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Myeloid-Derived Suppressor Cells Suppress Antitumor Immune Responses through IDO Expression and Correlate with Lymph Node Metastasis in Patients with Breast Cancer

Jinpu Yu,* Weijiao Du, † Fang Yan,*‡ Yue Wang,* Hui Li, † Shui Cao, † Wenwen Yu,* Chun Shen,*‡ Juntian Liu,§ and Xiubao Ren*†

Myeloid-derived suppressor cells (MDSCs) represent a heterogeneous population of myeloid cells that are present in cancer, inflammation, and infection. The inhibitory effects of MDSCs on innate and adaptive immunity lead to blocking immune surveillance and preventing the immune system from eliminating newly transformed cells (1). MDSCs impair immunity through multiple mechanisms that include inhibiting the activation of CD4+ (2) and CD8+ (3, 4) T cells, attenuating the cytotoxicity of NK cells (5), blocking L-selectin expression and upregulation of CD15 (6), and polarizing immunity toward a tumor-promoting type 2 phenotype through downregulation of IFN-γ and upregulation of IL-10 (7). MDSCs have been recognized by expression of CD11b and GR-1 in mice (7, 8). However, human MDSCs are less defined but are generally considered to be myeloid derived, nonlinearly determined, and with poor Ag presentation capacity. They are often characterized as Lin−CD33+CD11b+HLA-DR− in humans (9, 10). Furthermore, obvious disparity of the phenotypes of MDSCs isolated from different types of cancer patients has been identified, including hepatic cancer, renal cancer, colon cancer, and melanoma. This evidence implies that differentiation and accumulation of MDSCs may be cancer cell type dependent (11–14). Thus, the variation of the phenotypes of MDSCs limits the investigation of the suppressive mechanism of MDSCs in human cancers.

Previous studies have revealed a variety of mechanisms underlying suppression of T cells by MDSCs, including arginase-1 (ARG1)-mediated local depletion of arginine and cysteine, inducible NO synthase (iNOS), and NADPH oxidase–dependent production of reactive oxygen species and reactive nitrogen species, and activation and expansion of Ag-specific regulatory T cells (Tregs) (15–17). Recent studies demonstrated a distinct correlation between IDO, a rate-limiting enzyme in the catabolism of 1-methyl-L-tryptophan, and tumor-induced immunosuppression (18–20). IDO expression was significantly upregulated in MDSCs isolated from fresh breast cancer tissues (fresh MDSCs [fMDSCs]), which correlated with increased infiltration of Foxp3+ regulatory T cells in tumors and lymph node metastasis in patients. fMDSCs IDO expression was significantly upregulated in MDSCs isolated from healthy peripheral blood (hPB) (21), with L-selectin expression and Th1 polarization but stimulated apoptosis in T cells in an IDO-dependent manner. CD33+ progenitors isolated from healthy donors’ umbilical cord blood were cocultured with breast cancer cell line MDA-MB-231 cells to induce MDSCs. IDO expression was upregulated in induced MDSCs, which required phosphorylation of STAT3, but not STAT1. IDO was required for induced MDSCs’ immunosuppressive activity on T cells, which was blocked by IDO inhibitor 1-methyl-l-tryptophan or STAT3 antagonist JSI-124. Consistently, increased STAT3 phosphorylation level was found in fMDSCs. Together, our findings suggest that STAT3-dependent IDO expression mediates immunosuppressive effects of MDSCs in breast cancer. Thus, inhibition of MDSC-induced T cell suppression by blocking IDO may represent a previously unrecognized mechanism underlying immunotherapy for breast cancer. The Journal of Immunology, 2013, 190: 3783–3797.
response and antitumor cytotoxicity of activated T cells, and to increase CD4+CD25+ Tregs (21–23). It has been reported that several human cells express this molecule, including activated dendritic cells (DCs), macrophages, endothelial cells, fibroblasts, and multiple malignant cells. We reported that upregulated expression of IDO in primary breast cancer correlates with increased infiltrated Tregs in situ and lymph node metastasis (24). We also found that certain myeloid cell-like karyocytes expressed IDO in both primary tumor tissues and tumor-draining lymph nodes in invasive human breast carcinoma (24).

