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Research Paper

STAT3 inhibition by STA21 increases cell surface expression of MICB and the release of soluble MICB by gastric adenocarcinoma cells

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ABSTRACT

NKG2D is an activating receptor expressed on NK cells that binds to a variety of ligands, including MICA and MICB. These cell surface glycoproteins are overexpressed under cellular transformation, thus playing an important role in cell-mediated immune response to tumors. STAT3 is a transcription factor that is constitutively active in cancer. It negatively regulates MICA expression on target cells, while its inhibition enhances NK cell cytotoxicity against tumors. In this work, we aimed to describe the effect of STAT3 signaling inhibition by STA21 on the regulation of MICB expression in gastric adenocarcinoma cells and its effect on the cytotoxic function of NK cells. Treatment of gastric adenocarcinoma cells with STA21 induced an increase in MICB expression and soluble MICB secretion, as well as a variable pattern on effector cell degranulation. Soluble MICB secretion by gastric adenocarcinoma cells was not affected by metalloprotease inhibition. We also observed that primary gastric adenocarcinoma tissue released soluble MICB into the extracellular milieu. Recombinant MICB induced a significant decrease in the levels of NKG2D receptor on effector NK and CD8+ T cells, which correlated with an impaired cytotoxic function. Altogether, our data provide evidence that STAT3 signaling pathway regulates MICB expression on gastric adenocarcinoma cells and that recombinant soluble MICB compromises the cytolytic activity of NK cells.

1. Introduction

The major histocompatibility complex class I chain-related molecules A and B (MICA/B) are ligands of the natural killer group 2, member D (NKG2D) activation receptor of NK cells and other cytolytic lymphocytes, which play a crucial role in immune surveillance against tumors (Bauer et al., 1999; Raulet, 2003). The presence of NKG2D ligands (NKG2DLs) on tumor cells at early stages of cancer development triggers the cytotoxic activity of effector cells, contributing to tumor elimination (Huergo-Zapico et al., 2014). Indeed, the levels of tumor-associated NKG2DLs have been correlated with improved survival in patients with colorectal and early breast cancer (Watson et al., 2006; de

Abbreviations: ADAM, a disintegrin and metalloproteinase; APC, allophycocyanin; LDH, lactate dehydrogenase; MICA, major histocompatibility complex class I chain-related molecules A; MICB, major histocompatibility complex class I chain-related molecules B; NKG2D, natural killer group 2 member D receptor; NKG2DLs, NKG2D ligands; rMICA, recombinant MICA; rMICB, recombinant MICB; sMICA, soluble MICA; sMICB, soluble MICB; sNKG2DLs, soluble NKG2DLs; ULBP, UL16 binding protein

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Kruijf et al., 2012). In contrast, in ovarian cancer and advanced gastric cancer, the levels of NKG2DLs expression on tumor tissue predicts a poor prognosis of the disease (McGilvray et al., 2010; Ribeiro et al., 2016), suggesting that tumor expression of NKG2DLs may play an opposing role in immune surveillance depending on the tumor type and stage.

Tumor cells can also release soluble forms of NKG2DLs (sNKG2DLs) into the extracellular microenvironment (Holdenrieder et al., 2006a,b). Accordingly, high levels of sNKG2DLs are found in cancer patients' sera, which have been correlated with a more advanced clinical stage and with a poor prognosis of the disease (Holdenrieder et al., 2006a,b; Salih et al., 2006). It has been described that high serum levels of soluble MICA (sMICA) interfere with anti-tumor immunity through the down-regulation of the NKG2D receptor on cytolytic cells (Groh et al., 2002; Jinushi et al., 2005; Duan et al., 2011; Wu et al., 2004). Therefore, deficiency in the NKG2D-mediated effector functions by NK cells and $CD8^+$ T lymphocytes has been proposed as one of the mechanisms by which immune-mediated tumor elimination is compromised (Groh et al., 2002).

The mechanisms that regulate NKG2DLs expression on target cells are not completely understood. STAT3 is a member of the intracellular signal transducers and activators of transcription (STAT) family. STAT3 pathway is constitutively active in several types of tumors, including gastric cancer (Kim et al., 2009), a feature that is also observed in tumor cell lines, such as gastric adenocarcinoma and prostate cancer cells (Kanda et al., 2004; Mora et al., 2002). STA21 is a small molecule with potent STAT3-inhibiting activity; it blocks STAT3 pathway activation in breast cancer cells that present constitutive STAT3 signaling (Song et al., 2005). Previous reports have demonstrated that STAT3 negatively regulates MICA expression on target cells, while inhibition of STAT3 activation enhances NK cell cytotoxicity against tumor cells (Bedel et al., 2011).

In this study, we aimed to investigate the role of STAT3 in the regulation of MICA and MICB expression on gastric adenocarcinoma cells. We also analyzed the effect of recombinant MICB on NKG2D receptor expression on effector cells and in their cytotoxic function.

2. Methods

2.1. Cell lines and primary cell cultures

Human gastric adenocarcinoma cell lines AGS (ATCC, Pennsylvania, PA, U.S.A) and MKN45 (Riken Bio Resource Center, kindly provided by Dr. Andrew Quest, Instituto de Ciencias Biomédicas, Universidad de Chile), and human K562 erythroleukaemia cells (ATCC), were maintained in RPMI 1640 culture medium (HyClone Thermo Scientific, Logan City, UT, U.S.A). Prostatic adenocarcinoma cell line LNCaP (ATCC, gently provided by Dr. Ricardo Moreno, Department of Physiology, Pontificia Universidad Católica de Chile) was maintained in Dulbecco's modified Eagle's medium, D-MEM/F-12 (Invitrogen, Carlsbad, CA, U.S.A). Cell media were supplemented with 10% fetal calf serum (FCS), 1 mM glutamine and 100 mg/mL penicillin-streptomycin (HyClone Thermo Scientific) at 37 °C in a humidified atmosphere of 5% CO_2. For all experiments performed, cell lines were used until passage 10.

Five patients treated at the Department of Gastrointestinal Surgery, Hospital del Salvador (Santiago, Chile) and pathologically diagnosed with gastric adenocarcinoma, were enrolled in this study. Gastric tumor and matched mucosal tissues (1 g, approximately) were incubated in RPMI-3% FCS medium for 24 h. The levels of soluble MICA and MICB in the culture medium were determined by capture ELISA, as described below. sMICA and sMICB concentrations were normalized by tissue weight.

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood from healthy donors by separation with Ficoll–Hypaque (GE Healthcare AB, Stockholm, Sweden). Isolated PBMC was treated with 150 U/mL of recombinant human IL-2 (R & D Systems, Minneapolis, MN, U.S.A) at 37 °C in a humidified atmosphere of 5% CO_2 for 18 h before experimental procedures.

Written informed consent for gastric tissue and blood donation was obtained from all individual participants included in this work. This study was approved by the Committee on Human Ethics Investigation of the Faculty of Medicine, Universidad de Chile, and the Committee on Scientific Ethics of the Metropolitan Health Service of the Chilean Government.

