Novel Role for STAT3 in Transcriptional Regulation of NK Immune Cell Targeting Receptor MICA on Cancer Cells

Romain Bedel1,2,3, Antoine Thiery-Vuillemin1,2,4, Camille Grandclement1,2,3, Jeremy Balland1,2,3, Jean-Paul Remy-Martin1,2,3, Bernadette Kantelip5, Jean-René Pallandre1,2,3, Xavier Pivot1,2,4, Christophe Ferrand1,2,3, Pierre Tiberghien1,2,3, and Christophe Borg1,2,3,4

Abstract

The role of natural killer group 2, member D receptor (NKG2D)–expressing natural killer (NK) cells in tumor immunosurveillance, leading to cancer persistence in immunocompetent hosts. STAT3 plays a pivotal role both in oncogenic functions and in immunosuppression. In this study, we investigated the role of STAT3 in suppressing NK cell–mediated immunosurveillance. Using a colorectal cancer cell line (HT29) that can poorly activate NK, we neutralized STAT3 with pharmacologic inhibitors or siRNA and found that this led to an increase in NK cell-mediated immunosurveillance. On this basis, we investigated the expression of NKG2D ligands after STAT3 activation in HT29, mesenchymal stem cells, and activated lymphocytes. The NK cell recognition receptor MHC class I chain–related protein A (MICA) was upregulated following STAT3 neutralization, and a direct interaction between STAT3 and the MICA promoter was identified. Because cross-talk between DNA damage repair and NKG2D ligand expression has been shown, we assessed the influence of STAT3 on MICA expression under conditions of genotoxic stress. We found that STAT3 negatively regulated MICA expression after irradiation or heat shock, including in lymphocytes activated by CD3/CD28 ligation. Together, our findings reveal a novel role for STAT3 in NK cell immunosurveillance by modulating the MICA expression in cancer cells. Cancer Res; 71(5); 1615–26. ©2011 AARC.

Introduction

The immune system can detect and suppress emerging tumors (1). In addition to their role in pathogen immunity, natural killer (NK) cells have been implicated in tumor surveillance in both mice and human models (1–6). Among NK–activating receptors, natural killer group 2, member D receptor (NKG2D) is a C-type lectin-like transmembrane glycoprotein recognizing self-molecules (referred as NKG2D ligands; NKG2DLs) that emerged as a pivotal signaling pathway supporting cancer immunosurveillance. Indeed, transfect cell lines expressing NKG2DLs are rejected in vivo in an NKG2D-dependent manner (7, 8).

Recently, generation of NKG2D-deficient mice confirmed the critical role of these stimulatory NK receptors in immunosurveillance of spontaneous prostate cancer and lymphoma models (9). Contrary to prostate cancer arising in NKG2D-deficient mice, tumor cells isolated from fast-growing carcinoma in control mice (and not in smaller, late-arising tumors) lacked NKG2DLs, suggesting an NKG2D-dependent immunnoediting (9) and supporting the hypothesis that oncogenic pathways associated with cancer progression might negatively regulate NKG2DLs.

MHC class I chain–related A and B (MICA and MICB) or UL16 binding proteins (ULBP; refs. 7, 10, 11) are NKG2DLs, weakly expressed on normal cells and upregulated in cancers (12–16). Nonetheless, molecular mechanisms leading to NKG2DL regulation are poorly defined.

The enhanced incidence of colorectal cancer (CRC) in patients affected by inflammatory bowel disease (IBD) had established chronic inflammation as a cornerstone mechanism in tumor suppressor checkpoint subversion (17, 18). Particularly, interleukin 23 (IL-23) was shown to increase tumor incidence in mice (19) and to decrease cancer immunosurveillance through STAT3 (20), which is a transcription factor activated in IBD (21, 23) and directly involved both in intestinal inflammation and in cancer progression (24, 25).

In this study, we aimed to investigate the role of STAT3 activation in the regulation of NKG2DL expression and...
recognition of tumor cells by NK cells. We showed that STAT3 ablation in tumor cells modulates NK2D-mediated NK cell activation. STAT3 directly interacts with MICA promoter to repress MICA transcription. These results shed light on the negative regulation exerted by STAT3 on MICA expression in different cell types submitted to DNA damage or cellular stress.