Therefore, this study was designed to determine the IDO expression in MDSCs and the effects of IDO expression in MDSCs on immunosuppression of T cells, and to evaluate the relationship between MDSCs with increased IDO expression and lymph node metastasis in patients with breast cancer. We found that the majority of CD33+ myeloid cells isolated from the breast tumor tissues displayed the characteristic phenotype of CD45+CD13+CD33+CD14−CD15+. Thus, we defined this subset of poorly differentiated myeloid cells isolated from fresh tumor tissues as fresh MDSCs (fMDSCs). The IDO expression level in fMDSCs correlated with lymph node metastasis in patients with breast cancer. IDO mediated fMDSC-induced inhibition on T cell proliferation and Th1 polarization, and promotion of T cell apoptosis and secretion of immunosuppressive cytokines. By using a malignant breast cancer cell–normal myeloid progenitor coculture system, we showed that STAT3 was required for upregulation of IDO in breast cancer cell–induced MDSCs. This subset of MDSCs was defined as induced MDSCs (iMDSCs). Our results imply that target therapy against IDO by either inhibiting the IDO activity or blocking IDO expression to inhibit MDSC-related T cell suppression may represent a previously unrecognized immunotherapy for breast cancer.

Materials and Methods

Patients and healthy donors

In this study, clinical samples were collected from 85 primary breast cancer patients who received radical mastectomy at the Department of Breast Oncology of Tianjin Medical University Cancer Institute and Hospital from January 2009 to December 2009. All patients were women with a median age of 49.78 y old (29–68 y old) and pathologically diagnosed as having invasive ductal carcinoma. According to the sixth edition of the AJCC Cancer Staging Manual and Handbook, our study included 24 patients with the clinical stage I, 33 patients with stage II, and 28 patients with stage III. Among these 85 patients, there were 40 cases with tumor diameter ≥2 cm and 45 cases with tumor diameter ≤2 cm. We collected fresh breast cancer tissues and corresponding adjacent normal tissues from 35 patients, and paraffin-embedded breast cancer tissues and corresponding adjacent normal tissues from the other 50 patients.

Peripheral blood samples from the above 35 patients and 10 healthy donors, and umbilical cord blood (UCB) samples from 5 healthy donors (obtained from Tianjin Blood Center) were prepared to enrich CD33+ myeloid progenitors and CD3+ T cells. This project was approved by the Ethics Committee of Tianjin Medical University. All experiments involving humans were performed in accordance with the principles of Declaration of Helsinki. Written consents were obtained from each patient and healthy donor.

Isolation of CD33+ cells from breast tissues and PBMCs

Thirty-five cases of primary breast cancer tissues and adjacent normal tissues were dissected during surgery. Tumors diagnosed as invasive ductal carcinoma were collected for analyses. Tumor samples were minced into small pieces before they were dissociated into single-cell suspension using the enzyme digestion method (25). PBMCs were isolated from peripheral blood and UCB both of patients and healthy donors using Ficoll separation. Cell viability analysis was performed on the single-cell suspension of peripheral blood and gland blood PBMCs using trypan blue staining method before immunophenotyping and cell isolation. CD33+ cells were enriched using human CD33 MicroBeads (130-045-501; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. In brief, 107 cells were resuspended in 80 μl separating buffer with 20 μl MicroBeads. The cell suspension was incubated at 4˚C for 15 min before loaded onto an LS column for separation. The unlabeled cells passed through the column and were collected as the negative effluent. The magnetically labeled (CD33+) cells were eluted from the column using the plunger.