2.2. Cell treatment with STA21

Cells were incubated in RPMI-3% FCS with 30 μM STA21 (Enzo Life Science, Farmingdale, NY, U.S.A) or dimethylsulfoxide (DMSO), as vehicle, for 24 h. The adherent cells were recovered with phosphate-buffered saline (PBS)–2 mM EDTA solution, washed three times with PBS and prepared for either flow cytometry staining, CD107a mobilization assay, lactate dehydrogenase (LDH) cytotoxic assay or analysis of soluble MICA and MICB released into the culture medium.

2.3. Flow cytometry analysis

Cell lines were incubated with either purified mouse anti-human MICA or MICB monoclonal antibodies (mAb) (R & D Systems) followed by incubation with goat anti-mouse FITC-conjugated antibody (KPL, Gaithersburg, MD, U.S.A), as previously described (Serrano et al., 2011). Purified mouse IgG2b (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A) was used as the isotype control. For intracellular staining, cells were fixed and permeabilized using BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution (BD Biosciences, San Jose, CA, U.S.A) and cell staining was performed according to the manufacturer's instructions. Flow cytometry was carried out using a FACSAria flow cytometer (BD Bioscience) and the data were analyzed with FlowJo v8.7 software (Treestar Inc, Ashland, OR, U.S.A).

2.4. Treatment of PBMC with recombinant MICA and MICB

PBMC isolated from healthy donors (3×10^5 cells) were incubated in RPMI-3% FCS with recombinant MICA (rMICA) (ab182709, Abcam, Cambridge, MA, U.S.A) at 100 ng/mL or recombinant MICB (rMICB) (841623, R & D Systems) at 50 ng/mL for 24 h at 37 °C. In order to evaluate NKG2D receptor expression on effector cells, PBMC were then stained for 30 min at 4 °C with a mixture of anti-human mAbs: CD56-PE (eBioscience, San Diego, CA, U.S.A), CD3-PE/Cy7 (Biolegend, San Diego, CA, U.S.A), CD8-FITC (Biolegend), and NKG2D-APC (BD Bioscience). Cells were further assessed by flow cytometry.

2.5. CD107a mobilization assay

Target tumor cells and PBMC, previously treated with STA21 or recombinant MICA or MICB, respectively, were incubated at a 10:1 effector:target cell ratio in the presence of APC/Cy7-conjugated anti-CD107a mAb (Biolegend) and 3 μM of BD GolgiStop™ Protein Transport Inhibitor (BD Biosciences). Cells were then co-cultured for 5 h at 37 °C. Cells were stained for CD56, CD3 and CD8 and analyzed by flow cytometry as previously described. Alternatively, cells were fixed and permeabilized with BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) and intracellular IFN-γ was detected with AlexaFluor700-conjugated anti-human IFN-γ mAb (Biolegend) for additional 30 min at 4 °C, followed by flow cytometric analysis.

2.6. Lactate dehydrogenase (LDH) cytotoxic assay

After 5 h of effector-target cell co-culture, cells were centrifuged and the supernatants were analyzed for the quantification of lactate dehydrogenase (LDH) release using Cyto Tox96® Non-radioactive

Cytotoxicity Assay Kit (Promega, Fitchburg, WI, U.S.A). Experiments and analyses were performed according to the manufacturer's instructions.

2.7. Real-time quantitative PCR (qRT-PCR)

Total RNA was extracted using E.Z.N.A.® Total RNA kit I (Omega Bio-TekInc, Norcross, GA, U.S.A) and reversed transcribed using AffinityScript reverse transcriptase (Agilent Technologies, Santa Clara, CA, U.S.A). Samples were subjected to qRT-PCR using Stratagene MX3000P real-time system (Agilent Technologies). mRNAs were quantified using primers for MICA 5′-TGTAAG AAGAAAACATCAGCT-3′ (sense) and 5′-ATAGGTCAGGAAACTGAGGCAC-3′ (antisense) and for MICB 5′- TCACGGGTTTCATTCAGTTGG-3′ (sense) and 5′- ACA GCACCATGAGGTTGTAACG-3′ (antisense). Relative mRNA expression was calculated using the ΔΔCt method using RPL27 mRNA as the endogenous control.

2.8. Treatment with protease inhibitors

Tumor cell lines were incubated in RPMI-3% FCS or D-MEM/F-12-3% FCS with the broad-spectrum metalloprotease inhibitors Batimastat (BB94) (Merck, Darmstadt, Germany) at 10 μM and TNF-α protease inhibitor I (TAPI-1) (Merck) at 5 μM for 24 h. sMICA and sMICB levels in the supernatants of cell cultures were then determined by capture ELISA.

2.9. Capture enzyme-linked immunosorbent assay (ELISA)

Plates were coated with the capture anti-MICA mAb AMO1 (BamOmaB, Gräfelfing, Germany) at 3 μg/mL in PBS at 4 °C overnight. Plates were blocked by adding 1% w/v bovine serum albumin (BSA) in PBS for 2 h at 37 °C. Samples were incubated at 4 °C overnight, followed by incubation with a biotinylated goat anti-mouse MICA detection antibody (R & D Systems) at 400 ng/mL for 2 h at 37 °C. This reagent was diluted in 1% BSA/PBS and premixed with normal goat serum at 2% v/v (Gibco Invitrogen) 2 h prior to use. Next, plates were incubated with streptavidin-horseradish peroxidase (HRP)-conjugate (Sigma-Aldrich, St. Louis, MO, U.S.A) 1:2000 in 1% BSA/PBS for 30 min at room temperature. Each step was followed by washing 5 times with PBS/0.05% Tween 20 v/v. Finally, plates were developed using 1-Step Ultra TMB-ELISA Substrate Solution (Thermo Scientific), and absorbance was measured at 450 nm.

Soluble MICB was determined using Human MICB Duoset Kit (R & D Systems), following the manufacturer's instructions.

2.10. Statistical analysis

Where indicated, differences between two groups were calculated by the two-tailed paired or unpaired *t*-test. One-way ANOVA with Dunnett's multiple comparison test was used to analyze the differences among 3 or more groups. $p < 0.05$ was considered statistically significant.

3. Results

3.1. STAT3 inhibition increases the expression of MICB on gastric adenocarcinoma cell lines

MKN45 and AGS gastric adenocarcinoma cells express MICA and MICB (Fig. 1A), as well as other NKG2DLs, such as ULBP molecules, on their surface (data not shown). It has been reported that STAT3 inhibition in HT29 colorectal adenocarcinoma cells increases MICA expression, but no change in MICB expression was observed (Bedel et al., 2011). Since STAT3 is constitutively activated in AGS and MKN45 cell lines (Kanda et al., 2004) (data not shown), we decided to evaluate the effect of STAT3 inhibition, using the specific inhibitor STA21, on the expression of MICA and MICB in these gastric adenocarcinoma cell lines.

To evaluate the effect of STA21 on the expression of NKG2DLs, gastric adenocarcinoma cell lines were incubated with STA21, and MICA and MICB mRNA levels were estimated by qRT-PCR. When MKN45 and AGS cells were treated with the inhibitor, a significant increase in MICA ($p = 0.0183$ and $p = 0.0026$, respectively) and MICB ($p = 0.0083$ and $p = 0.0112$, respectively) transcript levels was observed (Fig. 1B). To confirm these results, we analyzed the effect of STA21 on MICA and MICB expression by flow cytometry. A significant increase in MICB expression was found both, on the cell surface of MKN45 and AGS cells (72%, $p = 0.0024$ and 23%, $p = 0.017$, respectively) (Fig. 1C), and in total protein expression on the same cell lines (28% and 30%, respectively) (Fig. 1D). Interestingly, total and cell surface expression of MICA did not change in gastric adenocarcinoma cells upon treatment with STA21 (Figs. 1E and F).