Materials and Methods

Reagents
The following antibodies were used: anti-human MICA (BZ-26; Diaclone); anti-human CD107a (LAMP-1; HA43), isotype PE (MOPC-21; BD Biosciences); anti–MICA-PE (2C10), MICB (9847-1), ULBP1 (Z-9), ULBP2 (F16), ULBP3 (2F9), TGF-β1 (C-16; Santa Cruz Biotechnology), anti-human STAT3 (79D7) and phospho-STAT3 (Tyr705, 3E2; Cell Signaling), neutralizing anti-MICA (clone 159227; RnDsystems). STA-21, a selective inhibitor of STAT3, was purchased from BIOMOL International. Oncostatin M (PeproTech) was used in some experiments. The TGF-β1 receptor inhibitor SB-431542 (Tocris Biosciences) was used in some experiments. Dynabeads Human T-Expander CD3CD28 (Invitrogen) were used for stimulation of peripheral blood lymphocytes (PBL).

Cell lines and primary cells cultures
HT29 (ATCC, HTB-38), SW620 (ATCC, CCL-227), Colo320 (ATCC CCL-220), K562 (ATCC CCL-243), MDA-MB231 (ATCC HTB26), U87 (ATCC HTB-14), and 293T (DSMZ ACC-635) cells were verified by morphology, tested for Mycoplasma, and conserved in master cell bank on reception. Cells were never used above passage 10. The stroma cell line SV56 was established as previously described (26). Cells were maintained in either RPMI 1640 (K562 and SV56) or Dulbecco’s modiﬁed Eagle’s medium (DMEM; HT29, SW620, and Colo320; Lonza) supplemented with 10% fetal calf serum (Invitrogen). NK cells were purified from blood by magnetic selection (Stem Cell). The purity of CD56/CD3 NK cells was assessed by flow cytometry and ranged from 90% to 98%. NK cells were activated for 24 hours with IL-2 (1,000 UI/mL) and IL-12 (100 UI/mL) and then treated with 1 mol/L glycine for 5 minutes at room temperature and then treated with 1 mol/L glycine for 5 minutes at room temperature. Cells were harvested and after 2 washing steps with ice-cold PBS, lysed in 500 μL of lysis buffer [50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% (v/v) NP40, 0.5% (m/v) Na deoxycholate, 2 mmol/L EDTA, 2 mmol/L NaF, 1 mmol/L vanadate, proteases inhibitor mixture]. A total of 200- to 1,000-bp DNA fragments were generated with 5 times

RNA silencing and plasmids constructs
Specific STAT3 siRNA (sense, 5′-AAAGAACTCTAGAC-CCGTCACAAAC-3′; antisense, 5′-AAATTTGTTGACCGGTT-CTGAAAGTT-3′) and scramble siRNA (sense, 5′-AAAGGAGG-GGCATGCAGCCGTT-3′; antisense, 5′-AAACCAACGTGG-CATGCCCCCT-3′) sequences were annealed, and cloned into the BbsI site of the 3′-LTR of pFIV-H1/U6 vector according to manufacturer’s instructions (System Biosciences). Lentiviral supernatant production and subsequent infection of cell lines were realized according to manufacturer’s instructions. Human STAT3C in pBabe vector was provided by Dr. J. Bromberg (27). pGL3-MICA-pro vector was previously described and kindly given by Dr. Jack D. Bui (28).

Site-directed mutagenesis
STAT3 binding site-directed mutagenesis was done according to manufacturer’s protocol (QuikChange II XL Site-Directed Mutagenesis Kit; Stratagene). Four base pairs within the STAT3 binding site were predicted to disrupt STAT3 binding, when mutated, without introducing or removing other binding sites. These changes were as follows: (T/C) (T/G) (C/A) (T/T) turning the normal TTCCCTTCCAGGAC STAT3 consensus binding sequence into TTCCCGATAGGAC. Two primers were designed to generate the mutated STAT3 binding site in the MICA promoter region of the pGL3-MICA vector. The sequences were the following: MutA-MICA-sense, 5′-cgtggctgtgtcagaaagaacagagctg-3′; MutA-MICA-antisense, 5′-cactggttgcttcacctagacagacacgcg-3′.

