Novel role for STAT3 in transcriptional regulation of NK immune cell targeting receptor MICA on cancer cells

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PRECIS : Findings reveal a novel mechanism by which STAT3 modulates immunosurveillance by NK immune cells, by repressing the expression of a key NK cell recognition molecule on cancer cells.
Abstract: The role of NKG2D-expressing Natural Killer (NK) cells in tumor immune surveillance is now well established. Nevertheless, tumor progression occurs despite tumor immune surveillance leading to cancer persistence in immune competent hosts. STAT3 plays a pivotal role both in oncogenic functions and immune suppression. In this study, we investigated the role of STAT3 in suppressing NK cell-mediated immunosurveillance. Using a colorectal cancer cell line (HT29) that is poorly able to activate NK, we neutralized STAT3 with pharmacological inhibitors or siRNA and found that this led to an increase of NK degranulation and interferon-gamma production in a TGF-β1 independent manner. Exposure to NKG2D neutralizing antibodies partially restored STAT3 activity, suggesting that it prevented NKG2D-mediated NK cell activation. 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 MICA was upregulated following STAT3 neutralization and a direct interaction between STAT3 and the MICA promoter was identified. Since crosstalk between DNA damage repair and NKG2D ligand expression has been demonstrated, 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.
Introduction

The immune system can detect and suppress emerging tumors (1). In addition to their role in pathogen immunity, Natural Killer cells (NK) 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 immune surveillance. Indeed, transfected tumor 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 immune surveillance 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 a NKG2D-dependent immune editing (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 ULBP proteins (7, 10, 11) are NKG2DLs, weakly expressed on normal cells and up-regulated in cancers (12-16). Nonetheless, molecular mechanisms leading to NKG2DLs 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 corner stone 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 immune surveillance.
through signal transducer and activator of transcription-3 (STAT3) (20). STAT3 is a transcription factor activated in IBD (21-23) and directly involved both in intestinal inflammation and cancer progression (24, 25).

In the present study, we aimed to investigate the role of STAT3 activation in the regulation of NKG2DLs expression and recognition of tumor cells by NK. We showed that STAT3 ablation in tumor cells modulates NKG2D-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, Besançon, France); anti-human CD107a (LAMP-1) (H4A3), isotype PE (MOPC-21) (BD Biosciences, Le Pont de Clay, France); anti-MICA-PE (2C10), MICB (9847-1), ULBP1 (Z-9), ULBP2 (F16), ULBP3 (2F9), TGF-β1 (C-16) (Santa Cruz Biotechnology, Heidelberg, Germany), anti-human STAT3 (79D7) and phospho-Stat3 (Tyr705) (3E2) (Cell Signaling, Beverly, USA), neutralizing anti-MICA (clone 159227, RnDsystems, Minneapolis, USA). STA-21, a selective inhibitor of STAT3 was purchased from BIOMOL International (Plymouth, USA). Oncostatin M (Peprotech, France) was used in some experiments. The TGF-β1 receptor inhibitor SB-431542 (Tocris Biosciences, Bristol, UK) was used in some experiments. Dynabeads® Human T-Expander CD3/CD28 (Invitrogen, Cergy-Pontoise, France) were used for stimulation of 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 upon 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 DMEM (HT29, SW620 and Colo320) medium (Lonza, Paris, France) supplemented with 10% Fetal Calf Serum (FCS) (Invitrogen, Cergy-Pontoise, France). NK were purified from healthy donor peripheral blood mononuclear cells (PBMC) using a negative magnetic selection (Stem Cell, Vancouver, Canada). The
purity of CD56/CD3 NK cells was assessed by flow cytometry and ranged from 90% to 98%. NK cells were maintained in RPMI 1640 Medium (Lonza, Paris, France) supplemented with 10% Human Serum (Invitrogen, Cergy-Pontoise, France).