Immunophenotyping

Expression of membrane markers on CD33+ cells isolated from primary cancer tissues was detected using PE-labeled anti-human CD13, CD14, CD15, IL-4Ra, and CD66b, PerCP-labeled anti-human CD45 and HLA-DR, and allophycocyanin-labeled anti-human vascular endothelial growth factor receptor (VEGFR). The distribution of CD45+CD13+CD33+CD14+CD15- cells in primary cancer tissues, corresponding adjacent normal tissues, patients’ and healthy donors’ peripheral blood, and UCB-derived CD33+ progenitors with or without breast cancer cell induction were detected using flow cytometry. Abs used for phenotyping included PE-labeled anti-human CD33 and CD13, FITC-labeled anti-human CD14 and CD15, and PerCP-labeled anti-human CD45 mAbs (BD Pharmingen, San Diego, CA). The percentage of MDSCs in blood leukocytes was measured by gating the leukocyte population using CD45 versus side scatter and defining MDSCs as the subpopulation of leukocytes that were positive for CD33 and CD14, but negative for CD14 and CD15 expression. The absolute numbers of MDSCs in peripheral blood were detected using the functional dual-platform technology (flow cytometry plus hematology), in which total leukocytes per milliliter detected by trypan blue staining were multiplied with the percentage of MDSCs in blood leukocytes. The average cell viability of single-cell suspension of tissues and blood PBMC samples detected by trypan blue staining was >90%. Viable cells were selected by gating using forward and side scatter characteristics. The isotype-matched IgG1 was used as a negative control. All samples were analyzed using a FACSaria flow cytometer (Becton Dickinson, Mountain View, CA) in 3 replicates, and at least 50,000 events were acquired for each analysis. Because of limited volume of tumor samples and complex phenotypic features, it is difficult to isolate sufficient CD45+CD13+CD33+CD14+CD15- cells from primary cancer tissues. Considering CD45+CD13+CD33+CD14+CD15- cells dominated in the CD33+ cells isolated from cancer tissues using CD33 MicroBeads at frequency of 82.3 ± 3.1%, we defined tumor-derived CD33+ cells as iMDSCs and investigated their immunosuppressive activity and underlying mechanisms in further analysis.

Preparation of iMDSCs by coculturing CD33+ myeloid progenitors with breast cancer cells in vitro

CD33+ myeloid progenitors (2 × 10^7/ml) isolated from healthy UCB were plated in the lower chamber of a Transwell inserted plate (CLS3412; Costar Corning) and cocultured with MDA-MB-231 breast cancer cells, which were plated on the upper chamber at a ratio of 1:5 for 48 h in RPMI 1640 medium supplemented with 10% FBS, in the presence or absence of a specific STAT3 antagonist, Cucurbitacin I (JSI-124; Sigma-Aldrich, St. Louis, MO), to block the activation of Jak2/Stat3. Culture of CD33+ myeloid progenitors without the breast cancer cells was used as the negative control. After 48 h of coculture, the CD33+ cells in the lower chamber were harvested and the corresponding supernatants were collected to detect the levels of multiple cytokines by ELISA assays. The phenotype of harvested cells was examined using flow cytometry as described earlier. The cells that displayed the characteristic phenotype of CD45+CD13+CD33+CD14+CD15- as iMDSCs were defined as breast cancer cell–induced MDSCs and sorted by flow cytometry for further analysis.