Real-time quantitative PCR
Total RNA was extracted using RNeasy Mini Kit (Qiagen) and reverse transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase (Life Technologies). Duplicate samples were subjected to real-time quantitative PCR (RT-qPCR). mRNAs were quantified using primers listed as follows: MICA (Hs00792193_m1; Applied Biosystems). ABL mRNA from each sample was quantified as an endogenous control. Relative mRNA expression was calculated using the ΔΔCt method, and untreated cells were used as the calibrator.

Luciferase assay
HT29 and 293T cells were transfected using Lipofectamine LTX (Invitrogen). In all conditions, Renilla luciferase (pRL-TK) and firefly luciferase (triggered by MICA or mutated-MICA promoters in pGL3-MICA vectors) were cotransfected. Firefly luciferase light values were divided by Renilla luciferase light values.

ELISA
IFN-γ was detected using commercial ELISA kits (Diacline). The sensitivity of the human IFN-γ kit was 4.7 pg/mL. MICA was detected using ELISA kits (Diacline). The sensitivity of the human MICA ELISA kit was 123 pg/mL. All concentrations are expressed as mean ± SEM of triplicates.

NK degranulation assay
NK cells were activated for 24 hours with IL-2 (1,000 UI/mL) and then cocultured in the presence of target cells for 4 hours at 10:1 E:T ratio. Degranulation of NK cells was analyzed by flow cytometric analysis of CD107a expression as previously described (29).

Chromatin immunoprecipitation assay
HT29 or 293T cells (5 × 10⁶) were cross-linked with 1% formaldehyde in the presence of protease inhibitors (Complete Mini EDTA Free; Roche) for 15 minutes at room temperature and then treated with 1 mol/L glycine for 5 minutes at room temperature. Cells were harvested and after 2 washing steps with ice-cold PBS, lysed in 500 μL of lysis buffer [50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% (v/v) NP40, 0.5% (m/v) Na deoxycholate, 2 mmol/L EDTA, 2 mmol/L NaF, 1 mmol/L vanadate, proteases inhibitor mixture]. A total of 200- to 1,000-bp DNA fragments were generated with 5 times
10-second sonication, using a Vibra Cell sonicator (Sonics & Materials). An aliquot of 100 μL was conserved (total input). Chromatin was immunoprecipitated overnight at 4°C with anti-human STAT3 (clone 79D7) or control rabbit immunoglobulin G (IgG). After a 2 hours incubation with Dynabeads Protein G (Invitrogen), beads were washed twice with wash buffer 1 (0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris, 150 mmol/L NaCl) and then submitted to another washing step with wash buffer 2 (0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris, 500 mmol/L NaCl) and finally 2 washing steps with TE buffer. Beads (and the total input DNA) were subsequently incubated at 65°C overnight to reverse cross-linking. Incubation with Proteinase K (Invitrogen) for 30 minutes at 55°C was done, and DNA samples were purified using QiAmp DNA Mini Kit (Qiagen), collected in 200 μL TE buffer and then assessed by PCR.

Statistical analysis
Results are expressed as the mean ± SEM. Group comparisons were done using Student’s t test. Differences were considered significant at P < 0.05.

Results
Role of STAT3 in colon cancer cell line susceptibility to NK cells
The implication of STAT3 in chronic intestinal inflammation and cancer oncogenesis prompted us to investigate the ability of different colon cancer cell lines to activate NK cells (30–33). For this purpose, NK cells were purified from PBL of normal volunteers and incubated with colon cancer cell lines or with the NK-sensitive K562 cell line for 24 hours. These preliminary experiments indicated that HT29 is a weak activator of NK cell functions in vitro compared with Colo320, SW620, and K562 (Fig. 1A and B). The higher expression of STAT3 in HT29 than in K562, Colo320, or SW620 (Fig. 1C) prompted us to investigate the precise role of STAT3 in the recognition of HT29 by NK cells.