**RNA silencing and plasmids constructs**

Specific STAT3 siRNA (sense 5’-AAAGAAACTTCAGACCCGTCAACAAA-3’, anti-sense 5’ AAAATTTGTGACGGGTCTGAAGTT-3’) and scramble siRNA (sense 5’-AAAGGAGGGGATGCCACGTTGG-3’, anti-sense 5’-AAAACCAACGTGGCATGCCCTC8 3’) sequences were produced, annealed and cloned into the BbsI site of the 3’ LTR of pFIV-H1/U6 vector according to manufacturer’s instructions (System Biosciences, Mountain View, CA). 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 performed according to manufacturer’s protocol (QuickChange® II XL Site-Directed Mutagenesis Kit, Stratagene, USA). 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) (C/T) turning the normal TTCCCTTCAGGAC STAT3 consensus binding sequence into TTCCCCGATAGGAC. 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’-cgcgttgtctgtcctgtaaggaacaagccagtg-3’ Muta-MICA-anti-sense 5’-cactggcttgttccttacaggacagacaacgcg-3’.
Real Time-quantitative PCR (RT-qPCR).

Total RNA were extracted using Kit RNeasy mini (Qiagen, Courtaboeuf, France) and reverse transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Rockville, MD, USA). Duplicate samples were subjected to RT-qPCR. mRNA were quantified using primers listed below: MICA (Hs00792193_m1) (Applied Biosystems). ABL mRNA from each sample was quantified as an endogenous control. Relative mRNA expression was calculated using the Delta-Delta-Ct method and untreated cells were used as calibrator.

Luciferase Assay

HT29 and 293T cells were transfected using Lipofectamine LTX (Invitrogen, France). 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

Detection of IFN-γ was done using commercial ELISA kits (Diaclone, France). The sensitivity of the human IFN-γ kit was 4.7 pg/mL. Detection of MICA was done using ELISA kits (Diaclone, France). 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 24h with IL-2 (1000 UI/ml) and then cocultured in presence of target cells for 4 hours at 10:1 E:T ratio. Degranulation of NK cells was analyzed by flow cytometry analysis of CD107a expression as previously described (29).
Chromatin immunoprecipitation (ChIP) assay

HT29 or 293T cells \(5 \times 10^6\) were cross-linked with 1% formaldehyde in the presence of protease inhibitors (Complete Mini EDTA Free, Roche) for 15 min at room temperature then treated with Glycine 1 M for 5 min at room temperature. Cells were harvested and after two washing steps with ice-cold PBS, lysed in 500 µl of Lysis Buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% v/v NP40, 0.5% m/v Déoxycholate Na, 2 mM EDTA, 2 mM NaF, 1 mM Vanadate, proteases inhibitor mixture). 200 to 1000 bp DNA fragments were generated with 5x10⁶ sonication using Vibra Cell™ sonicator (Sonis & Materials, Newton, USA). 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 IgG. After a 2 hours incubation with Dynabeads Protein G (Invitrogen, Paris, France), beads were washed twice with Wash Buffer 1 (0.1 % SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, 150 mM NaCl) then submitted to another washing step with Wash Buffer 2 (0.1 % SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, 500 mM NaCl) and finally two washing steps with TE Buffer. Beads (and the total input DNA) were subsequently incubated at 65°C overnight to reverse the cross-link. Incubation with Proteinase K (Invitrogen) for 30 min at 55°C was performed and DNA samples were purified using QIAamp DNA Mini Kit (Qiagen), collected in 200 µl TE Buffer and then assessed by PCR.

Statistical analysis

Results are expressed as the mean plus or minus the standard error of mean. Group comparisons were performed 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 (30-33). For this purpose, NK were purified from peripheral blood lymphocytes 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 to Colo320, SW620 and K562 (Figure 1A and 1B). The higher expression of STAT3 in HT29 compared to K562, Colo320 or SW620 (Figure 1C) prompted us to investigate the precise role of STAT3 in the recognition of HT29 by NK.

In order to confirm the influence of STAT3 in colorectal cancer models, we generated STAT3 deficient cell lines using a lentivirus-mediated gene transfer to deliver a specifically designed siRNA for STAT3 into HT29 and to produce a stable cell line (HT29\textsuperscript{siRNA-STAT3}). Western blot analysis confirmed a reduced expression of STAT3 in the knock-down cell line HT29\textsuperscript{siRNA-STAT3} (Figure 2A). Moreover, the level of phospo-STAT3 was also decreased in HT29\textsuperscript{siRNA-STAT3} (Figure 2B).

