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Noncanonical NF-κB Activation Mediates STAT3-Stimulated IDO Upregulation in Myeloid-Derived Suppressor Cells in Breast Cancer

Jinpu Yu,*† Yue Wang,*† Fang Yan,*† Peng Zhang,* Hui Li,* Hua Zhao,* Chui Yan,* Fan Yan,* and Xiubao Ren*‡

Immunotherapy for cancer treatment is achieved through the activation of competent immune effector cells and the inhibition of immunosuppressive cells, such as myeloid-derived suppressor cells (MDSCs). Although MDSCs have been shown to contribute to breast cancer development, the mechanism underlying MDSC-mediated immunosuppression is unclear. We have identified a poorly differentiated MDSC subset in breast cancer—suppressing T cell function through STAT3-dependent IDO upregulation. In this study we investigated the mechanisms underlying aberrant expression of IDO in MDSCs. MDSCs were induced by coculturing human CD33+ myeloid progenitors with MDA-MB-231 breast cancer cells. Increased STAT3 activation in MDSCs was correlated with activation of the noncanonical NF-κB pathway, including increased NF-κB-inducing kinase (NIK) protein level, phosphorylation of cytoplasmic inhibitor of NF-κB kinase α and p100, and RelB-p52 nuclear translocation. Blocking STAT3 activation with the small molecule inhibitor JSI-124 significantly inhibited the accumulation of NIK and IDO expression in MDSCs. Knockdown of NIK in MDSCs suppressed IDO expression but not STAT3 activation. RelB-p52 dimers were found to directly bind to the IDO promoter, leading to IDO expression in MDSCs. IL-6 was found to stimulate STAT3-dependent, NF-κB–mediated IDO upregulation in MDSCs. Furthermore, significant positive correlation between the numbers of pSTAT3* MDSCs, IDO* MDSCs, and NIK* MDSCs was observed in human breast cancers. These results demonstrate a STAT3/NF-κB/IDO pathway in breast cancer–derived MDSCs, which provides insight into understanding immunosuppressive mechanisms of MDSCs in breast cancer. The Journal of Immunology, 2014, 193: 2574–2586.

Myeloid-derived suppressor cells (MDSCs), a heterogeneous cell population, are composed of multiple myeloid cells arrested at various stages of lineage development. They contribute to the immunosuppressive tumor microenvironment (1). MDSCs have been identified in mice based on positive expression of CD11b and Gr1 markers (1, 2). However, phenotypic markers for human MDSCs have not been well defined because MDSCs express different markers in distinct tumor types (1, 2). Human MDSCs are generally distinguished by their detectable immunosuppressive properties, definitive myeloid lineage marker (CD33*), and poor Ag presentation capacity (HLA-DRlow/−) (1, 2). CD14 and CD15 have been reported as markers for two distinct subtypes of MDSCs in humans, monocytic MDSCs (CD14*), and polymorphonuclear MDSCs (CD15*) (1, 2).

In addition to the disparity in the phenotype of MDSCs, the molecular mechanisms underlying MDSC-mediated immunosuppression vary in different tumor types due to tumor heterogeneity and the complexity of the tumor microenvironment. Several mechanisms have been reported to regulate the suppressive tumor milieu by MDSCs (1, 2). High expression levels of metabolic enzymes, arginase-1 and inducible NO synthase, and production of reactive oxygen and nitrogen species play a role in MDSC-mediated immunosuppressive effects (1). Other mechanisms include the activation and expansion of regulatory T cells, cysteine sequestration, production of suppressive cytokines, such as IL-10 and TGF-β, and inhibition of CD62L-mediated migration of T cells (1). Recently, our group and others have reported that upregulation of IDO might be an immunosuppressive mechanism employed by MDSCs in human solid tumors, such as breast cancer, and hematological malignancies (3, 4).

IDO, an intracellular enzyme, catalyzes tryptophan through the kynurenine pathway, resulting in suppression of T cell growth and activation of the cellular stress response pathway via general control nonderepressible 2 kinase (5). Furthermore, tolerogenic kynurenine metabolites bind to the aryl hydrocarbon receptor in T cells, resulting in inhibition of T cell–mediated anticancer immunity (5). Besides macrophages, dendritic cells (DCs), and granulocytes (6, 7), IDO has been detected in immature myeloid-derived cells (8). A subset of MDSCs with a high level of IDO

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Abbreviations used in this article: ChIP, chromatin immunoprecipitation; DC, dendritic cell; IKK, inhibitor of NF-κB kinase; MDSC, myeloid-derived suppressor cell; NIK, NF-κB–inducing kinase; RT-PCR, real-time PCR; siRNA, small interfering RNA; SOCS3, suppressor of cytokine signaling 3; UCB, umbilical cord blood.

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expression was reported in patients with hematologic malignancy after allogeneic hematopoietic stem cell transplantation therapy, in which upregulated IDO expression led to decreased IFN-γ release, CD3ζ-chain expression in T cells, and T cell proliferation (3). We have identified a population of MDSCs with IDO expression in breast cancer with the characteristic phenotype of CD13+CD33+CD14+CD15+. This subset of MDSCs suppresses T cell function in an IDO-dependent manner, and the distribution of IDO+ MDSCs positively correlates with both the infiltration of Foxp3+ regulatory T cells in situ and the extent of lymph node metastasis in breast cancer patients (4).