Detecting the suppressive effects of fMDSCs and iMDSCs on CD3+ T cells

iMDSCs enriched by MicroBeads from primary cancer tissues or iMDSCs sorted by flow cytometry from cancer cell–treated CD33+ progenitors were collected for viability test using trypan blue staining method. CD3+ T cells were prepared from 10 cases of healthy PBMCs using Human Pan T Cells Isolation Kit II (130-091-156; Miltenyi Biotec) and tested for cell viability. Both MDSCs and T cells with viability >95% were used for functional assays. Purified T cells (4 × 10^5) were plated in a 24-well plate and cocultured with fMDSCs, iMDSCs, or their corresponding controls at ratios of 1:1 and 1:3 in RPMI 1640 medium supplemented with 10% FBS, 50 or 500 IU/ml recombinant human IL-2 at 73°C in a 5% CO2 incubator. Two specific T cell proliferation amplification protocols, stimulation using IL-2 (500 IU/ml; PeproTech, Rocky Hill, NJ) and anti-CD3/CD28 mAb (at bead/cell ratio of 1:1; Dynabeads Human T-Activator CD3/CD28), Invitrogen, Carlsbad, CA), were used to evaluate the immunosuppressive...
mediated immunity. Cucurbitacin I (JSI-124), a specific STAT3 antagonist, tryptophan (1-MT; Sigma-Aldrich), a specific IDO inhibitor, was used at counts + stimulator control counts). To detect apoptosis of T cells, we calculated using the formula: SI = (experimental counts)/(responder control counts). mRNA levels of IDO and STAT3 genes in each sample were calculated for each tissue section were selected for histology evaluation. The positive control for fMDSCs; and 2) healthy, UCB-derived, untreated CD33+ cells were stained blue on the membrane using 5-bromo-4-chloro-3-indolyl phosphate/NBT as substrate for ALP. Sections were then counterstained with hematoxylin. All sections were observed using an Olympus BX51 microscope. Five representative high-power fields (300 magnification) were evaluated with 500 IU/ml IL-2 only were used as T cells control. 1-Methyl-L-tryptophan (1-MT; Sigma-Aldrich), a specific IDO inhibitor, was used at a concentration of 500 μM to block the regulatory effect of IDO on T cell-mediated immunity. Cucurbitacin I (JSI-124), a specific STAT3 antagonist, tryptophan (1-MT; Sigma-Aldrich), a specific IDO inhibitor, was used at counts + stimulator control counts. To detect apoptosis of T cells, we calculated using the formula: SI = (experimental counts)/(responder control counts). To detect apoptosis of T cells, we calculated using the formula: SI = (experimental counts)/(responder control counts). To detect apoptosis of T cells, we calculated using the formula: SI = (experimental counts)/(responder control counts). Tissue sections were incubated with mouse anti-human IDO (Millipore, Billerica, MA), Foxp3 (Bioscience, San Diego, CA), STAT3 (Santa Cruz, San Diego, CA), estrogen receptor (ER) and progesterone receptor (PR; DAKO, Carpinteria, CA) mAbs. The immunohistochemistry was performed with 500 IU/ml IL-2 only were used as T cells control. 1-Methyl-L-tryptophan (1-MT; Sigma-Aldrich), a specific IDO inhibitor, was used at a concentration of 500 μM to block the regulatory effect of IDO on T cell-mediated immunity. Cucurbitacin I (JSI-124), a specific STAT3 antagonist, tryptophan (1-MT; Sigma-Aldrich), a specific IDO inhibitor, was used at counts + stimulator control counts. To detect apoptosis of T cells, we calculated using the formula: SI = (experimental counts)/(responder control counts). Tissue sections were incubated with mouse anti-human IDO (Millipore, Billerica, MA), Foxp3 (Bioscience, San Diego, CA), STAT3 (Santa Cruz, San Diego, CA), estrogen receptor (ER) and progesterone receptor (PR; DAKO, Carpinteria, CA) mAbs. The immunohistochemistry was performed with 500 IU/ml IL-2 only were used as T cells control. 1-Methyl-L-tryptophan (1-MT; Sigma-Aldrich), a specific IDO inhibitor, was used at a concentration of 500 μM to block the regulatory effect of IDO on T cell-mediated immunity. Cucurbitacin I (JSI-124), a specific STAT3 antagonist, tryptophan (1-MT; Sigma-Aldrich), a specific IDO inhibitor, was used at counts + stimulator control counts. To detect apoptosis of T cells, we calculated using the formula: SI = (experimental counts)/(responder control counts). Tissue sections were incubated with mouse anti-human IDO (Millipore, Billerica, MA), Foxp3 (Bioscience, San Diego, CA), STAT3 (Santa Cruz, San Diego, CA), estrogen receptor (ER) and progesterone receptor (PR; DAKO, Carpinteria, CA) mAbs. The immunohistochemistry was performed with 500 IU/ml IL-2 only were used as T cells control. 1-Methyl-L-tryptophan (1-MT; Sigma-Aldrich), a specific IDO inhibitor, was used at a concentration of 500 μM to block the regulatory effect of IDO on T cell-mediated immunity. Cucurbitacin I (JSI-124), a specific STAT3 antagonist, tryptophan (1-MT; Sigma-Aldrich), a specific IDO inhibitor, was used at counts + stimulator control counts. To detect apoptosis of T cells, we calculated using the formula: SI = (experimental counts)/(responder control counts).
The population of CD45^+CD33^+CD13^+CD14^+CD15^2 cells increases in primary breast cancer tissues and patients’ peripheral blood. (A) Paraffin-embedded breast cancer tissues were collected from 50 patients to detect CD33^+fMDSCs in situ by IHC staining using anti-CD33 Ab. CD33^+fMDSCs (brown staining on the cell membrane) were detected in the stroma of breast cancer tissues (A1), but not in normal adjacent tissues (A2) (original magnification ×400). (B) Fresh primary breast cancer tissues were collected from 35 patients to isolate CD33^+fMDSCs using magnetic bead separation technique. Wright-Giemsa staining of fMDSCs displayed myeloid precursors alike in histological appearance (original magnification ×1000). (C) The phenotypes of purified fMDSCs were analyzed using flow cytometry and multiple anti-human mAbs against the indicated markers, including CD45, CD13, CD14, CD15, HLA-DR, IL-4R, CD66b, and VEGFR. (D) The distribution of the CD45^+CD33^+CD13^+CD14^+CD15^− subpopulation in 35 cases of primary cancer tissues, adjacent normal tissues, patients’ peripheral blood, and 10 cases of healthy donors’ peripheral blood was compared using flow cytometry. (1) The subpopulation was gated using anti-CD45 mAb; (2) isotype control was used; and (3) the CD33^+CD13^+CD14^+CD15^− subpopulation was distinguished in Q1. The representative flow cytometry graphs of different samples are shown. The proportion of the interested subpopulation significantly increased in cancer tissues and patients’ blood. (E) The percentages of CD45^+CD13^+CD33^+CD14^+CD15^− cells and other reported MDSC subpopulations (including CD11b^−HLA-DR^−, CD33^+HLA-DR^−, and CD14^+HLA-DR^−) in blood of 35 breast cancer patients were compared with the counterparts in 10 healthy donors.