3.2. The cytotoxic response of effector cells challenged with STA21-treated target cells depends on individual donor samples

To assess whether STAT3 pathway inhibition affects gastric adenocarcinoma cell susceptibility to lysis by effector cells, we treated MKN45 cells with STA21, and target cell cytolysis was further analyzed using a LDH release assay. We did not observe a statistically significant variation in the release of LDH upon effector cell challenge with STA21-treated target cells (Fig. 2A). We also assessed the expression of CD107a, by flow cytometry, on $CD3^{-}CD56^{+}$ cells present in the PBMC of healthy donors upon challenge with MKN45 cells. Among the subject samples analyzed, we did not observe a consistent pattern in CD107a expression on effector cells co-cultured with STA21-treated target cells, since CD107a expression increased in only two out of five donor samples, while the levels of this degranulation marker did not change in two other individuals, and in one of them the CD107a levels decreased on effector cells (Fig. 2B). These results suggest that STAT3 inhibition and its subsequent MICB increase in target cells affects differently CD107a degranulation by NK cells from diverse healthy donors.

3.3. STAT3 inhibition increases the release of soluble MICB by gastric adenocarcinoma cell lines

Since STAT3 inhibition induced an increase in MICB expression on gastric adenocarcinoma cells, both at the protein and mRNA levels, we further asked whether STAT3 also affects the release of sMICB into the culture supernatant. First, we assessed whether gastric adenocarcinoma cell lines were able to secrete soluble NKG2DLs in measurable quantities. We detected significant levels of sMICB spontaneously secreted by MKN45 (132 ± 1 pg/mL) and AGS cells (257 ± 42 pg/mL), as well as by a control prostatic cancer cell line, LNCaP (216 ± 1 pg/mL) (Fig. 3A). However, sMICA release by MKN45 and AGS was not detected under the same experimental conditions, in contrast to the control LNCaP cells (568 ± 7 pg/mL) (Fig. 3A). We also evaluated the release of sMICA and sMICB by fresh primary gastric tumor and non-tumor adjacent gastric mucosa obtained from patients with gastric cancer. Tumors secreted significantly higher levels of sMICA than their matched gastric mucosa. A similar trend for sMICB levels was detected, although no statistically significant differences could be observed between gastric tumor and mucosal samples (Fig. 3B).

Next, we addressed the effect of STAT3 inhibition on the levels of sMICA and sMICB released by gastric adenocarcinoma cell lines. Our results showed a significant 3- and 1.7-fold increase in sMICB levels in the presence of STAT3 inhibitor STA21 for MKN45 (297%, $p = 0.0003$) and AGS (212%, $p = 0.0003$) cell lines, respectively (Fig. 3C). Nevertheless, upon STA21 treatment, sMICA levels were not detectable in the

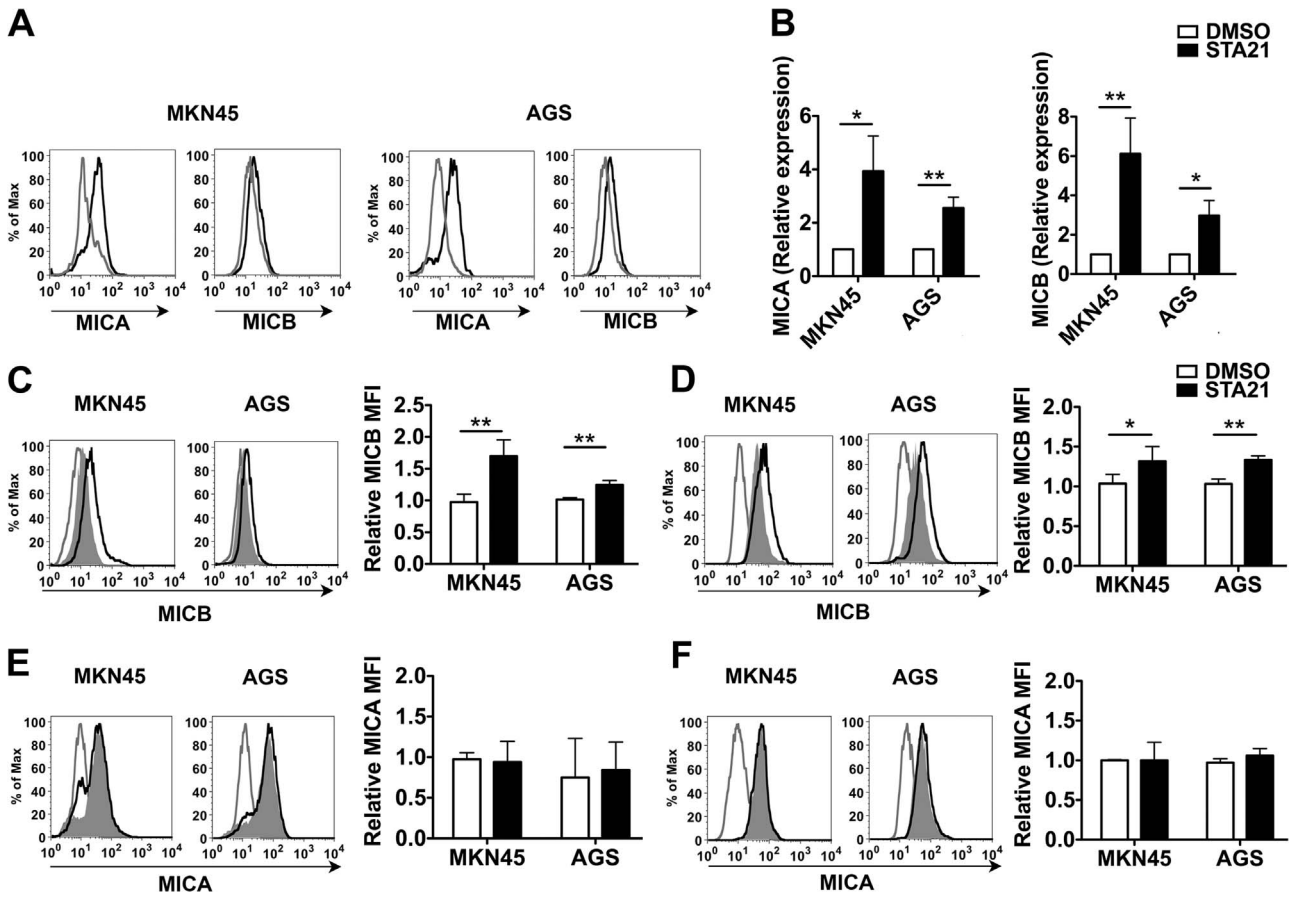

Fig. 1. STA21 increases the expression of MICB on gastric adenocarcinoma cell lines. (A) Surface expression of MICA and MICB on MKN45 and AGS cell lines was determined by flow cytometry. Black and grey histograms represent MIC expression and isotype control, respectively. Histograms are representative of three independent experiments. (B) Transcripts levels for MICA and MICB were assessed by qRT-PCR. Cells were incubated with vehicle alone (DMSO) or STA21. All determinations were carried out in triplicates and graphics represent 3 independent experiments. The two-tailed unpaired *t*-test was used for statistical analysis. Cells were incubated with STA21, and MICB and MICA surface expression (C and E, respectively) and total expression (D and F, respectively) were assessed by flow cytometry. Representative histograms show MICA and MICB expression in cell lines incubated with vehicle alone (DMSO) (grey solid histograms) or STA21 (black lines). Isotype control is represented by a grey line. Graphs represent 3 independent experiments for total MICB and MICA for all cell lines and 4 and 3 independent experiments for surface MICB and MICA for MKN45 and AGS cells, respectively. Data are presented as mean values ± S.D. The two-tailed unpaired *t*-test was used for statistical analysis. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