The molecular mechanism regulating aberrant expression of IDO in MDSCs remains unclear. IFN-γ is the most potent inducer of IDO expression in macrophages and DCs (9). IFN-γ-dependent IDO expression occurs primarily through the STAT1 pathway. STAT1 undergoes dimerization and translocates to the nucleus where it directly binds to the IDO gene promoter or indirectly induces IDO transcription by the IFN regulatory factor 1 protein (9). However, both Mougiakakos et al. (3) and our team (4) observed no change in IFN-γ expression in MDSCs from human breast cancer or hematological cancer. Only a low level of STAT1 phosphorylation and STAT1-dependent arginase-1 expression in MDSCs from human breast cancer has been observed (4). Therefore, STAT1 activation may not be the mechanism regulating aberrant upregulation of IDO in MDSCs in breast cancer. In this study, we investigated the mechanisms underlying regulation of IDO expression in MDSCs induced by coculturing healthy donor-derived CD33+ progenitors with MDA-MB-231 breast cancer cells. We demonstrate that increased STAT3 activation in MDSCs stimulates activation of the noncanonical NF-κB pathway, including increased NF-κB-inducing kinase (NIK) protein level, phosphorylation of cytoplasmic inhibitor of NF-κB kinase (IKK) α and p100, and nuclear translocation of RelB-p52, which subsequently induces the transcription of IDO by directly binding to the IDO promoter region. Furthermore, we found that IL-6 plays a role in promoting STAT3-dependent, NF-κB-mediated IDO up-regulation in MDSCs. Our study suggests a novel regulatory mechanism of IDO in MDSC-mediated immunosuppression and provides insight into developing more efficient immunotherapy for breast cancer.

Materials and Methods

Human samples

Umbilical cord blood (UCB) samples from 20 healthy donors (obtained from Tianjin Blood Center) were collected for isolation of CD33+ myeloid progenitors. This project was approved by the Ethics Committee of Tianjin Medical University. All experiments were performed in accordance with the principles of the Declaration of Helsinki. Written consents were obtained from healthy donors.

Induction of MDSCs in vitro

CD33+ cells were isolated from UCB of healthy donors using human CD33 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and cell viability analysis was performed usingtrypan blue exclusion. MDSCs were induced by coculturing CD33+ myeloid progenitors with the breast cancer cell line MDA-MB-231 for 48 h in vitro, and CD33+CD13+CD14+CD15+ cells were sorted by flow cytometry as described previously (4). MDSCs were treated with a specific STAT3 antagonist, JSI-124 (also called c ubicatin I; Sigma-Aldrich, St. Louis, MO), at a concentration of 10 µM to block STAT3 phosphorylation. Untreated CD33+ myeloid cells from healthy UCB were used as a negative control.

Small interfering RNA transfection

To define the regulatory effect of the NF-κB pathway on STAT3-induced IDO expression, two small interfering RNA (siRNA) candidates specifically targeting NIK mRNA sequence were synthesized (alias MAP3K14; siRNA1, sense, 5'-CGCCCAAUCAGCCAAUATT-3', antisense, 5'-UUUAUGGUUGCAGUUCUC-3'). siRNA2, sense, 5'-GUGAGAAGAAACCACAUAATTT-3', antisense, 5'-UUUAUGGUUGCAGUUCUC-3') and incorporated into our coculture system to inhibit NIK protein translation. A nontargeting siRNA (sense, 5'-UUUCUCGGACGUGUCCAGTUT-3', antisense, 5'-ACGGUGACGUGUCCAGAATT-3') was used to control for nonspecific interference. Mixed siRNAs targeting C/EBPβ (Santa Cruz Biotechnology, Santa Cruz, CA) were also purchased to determine whether the C/EBPβ pathway regulated IDO expression.

MDSCs were cultured in six-well plates without antibiotics for 24 h. Cells were then transfected with siRNA using Lipofectamine RNAiMAX reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. After 3 d, the cells were harvested for real-time PCR (RT-PCR) and Western blot analysis. The expression of C/EBPβ or NIK was evaluated to confirm full knockdown of both genes.

Western blot analysis

Western blot assay was performed to detect the expression levels of total protein and phosphorylated protein in MDSCs and CD33+ control cells using anti-human IDO (Millipore, Billerica, MA), STAT1, STAT3, IKKα, IKKβ, NIK, p-STAT1, p-STAT3, p-C/EBPβ, p-p100, p-IKKα/β, β-actin (Cell Signaling Technology, Danvers, MA), and p-IKKα (Signalway Antibody, College Park, MD) Abs as described previously (4). HRP-conjugated goat anti-mouse or anti-rabbit IgG Abs (Zhongshan jinqiao, Beijing, China) and a SuperSignal West Pico chemiluminescent substrate kit (Pierce Biotechnology, Rockford, IL) were used to visualize protein bands. The relative densities of protein were measured using the ChemiDoc XRS imaging system (Bio-Rad Laboratories, Hercules, CA) and normalized to β-actin, STAT1, STAT3, IKKα, or IKKβ bands.

RT-PCR analysis

The mRNA levels of IDO, NIK, C/EBPβ, and β-actin were analyzed using RT-PCR as previously described (4). The primers for IDO, NIK, C/EBPβ, and β-actin are listed in Table I. PCR products were analyzed using agarose gel electrophoresis.

Nuclear translocation assay

Nuclear extracts were prepared as previously described (10) to detect NF-κB nuclear translocation using the TransAM NF-κB family kit (Active Motif Europe, Rixensart, Belgium) according to the manufacturer’s instructions. A plate coated with immobilized oligonucleotides containing the consensus site (5'-GGGACTTTCC-3') specific for NF-κB binding was used to enrich Rel/NF-κB dimers. To detect NF-κB subunits bound to its target DNA, mouse anti-human p50, p52, RelA, and RelB mAbs were incubated with the plate and followed by HRP-conjugated goat anti-mouse secondary Ab. The OD was measured at 450 nm, and the relative level of NF-κB nuclear translocation was calculated by comparing the density-treated group to that of the CD33+ control.

DNA binding sites assay

A TransAM Flexi kit was used to define specific binding sites of NF-κB (Active Motif Europe). The biotinylated oligonucleotide probes containing putative binding sequences for NF-κB in the IDO promoter region were fixed in streptavidin-coated plates. After incubation of cell lysates in these plates, the presence of the DNA/protein complex was detected using anti-NF-κB (anti-p52, anti-p50, anti-RelA, and anti-RelB) and peroxidase-conjugated anti-rabbit Abs and measured by colorimetry (11). Four probes were generated using the Takara LA Taq PCR kit (Takara Bio, Kyoto, Japan). All probes were cocultured with nuclear extracts isolated from MDSCs. OD was measured at 450 nm and represented as relative binding activity of noncanonical NF-κB subunits (p52 and RelB) to certain probes. Identical oligonucleotide probes without biotinylation were used as competitive probes to eliminate nonspecific binding in this assay.