subpopulation significantly increased in cancer tissues and patients’ blood compared with that in adjacent normal tissues and healthy blood (p < 0.05). No significant difference of the proportion of the CD45^+CD33^+CD13^+CD14^+CD15^− subpopulation was observed in cancer tissues and in patients’ peripheral blood (p > 0.05). The mean absolute number of CD45^+CD33^+CD13^+CD14^+CD15^− subpopulation in patients’ peripheral blood was 531 ± 355 cells/µL, ranging from 117 to 1597 cells/µL, and the mean absolute number of CD45^+CD33^+CD13^+CD14^+CD15^− subpopulation in healthy peripheral blood was 82 ± 51 cells/µL, ranging from 11 to 151 cells/µL. Thus, the absolute numbers of CD45^+CD33^+CD13^+CD14^+CD15^− subpopulation significantly increased in patients’ blood compared with that in healthy blood (p < 0.01). However, after cell enrichment using CD33 MicroBeads,
the proportion of the CD45+CD33+CD13+CD14+CD15− subpopulation in the CD33+ cells in cancer tissues was significantly higher than that in patients’ peripheral blood (82.3 ± 3.1 versus 19.4 ± 8.7%; p < 0.05), which implied that the CD45+CD13+CD33+CD14−CD15− subpopulation dominated in cancer tissues of breast cancer patients.