culture supernatant of these cells (data not shown). These results indicate that STAT3 pathway is involved in the modulation of sMICB release by gastric cancer cells.

3.4. sMICB release by adenocarcinoma cell lines is not affected by metalloprotease inhibitors

It has been reported that ADAM10 and ADAM17 are the metalloproteases most commonly involved in MICA and MICB shedding (Salih

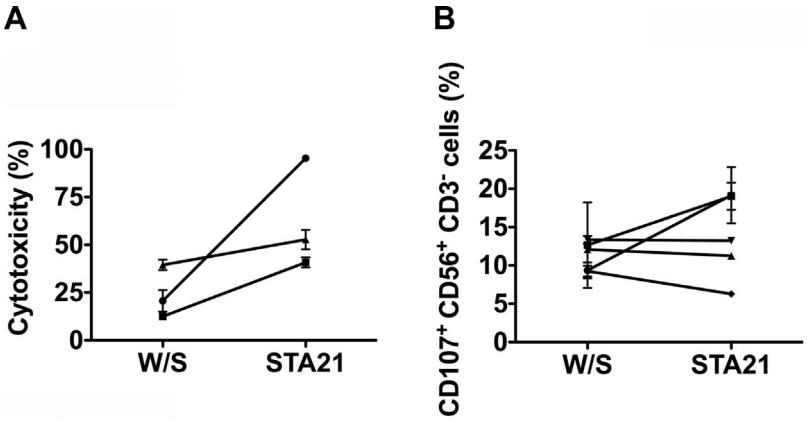

Fig. 2. Cytotoxic activity of effector cells against gastric adenocarcinoma cells treated with STA21. MKN45 cells were treated with STA21 and co-cultured with freshly isolated PBMC from healthy donors for 5 h at 10:1 effector:target cell ratio. (A) The susceptibility to cytolysis was determined by measuring the release of LDH into the supernatant. (B) Effector cell degranulation was analyzed according to their CD107a expression. For LDH release assay, 3 different donors were evaluated, and the assays were performed in triplicate wells. For the degranulation assay, 5 different donors were assessed, and all determinations were performed in triplicates. Data are presented as mean values ± standard deviation (S.D.). W/S, without stimulus.

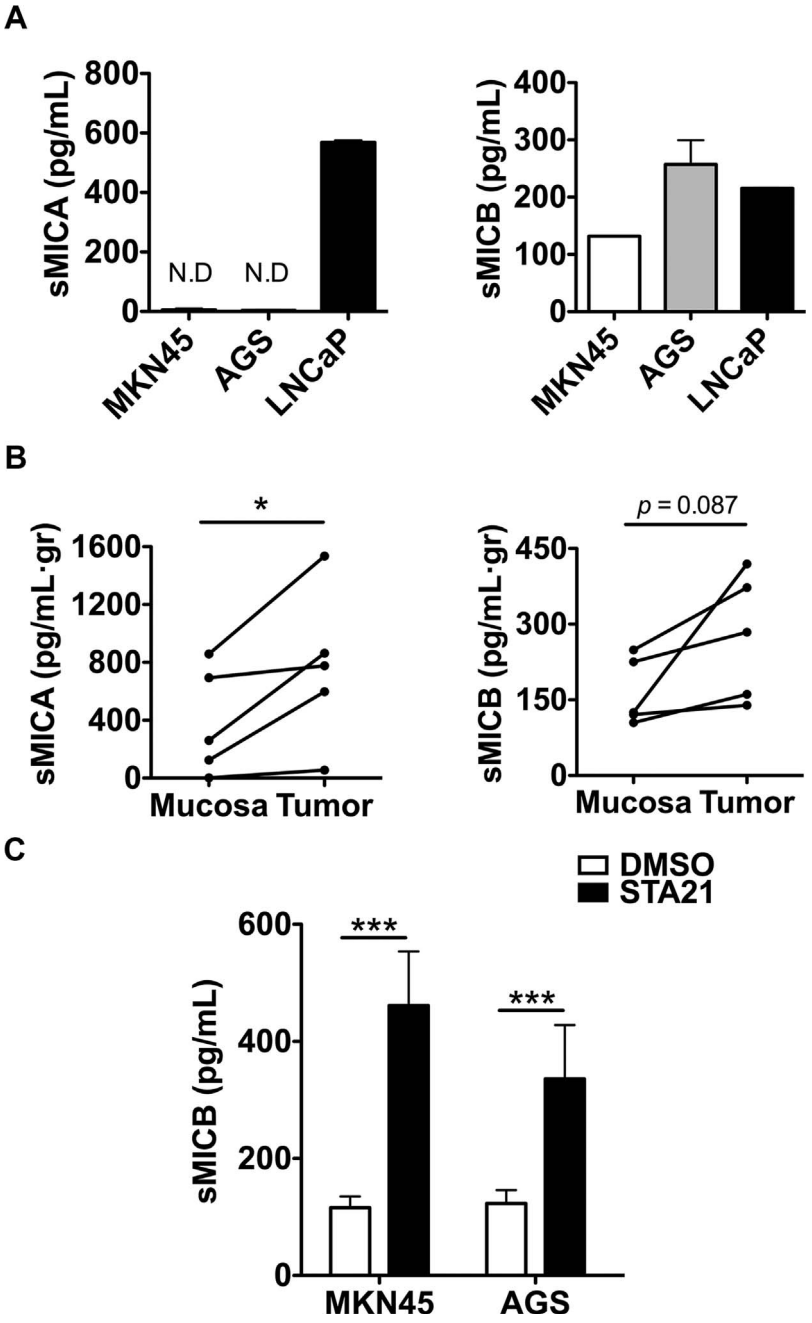

Fig. 3. STA21 increases the release of soluble MICB by gastric adenocarcinoma cell lines. (A) The release of sMICA and sMICB by cell lines was determined by ELISA. Graphs represent 3 independent experiments. (B) The release of sMICA and sMICB in freshly isolated gastric tumor and gastric mucosa from patients with gastric cancer was determined by ELISA, n = 5. sMIC concentration was normalized by tissue weight. The two-tailed paired *t*-test was used for statistical analysis. (C) sMIC concentration in the supernatants of cells incubated with STA21 was determined by ELISA. Graphs represent 4 and 6 independent experiments for MKN45 and AGS, respectively. The two-tailed unpaired *t*-test was used for statistical analysis. Data are presented as mean values ± S.D. $*p < 0.05$, $***p < 0.001$.

et al., 2006; Salih et al., 2002; Waldhauer et al., 2008; Boutet et al., 2009; Kohga et al., 2009; Chitadze et al., 2013). In order to analyze whether these enzymes participate in the shedding of MICB from gastric adenocarcinoma cell lines, these cells were incubated with the broad-spectrum metalloprotease inhibitors Batimastat (BB94) and TAPI-1. Surprisingly, the metalloprotease inhibitors did not affect the release of sMICB by MKN45 and AGS, although they did inhibit significantly the release of sMICA in LNCaP cells (79%, $p = 0.000007$) (Fig. 4). Hence, in the gastric cancer cell lines studied, proteolysis of MICB may be mediated by enzymes other than ADAM10 and ADAM17.