Chromatin immunoprecipitation–PCR assay

A chromatin immunoprecipitation (ChIP) assay was performed to identify the specific binding sequences in the IDO promoter region for the noncanonical NF-κB subunits p52, RelB, and STAT3 protein using an EZ-ChIP assay kit (Millipore/Upstate Biotechnology) according to the manufacturer’s instructions. MiaPaCa2 cells were used as a positive control of p52 and RelB binding to the skp2 gene (12, 13), and MDA-MB-231 cells served as a positive control of STAT3 binding to AKAP12 and HIC2 genes (14). The ChIP primers for detecting binding sequences are listed in Table I. Fragmented chromatin was immunoprecipitated with a ChIP-grade Ab against STAT3 (Cell Signaling Technology), RelB (Santa Cruz Biotechnology), or p52 (Abcam, San Francisco, CA). After reversal of the
cross-links and DNA precipitation, enriched DNA was analyzed by PCR amplification using primers specific for indicated regions. Quantification was performed by quantitative RT-PCR, and enrichment was presented as the percentage of the total density to the total input, as previously described (15).

Cytokine array and ELISA

The levels of 42 cytokines in the supernatants of MDSCs, CD33+ controls, and JSI-124–treated MDSCs were detected using the RayBio human cytokine Ab array (RayBiotech, Norcross, GA), as previously described (16). For validation, the levels of IL-1β, IL-6, IL-10, GM-CSF, IFN-γ, and TGF-β1 in the cell culture supernatants were detected using ELISA kits (Genzyme, Cambridge, MA), as previously described (4).

Cytokine stimulation and blocking assay

MDSCs were treated with recombinant cytokines IL-1β, GM-CSF, IL-6, and IL-10 (PeproTech, Rocky Hill, NJ) at a concentration of 1 μg/ml to examine the regulatory effects of these cytokines on IDO expression. MDSCs cultured with the supernatant of MDA-MB-231 cells were used as a positive control, and MDSCs cultured in PBS-supplemented RPMI 1640 medium were used as a negative control. To block the regulatory effects of cytokines on IDO expression, the specific neutralizing Abs against IL-1β (30 μg/ml; Millipore/Upstate Biotechnology), IL-6 (50 μg/ml; BioLegend, San Diego, CA), GM-CSF (90 μg/ml; BioLegend, San Diego, CA), and IL-10 (30 μg/ml; BioLegend, San Diego, CA) were added to the coculture system of CD33+ myeloid progenitors and MDA-MB-231 breast cancer cells. Expression of IDO, NIK, STAT3, and p-STAT3 were detected using Western blot analysis.

Immunofluorescence staining

Immunofluorescence staining was performed on 30 cases of formaldehyde-fixed, paraffin-embedded breast cancer tissues collected during radical mastectomy at the Department of Breast Oncology at Tianjin Medical University Cancer Institute and Hospital. All experiments involving humans were performed in accordance with the principles of Helsinki. Written consents were obtained from patients. Tissue sections were incubated with rabbit anti-human IDO (Millipore), rabbit anti-human p-STAT3 (Cell Signaling Technology), rabbit anti-human NIK (Santa Cruz Biotechnology), and mouse anti-human CD33 (Abcam) Abs overnight at 4°C, and Alexa Fluor goat anti-mouse or anti-rabbit IgG secondary Abs for 1 h. DAPI (Millipore) was used for nuclear counterstaining. Images were acquired using a Leica SP2 confocal microscope (Leica, Mannheim, Germany).

Breast cancer tissue sections were immunostained with p-STAT3 and CD33 for p-STAT3+ MDSCs, IDO and CD33 for IDO+ MDSCs, and NIK and CD33 for NIK+ MDSCs. Five representative high-power fields (×400 magnification) for each tissue section were selected for histological evaluation, and the percentages of p-STAT3+ MDSCs, IDO+ MDSCs, and NIK+ MDSCs were counted and analyzed.

Mice and treatment

4T1 mouse mammary carcinoma cells (3 × 10^6/mouse) were injected into the mammary fat pads of BALB/c mice. Five days following tumor cell implantation, mice were treated i.p. with JSI-124 at a dose of 1 mg/kg/d or vehicle control (PBS plus DMSO) for 12 d. JSI-124 was initially dissolved in DMSO at 1 mg/ml and stored at −20°C. Before treatment, JSI-124 was diluted with PBS. Mice were sacrificed on day 16 after the treatment. Tumor and lung tissues were collected for preparation of paraffin-embedded sections. Single-cell suspension of spleen was prepared for sorting MDSCs using mouse CD11b microbeads (Miltenyi Biotech). All animal experiments were performed according to protocols approved by the Experimental Animal Committee at Tianjing Medical University, Tianjin, China.

Statistical analysis

All data are presented as means ± SD. The statistical significance of the differences between mean values was determined using an SPSS 13.0 software package (SPSS, Chicago, IL). The one-way single-factor ANOVA and least significant difference method were used for comparison of the quantitative data. The correlation among the numbers of p-STAT3+ MDSCs, NIK+ MDSCs, and IDO+MDSCs in situ was analyzed using the linear regression method. The level of statistical significance was set at p < 0.05.

Results

STAT3 activation–induced IDO expression is independent of direct binding of STAT3 to the promoter region of IDO gene

To test whether STAT3 regulates IDO expression by directly binding to the IDO promoter, the whole DNA sequence of the IDO promoter region was used to screen potential STAT3 binding sites using online prediction programs, including two recommended eukaryotic transcription factors databases: TRANSFAC database and JASPAR database. We found five potential sequences in the IDO promoter for the binding of STAT3 (Tables I and II), four of which were identified as STAT3-binding sequences in other human genes (17–20).