To confirm the influence of STAT3 in CRC models, we generated STAT3-deficient cell lines by lentivirus-mediated gene transfer to deliver a specifically designed siRNA for STAT3 into HT29 and to produce a stable cell line (HT29siRNA-STAT3). Western blot analysis confirmed a reduced expression of STAT3 in the knockdown cell line HT29siRNA-STAT3 (Fig. 2A). Moreover, the level of phospho-STAT3 also decreased in HT29siRNA-STAT3 (Fig. 2B).

The next set of experiments was dedicated to assess whether the presence of STAT3 influences HT29 recognition by NK cells. Freshly purified NK cells were cocultured for 24 hours with HT29, HT29siRNA-CTRL, or HT29siRNA-STAT3 and harvested supernatants were then assessed for IFN-γ production (Fig. 2C). HT29siRNA-STAT3 triggered a significantly increased secretion of IFN-γ by NK cells compared with HT29siRNA-CTRL or HT29 (1.276 ± 82 pg/mL vs. 510 ± 2 pg/mL and 462 ± 38 pg/mL). To further study STAT3 implication on NK functions, we assessed NK degranulation according to STAT3 expression in HT29. Direct CD107a staining was done to reveal NK degranulation against HT29, HT29siRNA-CTRL, or HT29siRNA-STAT3 (Fig. 2D). We observed an increase in CD107a expression in cocultures done with HT29siRNA-STAT3 compared with HT29siRNA-CTRL, suggesting the active degradation of NK in the condition in which STAT3 is repressed (42% vs. 16% and 14% for HT29 and HT29siRNA-CTRL, respectively). Overall, we can hypothesize that STAT3 is implicated in HT29-altered sensitivity to NK functions.

STAT3 inhibition of HT29 recognition by NK cells involves a TGF-β1–independent mechanism
NK functions result from the integration of activating and inhibitory signaling related to different ligands recognized on target cells. TGF-β1, a cytokine produced by HT29 (34), has been previously characterized as a major inhibitory pathway limiting tumor cell recognition by NK cells (35). Moreover, we and others have shown that STAT3 can directly trigger TGF-β1 transcription (36, 37). Therefore, the expression of TGF-β1 in HT29siRNA-CTRL and HT29siRNA-STAT3 was analyzed by flow cytometry (Fig. 3A). We noticed a decreased expression of TGF-β1 in HT29siRNA-STAT3 compared with HT29siRNA-CTRL. The quantification of TGF-β1 by RT-qPCR supported previous results at the protein level (Fig. 3B). Indeed, TGF-β1 mRNA was repressed in HT29siRNA-STAT3, suggesting a direct link between STAT3 activity and TGF-β1 expression. As a consequence, we hypothesized that STAT3 activation in HT29 results in a constitutive inhibitory signal for NK cells. Therefore, the presence of a STAT3-dependent TGF-β1 expression in HT29 led us to investigate whether TGF-β1 signaling pathway accounted for STAT3-mediated inhibition of HT29 recognition by NK cells. For this purpose, freshly purified NK cells were cocultured with HT29siRNA-CTRL and HT29siRNA-STAT3 for 24 hours in the presence or absence of a specific pharmacologic inhibitor of TGF-β1 receptor, SB-431542 (38). SB-431542 treatment during 24 hours abrogated Smad2/3 phosphorylation, prevented TGF-β1 inhibition of IL-2–activated peripheral blood cell (data not shown), and did not influence NK cell–activating receptor expression (Supplementary Fig. 1). As shown previously, we observed a significant increase in IFN-γ production when we compared HT29siRNA-STAT3 with HT29siRNA-CTRL. TGF-β1 inhibition resulted in a weak upregulation of NK activation. In contrast, we could observe that STAT3 neutralization mediated by siRNA improved NK recognition of HT29 even when TGF-β1 signaling was blocked (Fig. 3C). These results suggest that TGF-β1 and STAT3 act independently to repress NK recognition of HT29. As NK activation is known to be the result of an integration of positive and negative signaling pathways following target cell recognition, it is plausible that in the absence of TGF-β1–mediated inhibitory signaling, STAT3-specific inhibition promotes the expression of NK-activating ligands on HT29.