3.5. Recombinant MICB decreases NKG2D receptor expression on NK cells and affects their cytotoxic capacity

Previous studies have shown that recombinant MICA (rMICA) induces endocytosis of the NKG2D receptor, thus decreasing its levels on the cell surface of effector cytolytic cells (Groh et al., 2002). In this study, we asked whether the exposure of effector cytolytic cells to recombinant MICB (rMICB) affects their NKG2D receptor expression and consequently influences their cytotoxic function. For this purpose, PBMC isolated from healthy donors were incubated with rMICB or rMICA, and NKG2D receptor expression was analyzed by flow cytometry on effector cells. A significant decrease in NKG2D receptor expression was observed on $CD3^{-}CD56^{+}$ NK cells (53%, $p = 0.00005$) and

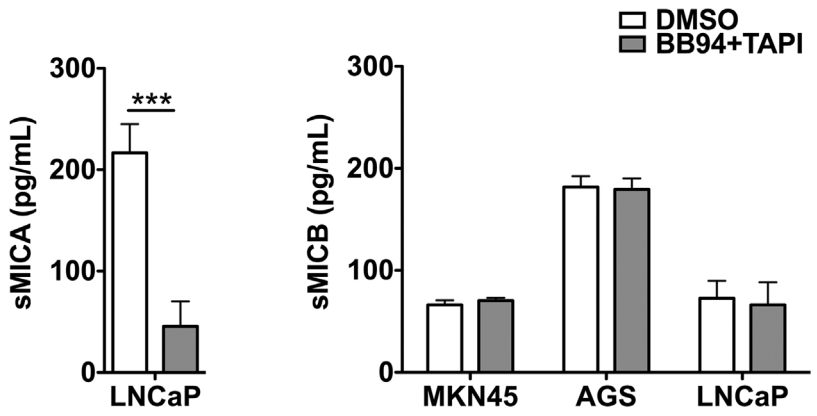

Fig. 4. sMICB release by adenocarcinoma cell lines is not affected by the use of metalloprotease inhibitors. sMICA and sMICB concentration in the supernatants of cells incubated with BB94 and TAPI-1 was determined by ELISA. Graphs represent 3, 3 and 4 independent experiments for MKN45, AGS and LNCaP cells, respectively. Data are presented as mean values ± S.D. The two-tailed unpaired *t*-test was used for statistical analysis. ***$p < 0.001$.

$CD8^+$ T lymphocytes (28%, $p = 0.019$) upon incubation with rMICB (Fig. 5A). Conversely, the levels of NKG2D receptor did not vary neither on NK cells nor on $CD8^+$ T lymphocytes when PBMC was incubated with rMICA (Fig. 5A).

To evaluate the functional consequences of NKG2D receptor down-regulation on NK cells by rMICB, PBMC from healthy donors were incubated with rMICB prior to co-culture with K562 target cells. CD107a expression and IFN-γ production were then evaluated on NK cells by flow cytometry. Effector cells incubated with rMICB showed significantly lower CD107a (30%) and IFN-γ (87%) levels upon challenge with K562 cells as compared with cells pre-incubated with vehicle alone (Fig. 5B). On the other hand, incubation of PBMC with rMICA did not change the levels of CD107a or IFN-γ on NK cells (Fig. 5B). We also evaluated the functional activity of $CD8^+$ T lymphocytes present in the PBMC treated with rMICB and challenged with K562 cells, and no alterations on CD107a or IFN-γ levels could be detected on these cells (data not shown). Therefore, our data suggest that rMICB induces a decrease on NKG2D expression on the surface of NK cells, impairing their cytotoxic function towards tumor cells.

4. Discussion

This work focused on the effect of STAT3 pathway inhibition in the regulation of the expression of MICA and MICB on gastric adenocarcinoma human cell lines. In a previous work, we demonstrated that IL-10 treatment of melanoma cell lines promotes a decrease of MICA levels on these cells (Serrano et al., 2011). Similar results have been observed in MKN45 and AGS gastric adenocarcinoma cell lines, as IL-10 decreases their cell surface expression of MICA (unpublished data). IL-10 is an anti-inflammatory cytokine that plays an important role in tumor immune evasion, whose signaling pathway involves STAT3 activation (Moore et al., 2001). STAT3 pathway is constitutively active in most cancers, and it has been associated with poor prognosis in patients with gastric cancer (Ji et al., 2016; Yu et al., 2016; He et al., 2015) and other tumor types (Xu and Lu, 2014). Due to its important role in cancer, STAT3 has been proposed as an anti-cancer target in several studies (Johnston and Grandis, 2011; Bharadwaj et al., 2015; Palagani et al., 2014).

Here, we inhibited STAT3 basal activation in gastric adenocarcinoma cell lines using 30 μM of STA21 for 24 h. The concentration used in this work is among the range commonly used in the literature (Song et al., PNAS, 2005 and Bedel et al., cancer research, 2011) to inhibit the STAT3 pathway. In addition, we did not observe differences in cell viability and cell number between STA21- or DMSO-treated cells within 24 h treatment with the inhibitor. We demonstrated an increase in the transcript levels of MICA and MICB on these cells. Accordingly, an increase in MICB expression on the cell surface and intracellularly was also observed after STAT3 inhibition, which was accompanied by a raise in the levels of sMICB. Conversely, MICA levels did not change in gastric adenocarcinoma cells after incubation with STA21. These results differ from what has been observed in colorectal adenocarcinoma cells, where STAT3 inhibition affects only MICA expression (Bedel et al., 2011). Interestingly, previous studies have shown the presence of a variety of microRNA molecules whose targets are MICA and/or MICB, which regulate the expression of these proteins (Tsukerman et al., 2012; Stern-Ginossar et al., 2008). Therefore, our results suggest that post-transcriptionally and/or post-translationally regulatory mechanisms for MICA and MICB exist in gastric tumor cells.