Furthermore, the proportions of other MDSC subpopulations with different markers reported in other cancer types (29) were analyzed in breast cancer patients (n = 35) and healthy donors (n = 10). The proportions of CD45+CD13+CD33+CD14+CD15− cells and CD33+HLA-DR− cells were significantly higher in blood of patients than healthy donors (p < 0.05), but no significant difference was observed in CD11b+HLA-DR− and CD14+HLA-DR+ subpopulations (Fig. 1E). This evidence suggests that CD45+CD13+CD33+CD14−CD15− cells might represent a unique phenotype of MDSCs in breast cancer.

Next, we evaluated the correlation between the proportion of CD45+CD13+CD33+CD14+CD15− cells in 35 cases of primary cancer tissues and patients’ clinicopathological features. The patients with advanced clinical stages and more lymph nodes metastasis had increased levels of fMDSCs in situ, which were 15.38% ± 5.69% in stage III versus 6.32 ± 4.2% in stages I and II (p < 0.0001) and 15.02 ± 5.87% in patients with ≥3 metastatic lymph nodes versus 6.82 ± 4.8% in patients with ≤3 metastatic lymph nodes (p = 0.0002). No significant correlation was identified between the proportion of CD45+CD13+CD33+CD14−CD15− cells and other clinicopathological features, including age, tumor diameter, histological grade, and expression of hormone receptors, such as ER, PR, and Her2 (Fig. 2, Tables I, II). Therefore, CD45+CD13+CD33+CD14−CD15− cells serving as a major subpopulation in CD33+ cells in cancer tissues was defined as MDSCs in breast cancer. MDSCs isolated from primary cancer tissues were termed as fMDSCs.

fMDSCs displayed immunosuppressive activity on T cells

T cells were stimulated with IL-2 or anti-CD3/CD28 mAb and proliferation of T cells was detected using BrdU labeling method to induce polyclonal amplification of T cells. IL-2 (500 IU/ml) or anti-CD3/CD28 mAb (at bead/cell ratio of 1:1)–induced T cell proliferation was significantly inhibited by fMDSCs at cell ratio (T:fMDSCs) of 1:1 (p < 0.05), compared with IL-2 or anti-CD3/CD28 mAb only treated group (Fig. 3A). No significant inhibition on T cell proliferation was found by the CD33+ cells isolated from healthy donors’ blood (healthy peripheral blood [hPB]-CD33, T:hPB-CD33 = 1:1) or patients’ blood (patient peripheral blood [pPB]-CD33, T:pPB-CD33 = 1:1; Fig. 3A).

We also detected the effects of fMDSCs and pPB-CD33 on proliferation of T cells treated with different levels of IL-2 and at different cell ratios. When the T cells were treated with IL-2 at 50 IU/ml at cell ratio of 1:1, no T cell amplification and no fMDSC-induced inhibition on proliferation was observed. When T cells were treated with IL-2 at 500 IU/ml, 73.9 ± 5.3% of T cell proliferation was suppressed by fMDSCs at the cell ratio of 1:1, which was significantly lower than that at cell ratio of 1:3 (82.1 ± 5.5%; p < 0.05; Fig. 3B). At both ratios, fMDSCs induced a more potent inhibitory effect on 500 IU/ml of IL-2–induced T cell proliferation than pPB-CD33 did (19.1 ± 17.9% at 1:1 and 25.8 ± 7.2% at 1:3; p < 0.05; Fig. 3B). These data suggested that fMDSCs suppressed proliferation of T cells in a dose- and cell ratio–dependent manner.