STAT3 knockdown restores NKG2DL expression
Rault and colleagues showed an increased tumor incidence in NKG2D−/− mice (9). As a consequence, avoidance of this specific NKG2D/NKG2DL pathway is a hallmark of many malignancies against NK immunity (28, 39, 40). Consequently, we choose to determine whether STAT3 was implicated in NKG2D-based tumor cell recognition. To clarify this point,
previous experiments were reproduced in the presence of neutralizing anti-NKG2D or IgG control antibodies. NK cells were pretreated for 30 minutes at 37°C with blocking antibodies and then cocultured with HT29siRNA-CTRL or HT29siRNA-STAT3 for 24 hours and harvested supernatants were assessed for IFN-γ production. As shown in Figure 4A (left), IFN-γ production by NK cells in the coculture with HT29siRNA-STAT3 was significantly decreased in the presence of NKG2D neutralization while not affected by control antibodies. Of note, NKG2D blockade reduced NK degranulation against HT29 (Fig. 4A, right). The magnitude of this inhibition by anti-NKG2D was significantly higher in the presence of specific STAT3 siRNA (Fig. 4A, right).

These results strongly suggested a role for NKG2DLs in the NK activation function conferred by STAT3 inhibition in HT29. Subsequently, expression of NKG2DLs was analyzed by flow cytometry in HT29siRNA-CTRL and HT29siRNA-STAT3 (Fig. 4B). Although MICA expression was influenced by STAT3 modulation, we failed to identify any variation in either MICB or ULBPs in HT29siRNA-CTRL and HT29siRNA-STAT3. To confirm this hypothesis, we carried out Western blotting analysis on total protein extracts from HT29, HT29siRNA-CTRL, and HT29siRNA-STAT3 to control the presence of MICA. These experiments revealed a marked increase in MICA protein in HT29siRNA-STAT3 (Fig. 4C).

Several authors mentioned that the shedding of MICA and the release of soluble protein (sMICA) are thought to promote tumor evasion (41, 42). Consequently, we assessed the supernatants coming from HT29siRNA-CTRL and HT29siRNA-STAT3 cultures by ELISA and did not observe a significant difference in sMICA quantified in both conditions (Supplementary Fig. 2). Thereafter, we decided to assess whether the correlation between the abrogation of STAT3 signaling and enhancement in MICA expression was detectable at the transcriptional level. RT-qPCR analyses were done on total mRNA extracts from HT29 cells treated with the STAT3 pharmacologic inhibitor STA21 at different time points (Fig. 4D). We detected a 9-fold increase in MICA mRNA expression in the presence of STA21. Similar results were obtained using the JAK2-specific inhibitor AG490 that predominantly repress STAT3 activity. These results show an inverse correlation between STAT3 activity and MICA expression.

Direct influence of STAT3 on MICA transcription

To extend previous results, we selected 2 tumor cell lines U87 and MDA-MB231 constitutively expressing an active form of STAT3 (Fig. 5A). MICA expression increased in U87 and MDA-MB231 cells treated with STA21 for 48 hours (Fig. 5B). The functional significance of STAT3 in tumor cell recognition by NK cells was studied in the coculture of NK with HT29, U87, and MDA-MB231 cells previously
treated during 48 hours with 2 different STAT3 pharmacologic inhibitors. These experiments confirmed that treatment of tumor cells with either STA21 or AG490 enhances NK IFN-γ production (Fig. 5C). As previous observations revealed that STAT3 influence seems to be restricted to MICA expression, we added anti-MICA–neutralizing antibodies in coculture experiments. Interestingly, MICA blockade decreased the IFN-γ production observed in NK cocultures with HT29, U87, or MDA-MB231 previously treated with STA21 or AG490 (Fig. 5C). To confirm the direct influence of STAT3 on MICA transcription in tumor cells, a pGL3-MICA vector containing the luciferase gene under the control of the 1-kb MICA promoter was transfected in HT29, U87, and MDA-MB231. Pharmacologic inhibition of STAT3 in all transfected cell lines resulted in an enhanced MICA promoter activity (Fig. 5D).