Here we showed that NK cells from healthy donors responded differently to target cell treatment with STA21, as observed in our degranulation assays. These results could be attributed to the high inter-individual variability that characterizes effector cells from healthy volunteers, as has been documented in a previous study (Shafi et al., 2011). In this work, the authors observed that healthy donors showed variable responses when their effector cells were challenged with target cells bearing specific MICA gene polymorphisms or different MICA levels on the cell surface. This variability was also observed for the non-polymorphic NKG2D ligand ULBP2. The authors hypothesized that the response of each individual is “tuned” for a specific amount of this ligand to induce an optimal response. Thus, we propose that the same variable effect may be observed for MICB, and that the increase on the cell surface levels of this ligand, induced by STA21, does not necessarily imply a higher cytotoxic response. We additionally demonstrate here that STA21 also increases MICB secretion into the extracellular medium. However, in the cytotoxic assay, target cells were washed thoroughly to remove STA21 inhibitor previous to co-culture with effector cells. These washing steps also removed the soluble MICB released in the supernatant. Therefore, soluble MICB levels released during the co-culture may have not affected the assay, since effectors and target cells remained together in culture for a short period of time, along with the low number of target cells used in the experiments.

In vivo, tumor cells release sNKG2DLs, and sera from patients with various types of cancer present elevated sNKG2DL levels (Holdenrieder et al., 2006a,b). Herein, we showed that primary gastric tumors also release sMICA and sMICB into the extracellular milieu. It has been shown that sMICA downregulates the expression of NKG2D receptor on cytolytic cells, leading to an impaired cytotoxic function (Groh et al., 2002; Jinushi et al., 2005; Hilpert et al., 2012; Wang et al., 2008). Indeed, a correlation between the concentration of sMICA and sMICB in the serum and cancer stage has been shown for several cancer types (Holdenrieder et al., 2006a,b; Salih et al., 2008). In this work, we observed that STAT3 inhibition by STA21 induced a significant increase in the release of sMICB in AGS and MKN45 cell lines. Since STAT3 activation has been shown to promote tumor immune evasion (Yu et al.,

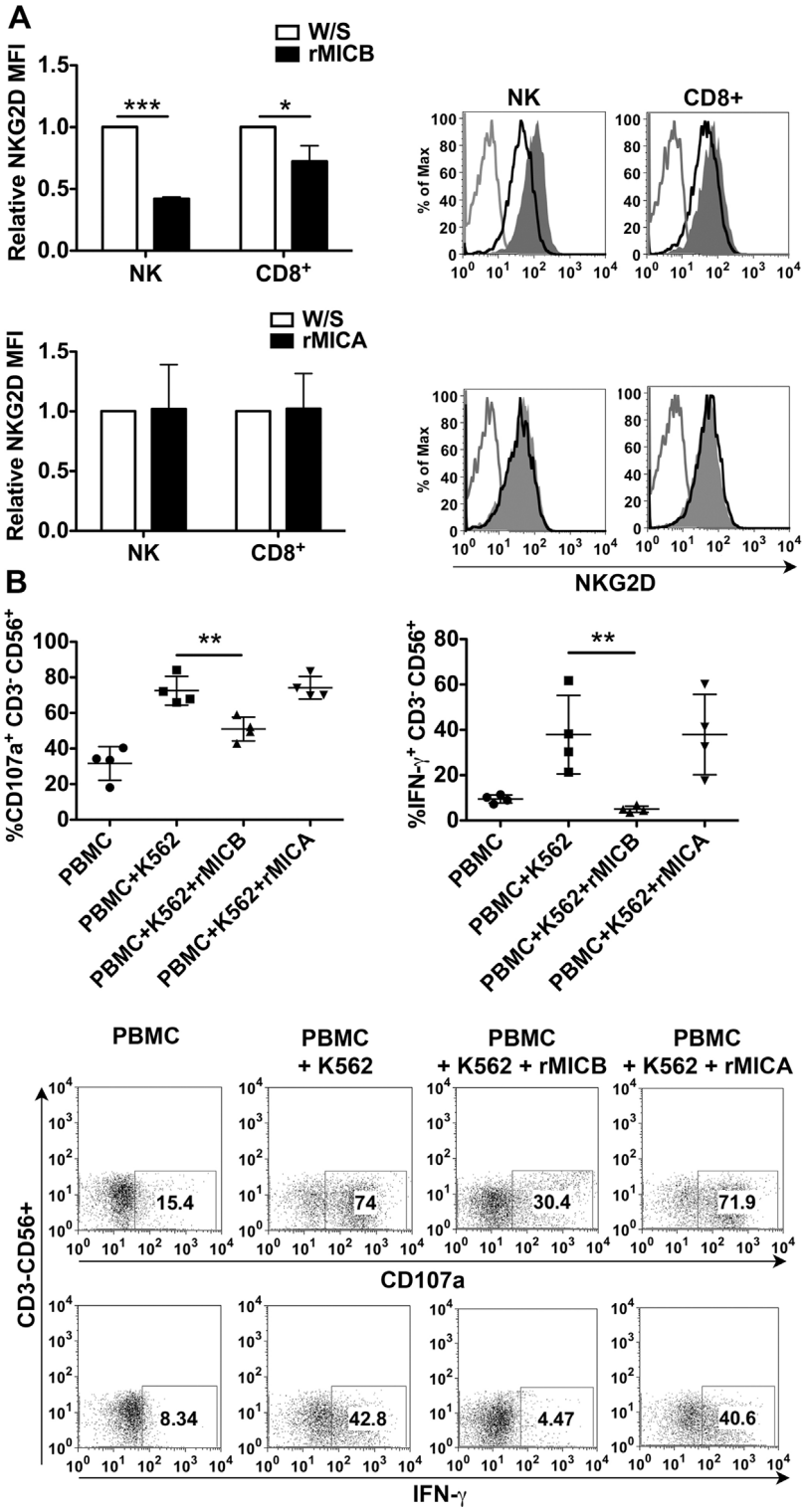

Fig. 5. rMICB decreases NKG2D receptor expression on $CD56^+CD3^-$ lymphocytes, affecting their cytotoxicity. PBMC from healthy donors was incubated with rMICB or rMICA for 24 h. (A) NKG2D receptor surface expression was assessed by flow cytometry. Graphs represent experiments performed with 3 different donors. Representative histograms show cells incubated with rMICB or rMICA (black line), cells incubated with vehicle alone (grey solid histogram), and the isotype control (grey line). (B) PBMC previously incubated with the recombinant proteins was co-cultured with the K562 cells for 5 h and levels of CD107 and IFN-γ were analyzed on $CD3^-CD56^+$ cells. Representative dot plots are shown, and graphs represent experiments carried out with 4 different donors. W/S, without stimulus. All determinations were made in triplicates. Data are presented as mean values ± S.D. The two-tailed paired *t*-test and one-way ANOVA with Dunnett's multiple comparison test were used for statistical analysis for A and B, respectively. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

2007), these results may seem contradictory, as the release of soluble NKG2DL by STAT3 inhibition is also associated with immune evasion mechanisms. STAT3 activation presents an inhibitory effect in MICB expression, which is in line with its role in tumor-induced immunosuppression. On the other hand, increased expression of MICA or MICB has also been associated with an immune evasion strategy. For instance, gastric cancer patients with large tumors that express MICA and MICB present a significant lower survival rates than patients bearing tumors that do not express any of these ligands (Ribeiro et al., 2016). Therefore, the role of STAT3 in MICB expression and its impact on cancer

immunosurveillance demonstrates such complexity that it requires further investigation. In addition, several studies have shown a role for ADAM10 and ADAM17 in the proteolytic shedding of MICA and MICB from tumor cells (Salih et al., 2006; Salih et al., 2002; Boutet et al., 2009; Chitadze et al., 2013; Kohga et al., 2010). Nevertheless, when gastric adenocarcinoma cells were treated with broad spectrum metalloprotease inhibitors to ADAM10 and ADAM17, sMICB levels in the supernatant did not change, suggesting that an enzyme, other than ADAM10 and/or ADAM17, is responsible for MICB cleavage from the gastric adenocarcinoma cell lines studied. On the other hand, the release of sMICA from LNCaP cells decreased significantly in the presence of these inhibitors, implying distinct mechanisms in the generation of soluble MICA and MICB in these cells.