The next set of experiments was dedicated to assess if the presence of STAT3 influences HT29 recognition by NK. Freshly purified NK were co-cultured for 24h with HT29, HT29\textsuperscript{siRNA-CTRL} or HT29\textsuperscript{siRNA-STAT3} and harvested supernatants were then assessed for IFN-γ production (Figure 2C). HT29\textsuperscript{siRNA-STAT3} triggered a significantly increased secretion of IFN-γ by NK compared to HT29\textsuperscript{siRNA-CTRL} or HT29 (1276 ± 82 pg/ml versus 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
performed to reveal NK degranulation against HT29, HT29siRNA-CTRL or HT29siRNA-STAT3 (Figure 2D). We observed an increase of CD107a expression in co-cultures performed with HT29siRNA-STAT3 compared to HT29siRNA-CTRL, suggesting an active degranulation of NK in the condition where STAT3 is repressed (42% versus 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 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. Transforming growth factor beta isoform 1 (TGF-β1), a cytokine produced by HT-29 (34), has been previously characterized as a major inhibitory pathway limiting tumor cell recognition by NK (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 (Figure 3A). We noticed a decreased expression of TGF-β1 in HT29siRNA-STAT3 compared to HT29siRNA-CTRL. The quantification of TGF-beta1 by RT-qPCR supported previous results at the protein level (Figure 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. Therefore, the presence of a STAT3-dependent TGF-β1 expression in HT29 led us to investigate if TGF-β1 signaling pathway accounted for STAT3 mediated inhibition of HT29 recognition by NK. For this purpose, freshly purified NK were co-cultured with HT29siRNA-CTRL and HT29siRNA-STAT3 for 24h in the presence or absence of a specific pharmacological...
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 (PBL) (data not shown) and did not influence NK cell activating receptor expression (Supplementary Figure 1). As shown previously, we observed a significant increase in IFN-γ production when we compared HT29siRNA-STAT3 to HT29siRNA-CTRL. TGF-β1 inhibition resulted in a weak up-regulation 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 (Figure 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 the TGF-β1-mediated inhibitory signaling, STAT3 specific inhibition promotes the expression of NK-activating ligands on HT29.

STAT3 knock-down restores NKG2DLs expression

Raulet DH et al. demonstrated an increased tumor incidence in NKG2D−/− mice (9). As a consequence, avoidance of this specific NKG2D/NKG2DLs 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 were pre-treated for 30 min at 37°C with blocking antibodies and then co-cultured with HT29siRNA-CTRL or HT29siRNA-STAT3 for 24h and harvested supernatants were assessed for IFN-γ production. As shown in Figure 4A, left panel, IFN-γ production by NK in the co-culture 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 (Figure 4A, right panel). The magnitude of this inhibition by anti-NKG2D was significantly higher in the presence of specific STAT3 siRNA (Figure 4A, right panel). These results strongly suggested a role for the 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 (Figure 4B). While MICA expression was influenced by STAT3 modulation, we failed to identify any variation of neither MICB nor ULBPs in HT29siRNA-CTRL and HT29siRNA-STAT3. In order to confirm this hypothesis, we performed 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 of MICA protein in HT29siRNA-STAT3 (Figure 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 Figure 2). Thereafter, we decided to assess if the correlation between the abrogation of STAT3 signaling and enhancement in MICA expression was detectable at the transcriptional level. RT-qPCR analyses were performed on total mRNA extracts from HT29 cells treated with the STAT3 pharmacological inhibitor STA21 at different time points (Figure 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 two tumor cell lines, U87 and MDA-MB231 constitutively expressing an active form of STAT3 (Figure 5A). MICA expression increased on U87 and MDA-MB231 cells treated with STA21 for 48h (Figure 5B). The functional significance of STAT3 on tumor cell recognition by NK was studied in coculture of NK with HT29, U87 and MDA-MB231 cells previously treated during 48 hours with two different STAT3 pharmacological inhibitors. These experiments confirmed that treatment of tumor cells with either STA21 or AG490 enhances NK interferon γ production (Figure 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 interferon γ production observed in NK colcultures with HT29, U87 or MDA-MB231 previously treated with STA21 or AG490 (Figure 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. Pharmacological inhibition of STAT3 in all transfected cell lines resulted in an enhanced MICA promoter activity (Figure 5D).