Figure 2. STAT3 knockdown restores NK cell activation by HT29. A, Western blot analysis for STAT3 on whole cell extracts from HT29, HT29siRNA-CTRL, or HT29siRNA-STAT3. β-Actin was used as a control of protein loading (20 μg per lane; representative experiment of n = 3). B, flow cytometric analysis of STAT3 and phospho-STAT3 expression by HT29siRNA-CTRL or HT29siRNA-STAT3. Gray and white histograms represent isotype or STAT3 staining, respectively. C, IFN-γ concentration assessed by ELISA of freshly purified NK cells cocultured with HT29, HT29siRNA-CTRL, or HT29siRNA-STAT3 for 24 hours in DMEM medium at E:T ratio (10:1). D, flow cytometric analysis of CD107a expression on IL-2–activated NK cells cocultured for 4 hours with HT29, HT29siRNA-CTRL, or HT29siRNA-STAT3 at E:T ratio (10:1; representative blot of n = 5). Bars, SD. *, P < 0.05.
STAT3 directly interacts with specific binding sites in MICA promoter

To exert its action as a transcriptional factor, STAT3 forms a cytoplasm homodimer, translocates into the nucleus, and interacts with functional transcription factor binding sites (TFBS) in the promoter of regulated genes. We sought to investigate the presence of STAT3 TFBSs in the MICA promoter to further elucidate the mechanisms governing previous observations. We used the predictive software MatInspector for TFBSs and found a significant match \((5\textsuperscript{\prime}-\text{TTC}T\text{TTC}C\text{AGG}C\text{AGACA}-3\textsuperscript{\prime})\) for a more recently discovered sequence (core nucleotide is underlined: TTCNGG; ref. 43). Complementary analyses were realized on MICB and ULBPs promoter sequences as well. We obtained specific sequences for MICB \((3\textsuperscript{\prime}-\text{TTC}T\text{TTC}T\text{GGG}C\text{AG}C\text{AGACS}-5\textsuperscript{\prime})\) and ULBP2 \((3\textsuperscript{\prime}-\text{CAT}C\text{TTC}\text{AGG}C\text{TCTC}C-5\textsuperscript{\prime})\) promoters, whereas no specific matches were retrieved from ULBP1 and ULBP3 promoter investigations (Supplementary Data 1 and 2). We initiated a chromatin immunoprecipitation (ChIP) assay to control whether STAT3 could indeed bind the TFBS given by MatInspector. HT29 cells were fixed with paraformaldehyde, sonicated, and total proteins were harvested. Specific immunoprecipitation with STAT3 monoclonal antibodies allowed for recovery and enrichment of STAT3-bound DNA. PCR, designed to amplify the sequence comprising NKG2DL potential TFBSs, was realized on immunoprecipitated DNA. An internal control for STAT3 binding was used with a PCR designed to amplify STAT3 TFBS in the IL-10 promoter (44). We noticed a signal for the MICA and MICB promoters in DNA isolated from STAT3 ChIP. Interestingly, there was no signal observed for the ULBP2 promoter analysis (Fig. 6A).

To further develop our hypothesis, the highly transfectable 293T cells, in which STAT3 activity also influence MICA promoter activity (Fig. 6B), was used to address the precise role of STAT3-TFBS on STAT3 and MICA promoter interactions.

