic activity of the effectors (48).

Interestingly, a pivotal role in this regulatory mechanism is mediated by the effect of GSK3 on STAT3 activation, a transcription factor recently described to specifically inhibit MICA gene expression in different cancer cell lines, via direct repression of its promoter activity (24). Moreover, STAT3 can be persistently activated in different types of human cancers, including MM, where it plays critical roles in the process of carcinogenesis and tumor survival (36, 49, 50), also sustaining local tumor-promoting stromal inflammation (51).
GSK3 activity has been shown to positively influence the Tyr\textsuperscript{705} phosphorylation and DNA-binding activity of STAT3, and inhibition of this kinase can strongly suppress the activating phosphorylation of STAT3 on Tyr\textsuperscript{705} and significantly modulate the expression of STAT3-regulated genes (5, 37, 39, 40, 47). Our data show that inhibition of GSK3 activity can decrease the constitutive Tyr\textsuperscript{705} phosphorylation of STAT3 in SKO-007(J3) cells; in this context, treatment of SKO-007(J3) cells with the STAT3 inhibitor STA-21 or with the JAK2-specific inhibitor AG490 (that predominantly repress STAT3 activity) can increase the expression of MICA, thus confirming the repressive action of active STAT3 on this gene in our experimental system. GSK3 inhibition can increase the transcriptional activity of the mica gene promoter (Fig. 4A) and reduce the interaction with STAT3, as revealed by quantitative ChIP assays on the promoter fragment encompassing the repressive MICA/STAT3-RE (24) (Fig. 5E). Moreover, overexpression of a constitutively active mutant form of STAT3 significantly inhibited MICA upregulation (Fig. 6), indicating that one of the mechanisms involved in GSK3-mediated regulation of mica gene expression could be related to the transcriptional activity of its promotor, where basal repression mediated by active STAT3 can be released by GSK3 inhibition.

At this regard, we could exclude that increased expression of \(\beta\)-catenin or the activation of HSF-1 (transcription factors specifically inhibited by active GSK3) is involved in the upregulation of MICA expression, by using RNA interference or viral transduction of constitutive active forms (Fig. 4C–G). We also investigated the possible cooperation between GSK3 inhibition and induction of MICA expression in chemotherapy-treated MM cells. As already described (19, 20), melphalan increased the expression of MICA in SKO-007(J3) cells, and this upregulation was further enhanced in the presence of LiCl, suggesting that GSK3 inhibition can cooperate with genotoxic drugs to increase MICA expression (Fig. 7). Interestingly, STAT3 constitutive activation was shown to prevent the induction of MICA following genotoxic stress (24). These observations suggest that
constitutive activation of this transcription factor in MM cells may interfere with pathways triggered by DDR; thus, GSK3 inhibition may favor MICA upregulation after melphalan treatment by reducing the repressive activity of STAT3. Finally, similar results were obtained using lenalidomide (Fig. 7), an antiangiogenic and antimyeloma molecule able to induce NK cell immune responses, and recently described to upregulate MICA in MM cells (52); however, the molecular mechanisms mediating these effects are unknown.

STAT3 is a positive regulator of the transcription of different genes; however, it can also act as a transcriptional repressor, as already described for the IL-8 and p53 genes. In this case, a direct binding of STAT3 to the IL-8 and p53 promoters was required for inhibition (53, 54). Further studies will be necessary to better clarify the transcriptional mechanisms involved in MICA upregulation mediated by GSK3/STAT3 inhibition, and to identify and dissect novel pharmacological cooperation with other modulators of NKG2D ligand expression to enhance NK cell activity against the tumor.

In conclusion, this study provides novel information about the therapeutic potential of GSK3 inhibition in MM; promotion of tumor immunosurveillance via enhancement of the NKG2D-ligand MICA expression could be considered an additional mechanism supporting the antimyeloma activity of GSK3 inhibitors and suggests their possible immunotherapeutic value.

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Disclosures
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References