STAT3 directly interacts with specific binding sites in MICA promoter

In order 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 (TFBSs) in the promoter of regulated genes. We sought to investigate the presence of STAT3 TFBSs in MICA promoter to further elucidate the mechanisms governing previous
observations. We used the predictive software MatInspector for TFBSs and found a significant match (5’-TTCTTCCAGGACAGACAA-3’) for a more recently discovered sequence (core nucleotide denoted in capitals: TTCCNGG) (43). Complementary analyses were realized on MICB and ULBPs promoter sequences as well. We obtained specific sequences for MICB (3’-TTCTTCCGGACAGACAA-5’) and ULBP2 (3’-CATCTTCCAGGCTCTCCCT-5’) promoters while no specific matches were retrieved from ULBP1 and ULBP3 promoter investigations (Supplementary Data 1 and 2). We initiated a ChIP assay to control whether STAT3 was indeed able to 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 NKG2DLs potential TFBSs, were 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 (Figure 6A).

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

Then, we performed a site-directed mutagenesis to remove STAT3-TFBS 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.
48h post transfection, cells were stimulated for 2 hours with OSM (100 ng/ml) at 37°C to amplify STAT3 phosphorylation. ChIP experiments were performed subsequently. PCR, designed to specifically amplify the pGL3 vector sequence, was realized on immunoprecipitated DNA (Figure 6C). We observed a specific band in the pGL3-MICA condition, suggesting a 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 both in HT29 and 293T cells (Figure 6D). Of 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.

Influence of STAT3 on MICA regulation by DNA damage pathways

DNA damage response pathway was reported to play a role in up-regulation of NKG2DLs and this molecular signaling is a possible candidate bridging cellular transformation and innate immune surveillance (16). For this purpose, the role of STAT3 in NKG2DLs 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 a down-regulation of MICA and prevented the induction of MICA following ionizing radiation or heat shock exposition (Figure 7A).
Furthermore, ATM mediated DNA damage response pathway also induces MICA expression on activated T cell lymphocytes (46). Since 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 or not to different STAT3 pharmacological inhibitors and activated by CD3/CD28 during 48h. As shown in Figure 7B, pharmacological inhibition of STAT3 promoted higher level of MICA expression in T lymphocytes following CD3/CD28 stimulation (Figure 7B). These results confer to STAT3 a pivotal role in MICA regulation, both in cancer and non malignant cells.

**Discussion**

NKG2D is an activating receptor shared by NK 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 the present study, we investigated the role of STAT3, a transcription factor harboring both oncogenic and immunosuppressive functions (27, 37), in NKG2DLs expression regulation.

In this study, we found that specific repression of STAT3 with RNA interference promoted the recognition of HT29 by NK. We demonstrated that this mechanism involves the activating receptor NKG2D (Figure 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 (Figure 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 ataxia-telangiectasia-mutated (ATM) and Ataxia telangiectasia and Rad3 related (ATR) could up-regulate the expression of NKG2DLs in epithelial cells, thus alerting 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 and not from tumors derived from NKG2D deficient mice highlighted the presence of NKG2DLs immunoediting.

Another mechanism was previously shown to prevent NKG2D-mediated recognition. Tumor-secreted metalloprotease-induce proteolytic shedding of MICA molecules and down-regulation of NKG2DLs expression (48). However, STAT3 modulation using siRNA or specific pharmacological inhibitors did not influence the level of soluble MICA detected in HT29 culture supernatants (Supplementary Figure 2).

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

Since 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 (Figure 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 colorectal cancer by 40–50% (17). Many studies revealed the IL-6/STAT3 signaling pathway to be critical for IBD development (51). Then, 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.