Then, we conducted a site-directed mutagenesis to remove STAT3-TFBSs in the promoter region of pGL3-MICA vector, displaying MICA promoter. After successful mutagenesis, we obtained a mutated version of pGL3-MICA (pGL3-MICAmut) that did not harbor any STAT3-TFBS. 293T cells were transfected with the normal or mutated luciferase construct. Forty-eight hours posttransfection, cells were stimulated for 2 hours with oncostatin M (OSM; 100 ng/mL) at 37°C to amplify STAT3 phosphorylation. ChIP experiments were done subsequently. PCR, designed to specifically amplify the pGL3 vector sequence, was realized on immunoprecipitated DNA (Fig. 6C). We observed a specific band in the pGL3-MICA condition, suggesting the binding of STAT3 to its target sequence on the vector. When STAT3-TFBS was mutated, there was no specific sequence amplification. These results support the specificity of the binding sequence in MICA promoter. Finally, 293T and HT29 cell lines were transfected with pGL3-MICA or pGL3-MICAmut vector. Specific mutation hampering STAT3 binding to MICA promoter increased luciferase activity in both HT29 and 293T cells (Fig. 6D). Note, similar results were obtained using U87 and MDA-231 cell lines (data not shown). Collectively, these results suggest that STAT3 regulates MICA expression at the transcriptional level.
Figure 4. STAT3 knockdown restores NKG2D ligands expression. A, freshly purified NK cells were cocultured for 24 hours with HT29iRNA-CTRL or HT29iRNA-STAT3 in the presence or absence of either an IgG or an NKG2D-blocking antibody (20 µg/mL for each condition) at E:T ratio (10:1). After 24 hours of cocultures, supernatants were harvested and IFN-γ concentration was assessed by ELISA (representative experiment of n = 2). B, flow cytometric analysis of CD107a expression on IL-2-activated NK after cocultures with HT29iRNA-CTRL or HT29iRNA-STAT3 (E:T = 10:1) for 4 hours in the presence or absence of an IgG or an NKG2D-blocking antibody (representative experiment of n = 3). C, flow cytometric analysis of MICA, MICB, ULBP1, ULBP2, and ULBP3 on HT29iRNA-CTRL or HT29iRNA-STAT3. Here, we show isotype (gray) versus specific staining (white). D, Western blot analysis for STAT3 and MICA on whole cell extracts from HT29, HT29iRNA-CTRL, or HT29iRNA-STAT3. β-Actin was used as a control of protein loading (20 µg per lane; representative experiment of n = 3). E, HT29 cells were treated with the STAT3 pharmacologic inhibitor STA21 (30 µmol/L) for 0, 24, and 48 hours. mRNA was extracted and MICA expression was assayed by RT-qPCR. Bars, SD; *, P < 0.05.

Influence of STAT3 on MICA regulation by DNA damage pathways

DNA damage response pathway was reported to play a role in upregulation of NKG2DLs and this molecular signaling is a possible candidate bridging cellular transformation and innate immunosurveillance (16). For this purpose, the role of STAT3 in NKG2DL induction by DNA damage response or heat shock was investigated in mesenchymal stem cells and activated lymphocytes known to express MICA (45, 46). The SV56 stroma cell line (26) was transduced with a retroviral vector containing a sequence for a modified and constitutive active form of STAT3 (STAT3C) or the empty vector (pBabe) and exposed to ionizing radiation or heat shock stress. STAT3 constitutive activation led to the downregulation of MICA and prevented the induction of MICA following ionizing radiation or heat shock exposition (Fig. 7A).

Furthermore, ataxia telangietasia mutated (ATM)-mediated DNA damage response pathway also induces MICA expression on activated T-cell lymphocytes (46). Because we and others have previously reported a STAT3 phosphorylation driven by CD28 costimulation in CD4+ lymphocytes (37, 47), we next investigated the influence of STAT3 on MICA expression following CD3/CD28-mediated activation of T lymphocytes. For this purpose, T-cell lymphocytes were purified, exposed with or without different STAT3 pharmacologic inhibitors, and activated by CD3/CD28 during 48 hours. As shown in Figure 7B, pharmacologic inhibition of STAT3 promoted higher level of MICA expression in T lymphocytes following CD3/CD28 stimulation (Fig. 7B). These results confer STAT3 a pivotal role in MICA regulation in both cancer and nonmalignant cells.
Figure 5. STAT3 pharmacologic inhibition specifically interferes with MICA transcription. A. Western blot analysis for STAT3 and STAT3-p on whole cell extracts from U87 and MDA-MB231 cell lines. β-Actin was used as a control of protein loading (20 μg per lane; representative experiment of n = 2). B. Flow cytometric analyses of MICA expression on U87 and MDA-MB231 treated with or without DMSO, STA21 (30 μmol/L) during 48 hours. C. IFN-γ concentration assessed by ELISA of freshly purified NK cells cocultured with HT29, U87, and MDA-MB231, in the presence or absence of IgG, anti-MICA, β, flow cytometric analyses of MICA expression on U87 and MDA-MB231 treated with or without DMSO, STA21 (30 μmol/L) during 48 hours before experiment (n = 2). D. Luciferase MICA promoter assay. HT29, U87, and MDA-MB231 were transiently transfected with pGL3-MICA vector. After 24 hours, cells were treated with or without STA21 at 30 μmol/L or AG490 (50 μmol/L). Results are presented as a ratio between the firefly luciferase activity and the Renilla luciferase activity for each condition (n = 2). Ctrl, control.