Next, the effects of fMDSCs at cell ratio of 1:1 on cytokine production by T cells were studied using ELISA method. A total of 500 IU/ml IL-2 or anti-CD3/CD28 mAb significantly stimulated production of multiple cytokines, including IFN-γ, TGF-β, IL-4, IL-10, and IL-12 by T cells (p < 0.05; Fig. 3C). fMDSCs inhibited IL-2–induced IFN-γ and increased TGF-β and IL-10 production by activated T cells (Fig. 3C), in which IFN-γ decreased from 179.7 ± 15.82 to 53.26 ± 1.62 pg/ml, IL-10 increased from 168.0 ± 19.00 to 311.3 ± 9.02 pg/ml, and TGF-β increased from 10.41 to 12.46 to 698.6 ± 25.63 pg/ml (p < 0.001; Fig. 3C1). Consistently, fMDSCs inhibited anti-CD3/CD28 mAb-induced IFN-γ production (from 724.9 ± 31.16 to 261.7 ± 38.09 pg/ml), increased IL-10 (from 240.5 ± 68.37 to 425.4 ± 13.76 pg/ml), and TGF-β (from 144.7 ± 16.39 to 355 ± 10.41 pg/ml; p < 0.05; Fig. 3C2). However, no significant effects of fMDSCs on production of IL-4 and IL-12 by T cells were observed. pPB-CD33 did not significantly affect IFN-γ, IL-10, and TGF-β production (p > 0.05) by T cells stimulated by 500 IU/ml IL-2 or anti-CD3/CD28 mAb (Fig. 3C).

To detect the effects of fMDSCs on T cell survival, we measured apoptosis of T cells cocultured with or without fMDSCs using Annexin V staining and flow cytometry method. Compared with
increased IDO in MDSCs suppresses immune responses

pPB-CD33, fMDSCs at cell ratio of 1:1 enhanced apoptosis in IL-2–induced T cells, which were 31.95 ± 2.03% versus 18.18 ± 1.98% (p < 0.05; Fig. 3D1). Consistently, fMDSCs at ratio of 1:1 also promoted apoptosis in anti-CD3/CD28 mAb–induced T cells, which were 34.65 ± 2.86% versus 21.50 ± 3.71% (p < 0.05; Fig. 3D2). These results suggested that fMDSCs possessed potent immunosuppressive activity on T cells in vitro.

IDO expression level in fMDSCs correlated with increased infiltration of Foxp3+ Tregs in situ and lymph node metastasis in breast cancer patients

We have found that IDO expression levels increased in primary tissues of invasive breast cancer (24); we further investigated whether IDO expression was upregulated in fMDSCs. IHC results showed that IDO+ karyocytes dispersed in the stroma between IDO+ cancer cell nests with brownish yellow staining in cytoplasm (Fig. 4A). We found karyocytes with coexpression of IDO and CD33 in 87.5% of breast cancer samples (28/32), which showed significantly higher than the rate of 36.36% (8/22) in IDO+ fMDSC negative counterparts (p = 0.002; Fig. 4D2).

To evaluate the clinical significance of IDO+ fMDSCs in situ, the correlation between the distribution of IDO and IDO+ fMDSCs in 50 cases of paraffin-embedded breast cancer tissues and multiple clinicopathological features of the corresponding patients was analyzed using cross-tab test and nonparametric correlation analysis. As shown in Table III, IDO+ tumors correlated with high risk for lymph node metastasis (p = 0.024). The IDO+ fMDSCs level was increased in cancer tissues from patients with detectable lymph node metastasis, compared with that in patients with no detectable lymph node metastasis (p = 0.027). These results implied that fMDSCs with IDO expression might correlate with lymph node metastasis of breast cancer tissues. In contrast, there was no significant correlation among the distribution of IDO+ fMDSCs and other clinicopathological features, such as age, menstrual status, tumor diameter, histological grade, and expression of hormone receptors, including ER, PR, and Her2.