We demonstrated that rMICB significantly decreases NKG2D expression on NK and $CD8^+$ T cells. On the other hand, we did not detect a variation on NKG2D receptor levels upon cell incubation with rMICA. In addition, the functional effect of soluble rMICB on cytolytic cells was analyzed by using the recombinant protein, which compromised NK cell function, as evidenced by a significant decrease in CD107a and IFN-γ levels on these effector cells. These results could be attributed to the decrease on the expression of NKG2D receptor on effector cell surface induced by rMICB. However, rMICA did not change the levels of CD107a or IFN-γ on NK cells, which is in line with the results observed in Fig. 5A, in which rMICA treatment did not induce a decrease in the levels of cell surface expression of NKG2D receptor on NK cells. The discrepancy between our results and previous studies may be explained by the different experimental conditions, the origin of soluble recombinant proteins, and polymorphisms of these molecules, among other factors (Salih et al., 2006; Groh et al., 2002).

In summary, our data provide evidence supporting a role for active STAT3 in the downmodulation of cell surface expression and soluble MICB release by gastric adenocarcinoma cells. This work also reveals an inhibitory effect of recombinant MICB on NK cells cytotoxic functions. These results increase the understanding of the regulation of MICB and the role that this soluble molecule may play in NK recognition of tumor cells.

Conflict of interest

None of the authors participating in the present work has any conflict of interest.

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References

Bauer, S., Groh, V., Wu, J., Steinle, A., Phillips, J.H., Lanier, L.L., Spies, T., 1999. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science 285, 727.

Bedel, R., Thiery-Vuillemin, A., Grandclement, C., Balland, J., Remy-Martin, J.P., Kantelip, B., Pallandre, J.R., Pivot, X., Ferrand, C., Tiberghien, P., Borg, C., 2011. Novel role for STAT3 in transcriptional regulation of NK immune cell targeting receptor MICA on cancer cells. Cancer Res. 71, 1615.

Bharadwaj, U., Eckols, T.K., Kolosov, M., Kasembeli, M.M., Adam, A., Torres, D., Zhang, X., Dobrolecki, L.E., Wei, W., Lewis, M.T., Dave, B., Chang, J.C., Landis, M.D., Creighton, C.J., Mancini, M.A., Tweardy, D.J., 2015. Drug-repositioning screening identified piperlongumine as a direct STAT3 inhibitor with potent activity against breast cancer. Oncogene 34, 1341.

Boutet, P., Aguera-Gonzalez, S., Atkinson, S., Pennington, C.J., Edwards, D.R., Murphy, G., Reyburn, H.T., Vales-Gomez, M., 2009. Cutting edge: the metalloproteinase ADAM17/TNF-alpha-converting enzyme regulates proteolytic shedding of the MHC class I-related chain B protein. J. Immunol. 182, 49.

Chitadze, G., Lettau, M., Bhat, J., Wesch, D., Steinle, A., Furst, D., Mytilineos, J., Kalthoff, H., Janssen, O., Oberg, H.H., Kabelitz, D., 2013. Shedding of endogenous MHC class I-related chain molecules A and B from different human tumor entities: heterogeneous involvement of the a disintegrin and metalloproteases 10 and 17. Int. J. Cancer 133, 1557.

de Kruijf, E.M., Sajet, A., van Nes, J.G., Putter, H., Smit, V.T., Eagle, R.A., Jafferji, I., Trowsdale, J., Liefers, G.J., van de Velde, C.J., Kuppen, P.J., 2012. NKG2D ligand tumor expression and association with clinical outcome in early breast cancer patients: an observational study. BMC Cancer 12, 24.

Duan, X., Deng, L., Chen, X., Lu, Y., Zhang, Q., Zhang, K., Hu, Y., Zeng, J., Sun, W., 2011. Clinical significance of the immunostimulatory MHC class I chain-related molecule A and NKG2D receptor on NK cells in pancreatic cancer. Med. Oncol. 28, 466.

Groh, V., Wu, J., Yee, C., Spies, T., 2002. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. Nature 419, 734.

He, S., Liao, G., Liu, Y., Huang, L., Kang, M., Chen, L., 2015. Overexpression of STAT3/pSTAT3 was associated with poor prognosis in gastric cancer: a meta-analysis. Int. J. Clin. Exp. Med. 8, 20014.

Hilpert, J., Grosse-Hovest, L., Grunebach, F., Buechele, C., Nuebling, T., Raum, T., Steinle, A., Salih, H.R., 2012. Comprehensive analysis of NKG2D ligand expression and release in leukemia: implications for NKG2D-mediated NK cell responses. J. Immunol. 189, 1360.

Holdenrieder, S., Stieber, P., Peterfi, A., Nagel, D., Steinle, A., Salih, H.R., 2006a. Soluble MICA in malignant diseases. International journal of cancer. J. Int. Cancer 118, 684.

Holdenrieder, S., Stieber, P., Peterfi, A., Nagel, D., Steinle, A., Salih, H.R., 2006b. Soluble MICB in malignant diseases: analysis of diagnostic significance and correlation with soluble MICA. Cancer Immunol. Immunother.: CII 55, 1584.

Huergo-Zapico, L., Acebes-Huerta, A., Lopez-Soto, A., Villa-Alvarez, M., Gonzalez-Rodriguez, A.P., Gonzalez, S., 2014. Molecular Bases for the Regulation of NKG2D Ligands in Cancer. Front. Immunol. 5, 106.

Ji, K., Zhang, L., Zhang, M., Chu, Q., Li, X., Wang, W., 2016. Prognostic value and clinicopathological significance of p-stat3 among gastric carcinoma patients: a systematic review and meta-Analysis. Medicine (Baltimore) 95, e2641.

Jinushi, M., Takehara, T., Tatsumi, T., Hiramatsu, N., Sakamori, R., Yamaguchi, S., Hayashi, N., 2005. Impairment of natural killer cell and dendritic cell functions by the soluble form of MHC class I-related chain A in advanced human hepatocellular carcinomas. J. Hepatol. 43, 1013.

Johnston, P.A., Grandis, J.R., 2011. STAT3 signaling: anticancer strategies and challenges. Mol. Interv. 11, 18.

Kanda, N., Seno, H., Konda, Y., Marusawa, H., Kanai, M., Nakajima, T., Kawashima, T., Nanakin, A., Sawabu, T., Uenoyama, Y., Sekikawa, A., Kawada, M., Suzuki, K., Kayahara, T., Fukui, H., Sawada, M., Chiba, T., 2004. STAT3 is constitutively activated and supports cell survival in association with survivin expression in gastric cancer cells. Oncogene 23, 4921.