Discussion

NKG2D is an activating receptor shared by NK cells and T-cell lymphocytes, now identified as a pivotal mechanism to prevent the emergence of cancer cells arising following DNA damage induction or spontaneous transformation (7, 9, 16). In this study, we investigated the role of STAT3, a transcription factor harboring both oncogenic and immunosuppressive functions (27, 37), in NKG2D expression regulation.

In this study, we found that specific repression of STAT3 with RNA interference promoted the recognition of HT29 by NK cells. We showed that this mechanism involves the activating receptor NKG2D (Fig. 4). We revealed an inverse correlation between STAT3 activation and expression of MICA. Finally, ChIP analyses and luciferase promoter assays have shown for the first time MICA as a target for STAT3 transcriptional regulation (Fig. 6).

The role of NKG2D in NK immunosurveillance has been well documented. NKG2D is involved in the prevention of cancer initiation (9) and control of tumor progression (8). DNA damage pathway checkpoints ATM and ATR (ataxia telangiectasia and Rad3 related) could upregulate the expression of NKG2DLs in epithelial cells, thus altering the immune system (16). These results established MICA/NKG2D axis as an early mechanism of tumor suppression. The higher incidence of spontaneous cancers observed in NKG2D-deficient mice confirmed the tumor-suppressive role of NKG2D in vivo (9). Moreover, the disappearance of NKG2DLs on tumor cells derived from aggressive tumors in mice expressing wild-type NKG2D, but not from tumors derived from NKG2D-deficient mice, highlighted the presence of NKG2DL immunoediting.

Another mechanism was previously shown to prevent NKG2D-mediated recognition. Tumor-secreted metalloprotease induce proteolytic shedding of MICA molecules and
downregulation of NKG2DL expression (48). However, STAT3 modulation using siRNA or specific pharmacologic inhibitors did not influence the level of soluble MICA detected in HT29 culture supernatants (Supplementary Fig. 2).

Furthermore, both the induction of MICA transcription following exposition to STAT3 pharmacologic inhibitors and direct binding of STAT3 on MICA promoter support a direct influence of STAT3 on the transcriptional regulation of MICA.

Because STAT transcription factors are expected to promote gene transcription, our findings unraveled a possible involvement of STAT3 in the negative regulation of transcription. Experiments using directed mutation of the STAT3 binding sites in MICA promoter indicated that STAT3 exerts its repressor activity on MICA transcription through DNA binding (Fig. 6). STAT3 has been shown to act as a transcriptional repressor of p53 and IL-8 (49, 50). Of note, a direct binding of STAT3 to p53 promoter was also required for STAT3-mediated inhibition of p53 transcription (49).

STAT3 has been described as a potential mediator in chronic inflammatory disorders and oncogenesis. The
relationship between chronic inflammation and tumor progression has been documented through clinical trials showing that long-term use of nonsteroidal anti-inflammatory drugs reduce the relative risk of developing CRC by 40% to 50% (17). Many studies revealed the IL-6/STAT3 signaling pathway to be critical for IBD development (51). Thus, it is plausible that STAT3 could be activated at the time of epithelial cell transformation or DNA damage exposition and binds MICA promoter to prevent its expression and promote escape to NKG2D-mediated immunosurveillance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Author Contributions

C. Borg designed the research, analyzed experiments, and wrote the manuscript. R. Bedel, C. Grandclement, J. Balland, J.-P. Remy-Martin, B. Kantelip, and A. Thiery-Vuillemin conducted research and analyzed experiments. C. Ferrand, X. Pivot, and P. Tiberghien contributed to the design and writing.

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STAT3 Negatively Regulates MICA Transcription


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