ChIP analysis was conducted to confirm the direct binding of STAT3 to the IDO promoter. Three pairs of primers were designed to amplify the regions containing five putative STAT3 binding sites (Fig. 1A1). MDA-MB-231 cells served as the positive control of STAT3 binding to AKAP12 and HIC2 genes. However, none of these putative binding sequences in the IDO promoter region was amplified in the PCR product (Fig. 1A2), which indicated that there is no direct binding between STAT3 and the IDO promoter. Thus, STAT3-induced IDO expression is independent of the direct binding of STAT3 to the IDO promoter region.

Stat3 activation–induced IDO expression does not require C/EBPβ

It has been reported that C/EBPβ, also known as liver-activating protein, is one of the most important downstream transcription factors of the STAT3 pathway that regulates myelopoiesis in healthy individuals and promotes differentiation of myeloid progenitors to functional MDSCs (21). Additionally, the IDO pro-

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDO</td>
<td>Forward: 5′-CATCTGAAAATCCTGACTAAG-3′&lt;br&gt;Reverse: 5′-CACGTGCAACTATACCTCCTC-3′</td>
</tr>
<tr>
<td>NIK</td>
<td>Forward: 5′-AACAGTTATATCTGACAGA-3′&lt;br&gt;Reverse: 5′-GGCAAGGACCATACATGAC-3′</td>
</tr>
<tr>
<td>C/EBPβ</td>
<td>Forward: 5′-TTACAGGCCGAGCTCGTG-3′&lt;br&gt;Reverse: 5′-GGAAAGTGTCTGCCAGCCT-3′</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward: 5′-TGCCACCCAGACCAATGAA-3′&lt;br&gt;Reverse: 5′-CTAATGCTAATGCCCTGCTAGA-3′</td>
</tr>
<tr>
<td>HIC2</td>
<td>Forward: 5′-GCGTACGCAAAGACAGC-3′&lt;br&gt;Reverse: 5′-ACGGAGCCCGCCGCTTC-3′</td>
</tr>
<tr>
<td>AKAP12</td>
<td>Forward: 5′-CGCGTTATTTATTTTTTCCAGGT-3′&lt;br&gt;Reverse: 5′-GTCGCCCAAGTCCAGAG-3′</td>
</tr>
<tr>
<td>skp2</td>
<td>Forward: 5′-ACGAGACCGAGCGGAACCTA-3′&lt;br&gt;Reverse: 5′-AGCTGTCGCCTCCCCAGAT-3′</td>
</tr>
</tbody>
</table>
The moter region contains binding sites for C/EBPβ (ATTCCCAAA, CTTCCTAAA, and CATTCCAAAA) (22). Therefore, we examined whether the C/EBPβ signaling pathway regulates STAT3-mediated IDO upregulation. Because phosphorylation of C/EBPβ at specific sites is essential for its activation and transcriptional activity (23), we measured the phosphorylation level of C/EBPβ in MDSCs. There was no significant difference in the phosphorylation level of C/EBPβ in MDSCs compared with that in CD33+ controls (Fig. 1B). JSI-124, a specific STAT3 antagonist, has been reported to produce significant selective inhibition of the JAK2/STAT3 pathway without off-target effects and has been used for inhibition of the function of the STAT3 pathway in MDSCs both in vivo and vitro (24, 25). JSI-124 treatment decreased the levels of pSTAT3 and pC/EBPβ in MDSCs (Fig. 1B). MDSCs transfected with siRNA against C/EBPβ, but not a nontargeting control siRNA downregulated the level of C/EBPβ mRNA, as detected by RT-PCR (Fig. 1C). Transfection of C/EBPβ-specific siRNA dramatically decreased the phosphorylation level of C/EBPβ, but only slightly decreased IDO expression in MDSCs (Fig. 1C, 1D), indicating that C/EBPβ activation is not likely the primary cause of IDO upregulation.

The noncanonical NF-κB pathway is activated in MDSCs

In inflammatory cells and immunocytes infiltrating into tumors, STAT3-induced activation of the NF-κB signaling pathway promotes an immunosuppressive microenvironment by regulating the expression of multiple genes involved in anti-apoptosis, proliferation, and immune response (26, 27).

### Table II. Suspected binding sites specific for STAT3 in the IDO promoter

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Position 5'</th>
<th>Position 3'</th>
<th>Sequence Length</th>
<th>Consensus STAT3-Binding Sequences in Human Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTCCCCAGAA</td>
<td>−3691</td>
<td>−3683</td>
<td>9</td>
<td>FCGR1A (17); PIM1 (18)</td>
</tr>
<tr>
<td>TTCCCCAGAA</td>
<td>−3656</td>
<td>−3648</td>
<td>9</td>
<td>FCGR1A (17); PIM1 (18)</td>
</tr>
<tr>
<td>TTCCCCAGAA</td>
<td>−3455</td>
<td>−3440</td>
<td>9</td>
<td>CDKN1A (19)</td>
</tr>
<tr>
<td>TTCCCCAGAA</td>
<td>−3456</td>
<td>−3440</td>
<td>9</td>
<td>IRF1 (20)</td>
</tr>
<tr>
<td>TTCCCCAGAA</td>
<td>−1119</td>
<td>−1111</td>
<td>9</td>
<td>Not found</td>
</tr>
</tbody>
</table>

*The position of suspected binding sites from the first coding DNA sequence.