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Figure legends

**Figure 1:** Investigation of colon cancer cell line susceptibility to NK cells. A. IFN-γ concentration assessed by ELISA of freshly purified NK cells co-cultured with tumor cell lines for 24h at E:T ratio (10:1) (representative experiment of n=4). B. Flow cytometry analysis of CD107a expression on IL-2 activated NK cells co-cultured for 4 hours with candidate tumor cells at E:T ratio (10:1) (representative blot of n=3). C. Western blot analysis for STAT3 on whole cell extracts. β-actin was used as a control of protein loading (20 µg per lane) (representative experiment of n=3). Bars, SD.

**Figure 2:** STAT3 knock-down 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 Cytometry analysis of STAT3 and phospho-STAT3 expression by HT29siRNA-CTRL or HT29siRNA-STAT3. Grey and white histograms represent isotype or STAT3 staining respectively. C. IFN-γ concentration assessed by ELISA of freshly purified NK cells co-cultured with HT29, HT29siRNA-CTRL or HT29siRNA-STAT3 for 24h in DMEM medium at E:T ratio (10:1). D. Flow Cytometry Analysis of CD107a expression on IL-2 activated NK co-cultured 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.

**Figure 3:** STAT3 inhibition of HT29 recognition by NK does not involve TGF-β1. Flow Cytometry analysis of TGF-β1 expression by HT29siRNA-CTRL or HT29siRNA-STAT3. Here we show isotype (grey) vs. specific staining (black). B. RT-qPCR analysis of total mRNA extracts from HT29siRNA-CTRL or HT29siRNA-STAT3. Raw data were analyzed with the ΔΔCt.
method and results are shown as relative expression to the control HT29siRNA-CTRL (representative experiment of n=2). B. Freshly purified NK were co-cultured for 24h with HT29siRNA-CTRL or HT29siRNA-STAT3 in the presence or absence of the TGF-β1 receptor inhibitor: SB-431542 (10 µM) at E:T ratio (10:1). Supernatants were harvested and IFN-γ concentration was assessed by ELISA (representative experiment of n=3). Bars, SD. *p<0.05.

Figure 4: STAT3 knock-down restores NKG2D ligands expression. A. Freshly purified NK cells were co-cultured for 24h with HT29siRNA-CTRL or HT29siRNA-STAT3 in the presence or absence of either a IgG or a NKG2D blocking antibody (20 µg/ml for each condition) at E:T ratio (10:1). After 24 hours of co-cultures supernatants were harvested and IFN-γ concentration was assessed by ELISA (representative experiment of n=2). B. Flow cytometry analysis of CD107a expression on IL-2 activated NK after co-cultures with HT29siRNA-CTRL or HT29siRNA-STAT3 (E:T ratio=10:1) for 4 hours in the presence or absence of a IgG or a NKG2D blocking antibody (representative experiment of n=3). C. Flow cytometry analysis of MICA, MICB, ULBP1, ULBP2 and ULBP3 on HT29siRNA-CTRL or HT29siRNA-STAT3. Here we show isotype (grey) versus specific staining (white). D. Western blot analysis for STAT3 and MICA 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). E. HT29 cells were treated with the STAT3 pharmacological inhibitor STA21 (30 µM) for 0, 24 and 48h. mRNA was extracted and MICA expression was assessed by RT-qPCR. Bars, SD. *p<0.05.
**Figure 5: STAT3 pharmacological 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 Cytometry analyses of MICA expression on U87 and MDA-MB231 treated or not with DMSO, STA21 (30µM) during 48 hours. C. IFN-γ concentration assessed by ELISA of freshly purified NK cells co-cultured with HT29, U87 and MDA-MB231, in the presence or absence of either IgG, anti-MICA or NKG2D blocking antibodies (20 µg/ml for each condition), for 24h in DMEM medium at E:T ratio (10:1). In some conditions, tumor cells were treated or not with DMSO, STA21 (30µM) or AG490 (50µM) 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 24h, cells were treated or not with STA21 at 30 µM or AG490 (50µM). Results are presented as a ratio between the firefly luciferase activity and the renilla luciferase activity (Ren/Luc) for each conditions (n=2).