Kim, D.Y., Cha, S.T., Ahn, D.H., Kang, H.Y., Kwon, C.I., Ko, K.H., Hwang, S.G., Park, P.W., Rim, K.S., Hong, S.P., 2009. STAT3 expression in gastric cancer indicates a poor prognosis. J. Gastroenterol. Hepatol. 24, 646.

Kohga, K., Takehara, T., Tatsumi, T., Miyagi, T., Ishida, H., Ohkawa, K., Kanto, T., Hiramatsu, N., Hayashi, N., 2009. Anticancer chemotherapy inhibits MHC class I-related chain a ectodomain shedding by downregulating ADAM10 expression in hepatocellular carcinoma. Cancer Res. 69, 8050.

Kohga, K., Takehara, T., Tatsumi, T., Ishida, H., Miyagi, T., Hosui, A., Hayashi, N., 2010. Sorafenib inhibits the shedding of major histocompatibility complex class I-related chain A on hepatocellular carcinoma cells by down-regulating a disintegrin and metalloproteinase 9. Hepatology 51, 1264.

McGilvray, R.W., Eagle, R.A., Rolland, P., Jafferji, I., Trowsdale, J., Durrant, L.G., 2010. ULBP2 and RAET1E NKG2D ligands are independent predictors of poor prognosis in ovarian cancer patients. Int. J. Cancer 127, 1412.

Moore, K.W., de Waal Malefyt, R., Coffman, R.L., O'Garra, A., 2001. Interleukin-10 and the interleukin-10 receptor. Annu. Rev. Immunol. 19, 683.

Mora, L.B., Buettner, R., Seigne, J., Diaz, J., Ahmad, N., Garcia, R., Bowman, T., Falcone, R., Fairclough, R., Cantor, A., Muro-Cacho, C., Livingston, S., Karras, J., Pow-Sang, J., Jove, R., 2002. Constitutive activation of Stat3 in human prostate tumors and cell lines: direct inhibition of Stat3 signaling induces apoptosis of prostate cancer cells. Cancer Res. 62, 6659.

Palagani, V., Bozko, P., El Khatib, M., Belahmer, H., Giese, N., Sipos, B., Malek, N.P., Plentz, R.R., 2014. Combined inhibition of Notch and JAK/STAT is superior to monotherapies and impairs pancreatic cancer progression. Carcinogenesis 35, 859.

Raulet, D.H., 2003. Roles of the NKG2D immunoreceptor and its ligands. Nat. Rev. Immunol. 3, 781.

Ribeiro, C.H., Kramm, K., Galvez-Jiron, F., Pola, V., Bustamante, M., Contreras, H.R., Sabag, A., Garrido-Tapia, M., Hernandez, C.J., Zuniga, R., Collazo, N., Sotelo, P.H., Morales, C., Mercado, L., Catalan, D., Aguillon, J.C., Molina, M.C., 2016. Clinical significance of tumor expression of major histocompatibility complex class I-related chains A and B (MICA/B) in gastric cancer patients. Oncol. Rep. 35 (1309).

Salih, H.R., Rammensee, H.G., Steinle, A., 2002. Cutting edge: down-regulation of MICA on human tumors by proteolytic shedding. J. Immunol. 169, 4098.

Salih, H.R., Goehlsdorf, D., Steinle, A., 2006. Release of MICB molecules by tumor cells: mechanism and soluble MICB in sera of cancer patients. Hum. Immunol. 67, 188.

Salih, H.R., Holdenrieder, S., Steinle, A., 2008. Soluble NKG2D ligands: prevalence, release, and functional impact. Front. Biosci. 13, 3448.

Serrano, A.E., Menares-Castillo, E., Garrido-Tapia, M., Ribeiro, C.H., Hernandez, C.J., Mendoza-Naranjo, A., Gatica-Andrades, M., Valenzuela-Diaz, R., Zuniga, R., Lopez, M.N., Salazar-Onfray, F., Aguillon, J.C., Molina, M.C., 2011. Interleukin 10 decreases MICA expression on melanoma cell surface. Immunol. Cell Biol. 89, 447.

Shafi, S., Vantourout, P., Wallace, G., Antoun, A., Vaughan, R., Stanford, M., Hayday, A.,

2011. An NKG2D-mediated human lymphoid stress surveillance response with high interindividual variation. Sci. Transl. Med. 3, 113ra124.
Song, H., Wang, R., Wang, S., Lin, J., 2005. A low-molecular-weight compound discovered through virtual database screening inhibits Stat3 function in breast cancer cells. Proc. Natl. Acad. Sci. U. S. A. 102, 4700.
Stern-Ginossar, N., Gur, C., Biton, M., Horwitz, E., Elboim, M., Stanietsky, N., Mandelboim, M., Mandelboim, O., 2008. Human microRNAs regulate stress-induced immune responses mediated by the receptor NKG2D. Nat. Immunol. 9, 1065.
Tsukerman, P., Stern-Ginossar, N., Gur, C., Glasner, A., Nachmani, D., Bauman, Y., Yamin, R., Vitenshtein, A., Stanietsky, N., Bar-Mag, T., Lankry, D., Mandelboim, O., 2012. MiR-10b downregulates the stress-induced cell surface molecule MICB, a critical ligand for cancer cell recognition by natural killer cells. Cancer Res. 72, 5463.
Waldhauer, I., Goehlsdorf, D., Gieseke, F., Weinschenk, T., Wittenbrink, M., Ludwig, A., Stevanovic, S., Rammensee, H.G., Steinle, A., 2008. Tumor-associated MICA is shed by ADAM proteases. Cancer Res. 68, 6368.
Wang, H., Yang, D., Xu, W., Wang, Y., Ruan, Z., Zhao, T., Han, J., Wu, Y., 2008. Tumor-derived soluble MICs impair CD3(+)CD56(+) NKT-like cell cytotoxicity in cancer patients. Immunol. Lett. 120, 65.
Watson, N.F., Spendlove, I., Madjd, Z., McGilvray, R., Green, A.R., Ellis, I.O., Scholefield, J.H., Durrant, L.G., 2006. Expression of the stress-related MHC class I chain-related protein MICA is an indicator of good prognosis in colorectal cancer patients. Int. J. Cancer 118, 1445.
Wu, J.D., Higgins, L.M., Steinle, A., Cosman, D., Haugk, K., Plymate, S.R., 2004. Prevalent expression of the immunostimulatory MHC class I chain-related molecule is counteracted by shedding in prostate cancer. J. Clin. Invest. 114, 560.
Xu, Y.H., Lu, S., 2014. A meta-analysis of STAT3 and phospho-STAT3 expression and survival of patients with non-small-cell lung cancer. Eur. J. Surg. Oncol. 40, 311.
Yu, H., Kortylewski, M., Pardoll, D., 2007. Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. Nat. Rev. Immunol. 7, 41.
Yu, S., Li, G., Wang, Z., Wang, Z., Chen, C., Cai, S., He, Y., 2016. The prognostic value of pSTAT3 in gastric cancer: a meta-analysis. J. Cancer Res. Clin. Oncol. 142, 649.