---

**FIGURE 1.** STAT3-induced IDO expression does not occur through the direct binding of STAT3 to the IDO promoter region and does not require C/EBPβ in MDSCs. (A) Binding of STAT3 to three different regions in the IDO promoter, which contains five STAT3-binding candidate sites, was measured by ChIP. (A1) Regions amplified are highlighted with bracketing the binding sites. (A2) ChIP-PCR assay was conducted to detect STAT3 binding to putative binding sequences. AKAP12 and HIC2 genes served as the positive control. Quantitative values are calculated using the ratio of ChIP-PCR product to input-PCR product (relative ChIP value, enrichment ratio). *p < 0.05. (B) Western blot was performed to detect the expression of pSTAT3, p-C/EBPβ, and IDO in the whole-cell extracts of CD33+ controls, MDSCs, and JSI-124–treated MDSCs (J-MDSCs). STAT3 or β-actin blots were used as protein loading controls. (C) The mRNA expression of C/EBPβ and IDO in MDSCs transfected with C/EBPβ-specific siRNA or negative control was examined using RT-PCR assay. (D) Western blot was performed to examine phosphorylation of STAT3, C/EBPβ, and IDO in MDSCs and C/EBPβ-specific siRNA-transfected MDSCs. Data in (A) are quantified from three separate experiments. Data in (B)–(D) are representative of three separate experiments. NCsiRNA, nontargeting control siRNA.
pathway plays an important role in manipulating the suppressive activity of MDSCs as well as IDO expression in DCs (28–30). Therefore, we examined the canonical and noncanonical NF-κB pathways in MDSCs. We detected the levels of p-IKKα and p-IKKβ, which represent the core signaling proteins in the noncanonical and canonical NF-κB pathways, respectively (31, 32). There was a significant increase in p-IKKα at Thr23 and Ser176/180 ($p < 0.05$) and p-IKKβ at Ser177/181 ($p < 0.05$) in MDSCs compared with CD33+ control cells and JSI-124–treated MDSCs (Fig. 2A). Increased p-IKKα and p-IKKβ are associated with increased STAT3 phosphorylation and expression of IDO in MDSCs (Fig. 2A).

Activation of the noncanonical NF-κB pathway is a complex process. High levels of NIK lead to the activation of IKKα, resulting in phosphorylation of p100 and nuclear translocation of RelB/p52 dimers (33). In untreated cells, NIK is constitutively degraded to prevent unnecessary activation (33, 34). In concordance, the level of NIK protein was low in CD33+ controls and significantly increased in MDSCs. Treatment of MDSCs with JSI-124 to block STAT3 activation completely blocked NIK accumulation (Fig. 2B). We also measured the level of p-p100, which plays an important role in the noncanonical NF-κB pathway (33). Consistently, the level of p-p100 notably increased in MDSCs, but not in CD33+ control cells or JSI-124–treated MDSCs (Fig. 2B). Using an ELISA-based nuclear translocation assay, we compared the levels of p50, p52, RelA, and RelB subunits in nuclear lysates of CD33+ control cells and MDSCs. There was a significant increase in the levels of noncanonical NF-κB subunits p52 and RelB, but not canonical NF-κB subunits p50 and RelA, in the nuclear lysates of MDSCs compared with those of CD33+ control cells (Fig. 2C, $p < 0.05$). Blocking STAT3 activation by JSI-124 dramatically decreased the levels of p52 and RelB in nuclei (Fig. 2C, $p < 0.05$). These data indicate that the noncanonical NF-κB pathway is activated in MDSCs and might be involved in STAT3-induced IDO expression.

**STAT3 activation induced IDO expression through NF-κB activation**

Next, we examined whether the activation of the noncanonical NF-κB pathway is responsible for the upregulation of IDO in MDSCs. First, we treated MDSCs with JSI-124 to block the STAT3 pathway and measured changes in STAT3 activation completely blocked NIK accumulation and IDO expression in MDSCs at different time points. The level of STAT3 phosphorylation in MDSCs was reduced at 30 min and completely suppressed at 4 h after treatment with JSI-124. Consistently, the level of NIK in MDSCs started to decline at 1 h

**FIGURE 2.** The noncanonical NF-κB pathway is activated in MDSCs. (A1) The phosphorylation of IKKβ and IKKα in CD33+ controls, MDSCs, and J-MDSCs was measured. (A2) The levels of p-IKKβ and p-IKKα were compared using the density ratio of phosphorylated protein to total protein. *$p < 0.05$. (B) The levels of NIK and p-p100 protein in CD33+ controls, MDSCs, and J-MDSCs were compared. STAT3 or β-actin blots were used as protein loading controls. (C) ELISA was conducted to compare the levels of p50, p52, RelA, and RelB subunits in nuclear extracts of CD33+ controls, MDSCs, and J-MDSCs. The transcriptional activity was measured using absorbance at 450 nm (A450). Data in (A) and (C) are quantified from separate separate experiments. Data in (B) are representative of three separate experiments. *$p < 0.05$. 

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and was completely blocked at 8 h after JSI-124 treatment. The level of IDO significantly decreased at 4 h after JSI-124 treatment (Fig. 3A). These data suggest that the accumulation of NIK and upregulation of IDO is a consequence of STAT3 signaling activation.

To determine whether noncanonical NF-κB activation is the upstream molecular event of IDO expression, MDSCs were transfected with NIK siRNAs to knockdown NIK expression. Two siRNA candidates efficiently attenuated NIK expression in MDSCs at the mRNA and protein levels compared with nontargeting control siRNA (Fig. 3B, 3C). In NIK knockdown MDSCs, the level of IDO protein was reduced significantly, but the level of pSTAT3 protein was not affected (Fig. 3B, 3C). In contrast, both IDO and NIK expression were reduced by JSI-124 treatment in MDSCs (Fig. 3A).

Taken together, these data suggest that STAT3 stimulates IDO expression via noncanonical NF-κB signaling in MDSCs, which is described as the STAT3/NF-κB/IDO pathway.

RelB/p52 dimers directly bind to the IDO promoter to regulate IDO expression in MDSCs

A recent study indicated that RelB/p52 dimers bind to the IDO gene promoter and promote IDO expression in mouse DCs (28). Considering the diversity in the IDO promoter region sequence between mice and humans, we investigated whether a similar molecular mechanism occurred in humans. We conducted a series of experiments to determine whether RelB/p52 dimers directly bind to the IDO promoter in human MDSCs. Bioinformatic analyses were performed to screen potential NF-κB binding sites in the IDO promoter region. In total, 11 putative RelB/p52 binding sites were screened within 3000 bp of the IDO promoter region (Table III). To further determine the specificity of these binding sites to both noncanonical NF-κB subunits p52 and RelB, four biotinylated detection probes containing putative binding sequences were prepared (Fig. 4A). Probes 2 and 3 displayed significantly stronger binding activity to RelB and p52 proteins (Fig. 4B, p < 0.05).