**Figure 6: STAT3 directly interact with specific binding sites in MICA promoter.**

A. PCR analysis was performed on DNA retrieved from specific STAT3 ChIP experiments. Potential site sequence is indicated along with the PCR results (representative of n=3) B. Luciferase MICA promoter assay. 293T were transiently transfected with pGL3-MICA vector. After 24h, cells were treated or not with STA21 at 30 µM. Results are presented as a ratio between the firefly luciferase activity and the renilla luciferase activity (Ren/Luc) for each conditions (n=2). C. 293T cells were transiently transfected with either the pGL3-MICA or the pGL3-MICAmut vector. After 48h, cells were treated with OSM (100 ng/ml) for 2h. After the incubation cells were fixed and a ChIP experiment was realized as previously described. PCR analysis were performed on DNA retrieved from specific STAT3 ChIP experiments.
D. 293T and HT29 were transiently transfected with pGL3-MICA or pGL3-MICAmut. After 48h, luciferase activity was quantified using the manufacturer’s protocol. Results are presented as a ratio between the firefly luciferase activity and the renilla luciferase activity (Ren/Luc) for each conditions (n=2).

**Figure 7: STAT3 influences MICA expression in non malignant cells.** A. Stroma cell lines SV56pBabe and SV56STAT3C were stimulated with either ionizing irradiation (25 Gy) or heat shock (45°C for 1h). 16h after treatment, MICA expression was assessed by flow cytometry analysis (Representative of n=3). B. PBL isolated from healthy donor were stimulated for 48h with CD3/CD28 beads at a bead/T cell ratio of 1:1 in the presence or absence of STA21 (30 µM) or AG490 (50 µM). MICA expression was assessed by flow cytometry analysis and reported as relative fluorescent intensity (RFI). RFI were calculated by dividing the mean fluorescent intensity of test antibody by the fluorescent intensity of isotype control treated cells from the same well (n=3).
A. 

![Graph showing IFN-γ production (pg/mL) for different cell lines.]

- Medium: 0 pg/mL
- K562: 2000 pg/mL
- SW620: 1500 pg/mL
- Colo320: 1200 pg/mL
- HT29: 500 pg/mL

B. 

![Fluorescence-activated cell sorting (FACS) plots for different cell lines with CD107a and SSC axes.]

- K562: 48%
- SW620: 27%
- Colo320: 25%
- HT29: 13%

C. 

![Western blot analysis for STAT3 and β-actin proteins.]

- HT29
- K562
- Colo320
- SW620

- STAT3: 79 kDa
- β-actin: 42 kDa
Figure A: Western blot analysis of STAT3 and β-actin in HT29, HT29 siRNA STAT3, and HT29 siRNA CTRL.

Figure B: Flow cytometry analysis of STAT3 and phospho-STAT3 (Y705) in HT29, HT29 siRNA CTRL, and HT29 siRNA STAT3.

Figure C: Bar graph showing IFN-γ production in HT29, HT29 siRNA CTRL, and HT29 siRNA STAT3. * indicates significant difference.

Figure D: Flow cytometry analysis of CD107a in HT29, HT29 siRNA CTRL, and HT29 siRNA STAT3. Numbers indicate percentage of activated cells.
A.  

<table>
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<tr>
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<th>H₂O</th>
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<td>IL-10 promoter (positive control)</td>
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B.  

![Graph showing firefly:renilla luminescence ratio for 293T cells treated with N.T. and STA21.](image)

C.  

![Graph showing firefly:renilla luminescence ratio for 293T cells treated with OSM (100 ng/ml).](image)

D.  

![Graph showing firefly:renilla luminescence ratio for HT29 and 293T cells treated with pGL3 MICA and pGL3 MICA mut.](image)
Novel role for STAT3 in transcriptional regulation of NK immune cell targeting receptor MICA on cancer cells

Romain Bedel, Antoine Thiery-Vuillemin, Camille Grandclement, et al.

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