ChIP analysis was then performed to test whether there is direct binding of p52/RelB to the candidate binding sequences in the IDO promoter region. Five pairs of primers were designed to amplify the regions containing six putative noncanonical NF-κB binding sequences (Fig. 4C1). The PCR results demonstrated detectable amplification of three regions containing four putative RelB/p52-specific binding sequences in the IDO promoter region after anti-human RelB and p52 Ab-specific immunoprecipitation (Fig. 4C). The results implied that STAT3 activation stimulates nuclear translocation of two noncanonical NF-κB subunits, p52 and RelB, which directly interact with the IDO promoter and initiates IDO transcription in MDSCs.
Increased STAT3 phosphorylation and NIK expression were correlated with upregulated IDO expression in situ in breast cancer

We investigated whether the levels of STAT3 phosphorylation and NIK expression were correlated with the IDO expression in MDSCs in situ in breast cancer using dual immunofluorescent staining (Fig. 5A). Immunofluorescence results showed that 64.2 ± 7.4% of MDSCs were IDO+ MDSCs (defined as IDO and CD33 double-positive karyocytes) in the stroma (Fig. 5). For MDSCs, 79.7 ± 8.2% were positive for STAT3 phosphorylation (p-STAT3+ MDSCs, defined as p-STAT3 and CD33 double-positive karyocytes) (Fig. 5). The percentage of NIK+ MDSCs (defined as NIK and CD33 double-positive karyocytes) was 65.8 ± 7.5% of MDSCs were IDO+ MDSCs (defined as IDO and CD33 double-positive karyocytes) in the stroma (Fig. 5). For MDSCs, 79.7 ± 8.2% were positive for STAT3 phosphorylation (p-STAT3+ MDSCs, defined as p-STAT3 and CD33 double-positive karyocytes) (Fig. 5). The percentage of NIK+ MDSCs (defined as NIK and CD33 double-positive karyocytes) was 65.8 ± 7.5%

Table III. Suspected binding sites specific for NF-κB factors within the IDO promoter

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Position 5′</th>
<th>Position 3′</th>
<th>Length</th>
<th>Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGGGTTTCAC</td>
<td>−2984</td>
<td>−2975</td>
<td>10</td>
<td>Prediction programs</td>
</tr>
<tr>
<td>GGGAGTTATT</td>
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<td>−2785</td>
<td>9</td>
<td>Identified binding sequence</td>
</tr>
<tr>
<td>AAAATTTCTTG</td>
<td>−2644</td>
<td>−2635</td>
<td>10</td>
<td>Prediction programs</td>
</tr>
<tr>
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<td>−2296</td>
<td>12</td>
<td>Prediction programs</td>
</tr>
<tr>
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<td>−2147</td>
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</tr>
<tr>
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</tr>
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<td>−1969</td>
<td>9</td>
<td>Identified binding sequence</td>
</tr>
<tr>
<td>GAGATGATT</td>
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<td>Identified binding sequence and prediction programs</td>
</tr>
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<td>−1489</td>
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<td>Identified binding sequence and prediction programs</td>
</tr>
<tr>
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<td>−1120</td>
<td>9</td>
<td>Identified binding sequence and prediction programs</td>
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<td>GAAAACACT</td>
<td>−616</td>
<td>−608</td>
<td>9</td>
<td>Identified binding sequence</td>
</tr>
</tbody>
</table>

a The position of suspected binding sites from the first coding DNA sequence.

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*FIGURE 4. Noncanonical NF-κB subunits RelB and p52 regulate IDO expression via direct binding to the IDO promoter region in MDSCs. (A) Four detection biotinylated probes covering 11 putative sequences were synthesized. (B) Detection probes were incubated with MDSC nuclear extracts with or without competitive probes to verify the specificity of NF-κB binding to the biotinylated detection probes. *p < 0.05. (C1) Binding of p52 and RelB to two detection biotinylated probes, which covered six putative noncanonical NF-κB binding sequences in the IDO promoter, was determined by ChIP-PCR assay. Regions amplified are highlighted, bracketing putative noncanonical NF-κB binding sequences. MiaPaCa2 cells served as the positive control for p52 and RelB binding to the skp2 gene promoter. All products were analyzed on agarose gels. (C2) Quantitative values are calculated using the ratio of ChIP-PCR product to input-PCR product (relative ChIP value, enrichment ratio). Data in (B) and (C) are quantified from three separate experiments. *p < 0.05.
These data suggest that the STAT3 signaling and noncanonical NF-κB pathways were activated in most MDSCs in situ in breast cancer. We next compared the correlation among the numbers of p-STAT3+ MDSCs, NIK+ MDSCs, and IDO+ MDSCs in primary cancer tissues. Significantly positive correlation was observed between the numbers of pSTAT3+ MDSCs and IDO+ MDSCs \((p < 0.05, r = 0.5414, \text{Fig. } 5\text{C1})\), which was consistent with our previous results (4). We also found a significant positive correlation between the numbers of p-STAT3+ MDSCs and NIK+ MDSCs, which implied that STAT3 phosphorylation correlated with activation of the noncanonical NF-κB pathway in MDSCs \((p < 0.05, r = 0.5597, \text{Fig. } 5\text{C2})\). Moreover, there was a significant correlation between NIK+ MDSCs and IDO+ MDSCs \((p < 0.05, r = 0.4691, \text{Fig. } 5\text{C3})\), which was consistent with our observation that activation of the noncanonical NF-κB pathway stimulates IDO upregulation in MDSCs in vitro. Taken together, these data implied an aberrantly activated STAT3/NF-κB/IDO pathway in breast cancer–derived MDSCs in situ.

Blocking STAT3 signaling inhibited tumor growth and metastasis in vivo

To investigate the regulatory effect of the STAT3/NF-κB/IDO pathway on MDSC development in vivo, JSI-124 was used to block the STAT3/NF-κB/IDO pathway in MDSCs in mouse 4T1 mammary tumor model. The tumor-bearing mice were treated with JSI-124 or vehicle control (PBS plus DMSO) 5 d after tumor implantation. Tumor growth was significantly inhibited after the JSI-124 treatment. On day 16 after the treatment, the average tumor size in the JSI-124–treated group had shrunk to \(<200 \text{ mm}^3\), which decreased \(>7\)-fold compared with tumors in the vehicle control group (Fig. 6A). The numbers and sizes of metastatic nodules in the lung dramatically reduced in JSI-124–treated mice compared with the controls (Fig. 6B), indicating that JSI-124 significantly inhibited 4T1 mammary tumor growth and metastasis in vivo. Simultaneously, a significant decrease in the numbers of CD11b+ MDSCs in the spleen and cancer tissues were observed in JSI-124–treated mice compared with the controls, which suggests that JSI-124 significantly inhibits MDSC development and accumulation (Fig. 6C). We isolated CD11b+ MDSCs from the spleen of JSI-124–treated mice and found decreased expression of p-STAT3, NIK, and IDO protein in CD11b+ MDSCs compared with those isolated from the controls (Fig. 6D). These data suggest that blocking STAT3 signaling significantly inhibits the activation of the STAT3/NF-κB/IDO pathway in MDSCs in vivo.

IL-6–stimulated STAT3-dependent NF-κB mediates IDO expression in MDSCs

Several reports have indicated that cytokines participate in the expansion and accumulation of MDSCs (1, 35). Thus, we determined whether cytokines trigger STAT3 activation and promote IDO expression during MDSC development and accumulation. The supernatants of MDSCs were collected to detect the levels of 42 cytokines using a human cytokine Ab array (Fig. 7A). The signal intensity of each cytokine in the combined supernatants of MDA-MB-231 cells and CD33+ control cells served as the baseline measurement. The fold change was calculated by comparing the signal intensity of each cytokine in the supernatant of MDSCs to the respective baseline level (Fig. 7B1). The levels of nine cytokines increased \(>2\)-fold in the supernatants of MDSCs (Fig. 7B2). After the JSI-124 treatment, eight of these cytokines decreased \(>2\)-fold in the supernatants of MDSCs (Fig. 7B2). Among them, GM-CSF, IL-6, IL-10, and IL-1β are well-defined inducers of the STAT3 signaling pathway.

FIGURE 5. Correlation among p-STAT3, NIK, and IDO protein expression in breast cancer–derived MDSCs in situ. Paraffin-embedded breast cancer tissues were collected from 30 patients. (A) Breast cancer tissue sections were immunostained with p-STAT3 and CD33 for p-STAT3+ MDSCs (A1), NIK and CD33 for NIK+ MDSCs (A2), and NIK and CD33 for IDO+ MDSCs (A3) (original magnification \(\times200\)). (B) The percentages of p-STAT3+ MDSCs, IDO+ MDSCs, and NIK+ MDSCs in the total MDSC population are shown. (C) The correlations between the distribution of p-STAT3+ MDSCs, IDO+ MDSCs, and NIK+ MDSCs were analyzed.
Next, we measured the secretion of six cytokines in the supernatants of MDSCs using the ELISA method, including GM-CSF, IL-6, IFN-γ, IL-1β, TGF-β, and IL-10, which were reported to induce STAT3 activation and IDO expression efficiently (9, 36, 37). Consistent with results of the cytokine array, the levels of GM-CSF, IL-6, and IL-10 dramatically increased in the supernatants of MDSCs compared with the controls (Fig. 7C, p < 0.05), which implied that these cytokines might be involved in the activation of STAT3 signaling and promotion of IDO expression in MDSCs.

Next, we treated MDSCs with recombinant cytokines IL-1β, GM-CSF, IL-6, and IL-10 to detect their regulatory effects on IDO expression. Increased IDO expression was found in IL-6–treated MDSCs compared with the other cytokine-treated MDSCs (Fig. 7D, p < 0.05). Furthermore, a specific IL-6–neutralizing Ab significantly decreased the levels of p-STAT3, NIK, and IDO in MDSCs (Fig. 7E, p < 0.05). These results indicate that IL-6 might be a dominant trigger to induce STAT3-dependent IDO expression in MDSCs via activation of the STAT3/IDO pathway.

**Discussion**

MDSCs play vital roles in attenuating the therapeutic effects of immunotherapy (1, 35). Progress has been made in the development of innovative drugs targeting MDSCs in recent years (1, 35). However, few therapies provide comparable antitumor efficiency in both hematological and solid malignancies (1) due to the complicated phenotypes of distinct, tumor-derived MDSC populations (3, 35).

In studies investigating MDSCs in breast cancer, most efforts have focused on mouse models rather than humans because of uncertainty of cell phenotypes and complicated regulatory mechanisms in human MDSCs (38–40). In a previous study, our group identified a poorly differentiated subpopulation of MDSCs with the characteristic phenotype of CD13+CD33+CD14-CD15- (4). This MDSC subset significantly inhibited T cell immunity in breast cancer via a STAT3-induced IDO-dependent manner. Therefore, elucidating concrete molecular mechanisms modulating the STAT3/IDO pathway in breast cancer–derived MDSCs may help in the development of novel therapies to reverse MDSC-mediated immunosuppression in breast cancer patients.

In this study, we used a coculture system composed of healthy, donor-derived CD33+ progenitors and the breast cancer cell line MDA-MB-231 in vitro to induce MDSCs with an identical phenotype and immunosuppressive activity as primary MDSCs isolated in situ and to define underlying molecular mechanisms regulating STAT3-dependent IDO expression in primary breast cancer–derived MDSCs (4). Because there is limited information regarding molecular mechanisms for regulation of the STAT3/IDO pathway in breast cancer–derived MDSCs may help in the development of novel therapies to reverse MDSC-mediated immunosuppression in breast cancer patients.

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FIGURE 7. IL-6 stimulates STAT3-dependent IDO expression in MDSCs. (A) Image of human cytokine Ab array. The spot density represents the levels of cytokines in supernatants. UC represents CD33+ controls isolated from primary cord blood. (B1) The levels of 42 cytokines in the supernatant of MDSCs, J-MDSCs, and the control cells are displayed. (B2) Nine cytokines increased at least 2-fold in MDSCs compared with baseline. (Figure legend continues)
It has been demonstrated that perturbation of STAT signaling plays a fundamental role in tumorigenesis (26). In the STAT family, STAT1 and STAT3 play important roles in regulating IDO expression (9). STAT1 can directly bind to the IDO gene promoter to induce rapid transcription in DCs (9). To define whether STAT3 induces IDO expression in MDSCs in a similar manner, the binding activity of p-STAT3 to the IDO gene promoter was evaluated. Five potential binding sequences for STAT binding in the IDO gene promoter region were screened, and four of them have been definitively identified as STAT3-binding sequences in other human genes (17–20). However, no direct binding was observed using ChIP analysis, which implied that transcription factors other than STAT3 regulates IDO expression in breast cancer-derived MDSCs.

We also measured the key downstream transcription factor of STAT3, C/EBPβ, which has been reported as a determining transcription factor in MDSCs similar to Foxp3 in regulatory T cells (37, 41). We found no significant differences of the levels of two phosphorylated C/EBPβ protein subunits, both of which are essential for transcriptional activity, between MDSCs and control cells (23), which is consistent with previous studies reporting that there is no change in C/EBPβ protein expression in human MDSCs compared with normal CD33+ cells controls (3, 41, 42). When we knocked down expression of C/EBPβ using a specific siRNA, only a slight decrease of IDO protein was detected. This implied that C/EBPβ is not involved in regulation of IDO expression.

The NF-κB family has been considered the central mediator of the inflammatory process and a key participant in innate and adaptive immune responses (43). There are two independent signal transduction pathways of NF-κB family, the canonical NF-κB pathway induces IκB-dependent IκB phosphorylation and nuclear translocation of p65-containing heterodimers. The noncanonical NF-κB pathway triggers IKKα-mediated phosphorylation of p100 and generation of transcriptionally active p52/RelB complexes (33). Both NF-κB pathways have been reported to participate in regulation of IDO expression in DCs (28–30). In the present study, we found that the noncanonical NF-κB pathway, but not the canonical NF-κB pathway, accounts for STAT3-mediated IDO up-regulation in MDSCs, which is consistent with findings in DCs and is described as the STAT3/NF-κB/IDO pathway (9).

Although the biological roles of the noncanonical NF-κB pathway have been extensively studied, its functions in specific cell types are obscure (34). In cancer, the roles of noncanonical NF-κB pathway has emerged as one of the most important drivers of tumor-promoting machinery (27, 44). Nadiminty et al. (27) reported that acetylation of STAT3 activates the noncanonical NF-κB pathway, accounts for STAT3-mediated IDO up-regulation and the monocytic (CD11b+Ly6G+Ly6Clo) populations. Ly6G and Ly6C are two distinct isoforms of the Gr1 protein. Therefore, CD11b+Gr1+ MDSCs is used as the conventional phenotype of well-differentiated MDSCs in mice (1). In our previous work, we have demonstrated that breast cancer-derived MDSCs are a poorly differentiated subset of MDSCs, which express low levels of CD14 and CD15. Therefore, we used CD11b+ rather than CD11b+ Gr1+ as the phenotypic marker to isolate poorly differentiated MDSCs. Our results implied that heterogeneous MDSCs might...
use separate mechanisms to regulate T cell immunity in the tumor microenvironment, as is reported in previous studies (1). The consistent conclusion from these studies and ours is that tumor-derived IL-6 is critical for IDO-mediated MDSC immunosuppressive activity. Thus, inhibition of tumor-derived IL-6 might be an efficient approach to inhibit the development and accumulation of MDSCs and reverse MDSC-mediated immunosuppression.

The trigger of the STAT3/NF-κB/IDO pathway in the microenvironment of breast cancer is not clear. Recently, Condamine and Gabrilovich (47) reported that increased secretion of different soluble factors, such as IL-6, GM-CSF, IL-10, and IL-1β, in tumor sites provided a beneficial microenvironment for MDSC development and function. Signaling pathways triggered by most of the above factors in MDSCs converge on STAT3 (35). Our study showed that among the above cytokines, IL-6 is the dominant cytokine to induce STAT3-mediated IDO expression in breast cancer–derived MDSCs. This result is consistent with published works in which IL-6 was reported to induce IDO upregulation in a neuroblastoma cell line in vitro (48). Moreover, it has been reported that CD33+ cells with potent suppressive capacity were generated in vitro by cytokines including IL-6 (42). Thus, we suggest that IL-6 might be a key factor to trigger the STAT3/NF-κB/IDO pathway in MDSCs in breast cancer.

Suppressor of cytokine signaling 3 (SOCS3) is an important downstream target of IL-6 for the activation of STAT1 and STAT3. In normal cells, such as DCs, IL-6 induces rapid upregulation of SOCS3, leading to the suppression of STAT activation and IDO expression, which is described as the SOCS3 feedback loop (49, 50). Dysfunctional silencing of SOCS3 is frequently observed in malignant cells, which could induce long-term activation of STAT3 signaling and resistance to apoptosis (51). It has been reported that in the absence of SOCS3, activation of STAT1 and STAT3 in DCs is markedly enhanced, leading to an increasing IDO expression (49, 50). In our pilot study, we observed a lack in SOCS3 protein in MDSCs compared with myeloid-derived DCs (data not shown), which implied that a defect in the SOCS3 feedback loop in breast cancer–derived MDSCs may contribute to STAT3 phosphorylation and upregulation of IDO expression. Therefore, we are now focusing on validating the correlation between absence of SOCS3 protein and activation of the STAT3/NF-κB/IDO pathway as well as identifying molecular mechanisms that regulate SOCS3 silencing in MDSCs.

In conclusion, our studies suggest that the noncanonical NF-κB pathway regulates STAT3-dependent upregulation of IDO expression in breast cancer–derived MDSCs, which displays potent immunosuppressive function on T cell immunity and efficiently promotes lymph node metastasis in patients with breast cancer. Therefore, specifically blocking the noncanonical NF-κB pathway in MDSCs might improve the clinical efficiency of immunotherapy.

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Disclosure

The authors have no financial conflicts of interest.

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