IDO expression was upregulated in fMDSCs, which is required for inhibition of T cell immunity in vitro

To define whether upregulated IDO expression was induced by tumor or constitutively expressed on CD33+ myeloid cells, we compared the IDO expression at mRNA and protein levels between tumor-derived fMDSCs and CD33+ cell derived from patients’ peripheral blood using RT-PCR assay and Western blot analysis. Significantly increased mRNA level and higher protein level of IDO were detected in fMDSCs compared with the CD33+ fMDSC negative counterparts (p < 0.05; Fig. 3D1). Consistently, fMDSCs at ratio of 1:1 enhanced apoptosis in anti-CD3/CD28 mAb–induced T cells, which were 31.95 ± 2.03% versus 21.50 ± 3.71% (p < 0.05; Fig. 3D2). These results suggested that fMDSCs possessed potent immunosuppressive activity on T cells in vitro.

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myeloid cells were recruited from the peripheral blood to the tumor microenvironment. Because Arg1 was reported as a characteristic biomarker of MDSCs in multiple cancer types that displayed similar enzyme activity as IDO, we compared the protein expression of these two enzymes in fMDSCs. The level of IDO was relatively higher than that of Arg1 in the same samples as Western blot implied (Fig. 5B). Therefore, we concluded that IDO, rather than Arg1, is the most predominant rate-limiting enzyme in tryptophan metabolism in fMDSCs.

In our previous study, IDO was demonstrated to inhibit T cell–based immunity by downregulating IL-2 and/or anti-CD3 mAb OKT3-induced T cell proliferation and activation via blocking phosphorylation of Vav1 protein in T cells (27). Therefore, we investigated whether fMDSC-mediated T cell immunosuppression was induced by increased IDO expression. Compared with patients’ PB-derived CD33+ cells control, fMDSCs significantly inhibited IL-2–induced (57.99 ± 6.09%) and anti-CD3/CD28 mAb–induced (33.81 ± 6.25%) polyclonal amplification of T cells (p < 0.05; Fig. 5C). Most importantly, the specific IDO inhibitor, 1-MT, abolished fMDSC-regulated inhibitory effect on T cell amplification, with the inhibitory rate as 9.06 ± 6.13% for IL-2 stimulation and 2.32 ± 3.79% for CD3CD28 mAb stimulation.

**FIGURE 3.** fMDSCs have immunosuppressive activity on T cells. T cells were isolated using Ficoll separation liquid for detection of proliferation (A, B) and apoptosis (D) using BrdU labeling and Annexin V staining, respectively. The T cell culture supernatant was collected for detecting the levels of cytokines using ELISA assay (C). (A) A total of 4 × 10^5 T cells stimulated with 500 IU/ml IL-2 or anti-CD3/CD28 mAb at bead/cell ratio of 1:1 were cocultured with fMDSCs and control cells at ratio of 1:1 in RPMI 1640 medium supplemented with 10% FBS for 3 d for detecting proliferation. Patients’ peripheral blood–derived CD33+ cells (pPB-CD33+) and healthy donors’ peripheral blood–derived CD33+ cells (hPB-CD33+) were used as the negative control for fMDSCs. (B) T cells were treated with either 50 or 500 IU/ml IL-2 at cell ratios of both 1:1 and 1:3 for detecting proliferation. (C) T cells stimulated with 500 IU/ml IL-2 stimulation (1) and anti-CD3/CD28 mAb at bead/cell ratio of 1:1 (2) were cocultured with fMDSCs and control cells at ratio of 1:1. Supernatants were collected for detecting indicated cytokine levels using ELISA assay. fMDSCs significantly inhibited the secretion of IFN-γ, but promoted secretion of TGF-β and IL-10. (D) T cells stimulated with 500 IU/ml IL-2 (1) and anti-CD3/CD28 mAb at bead/cell ratio of 1:1 (2) were cocultured with fMDSCs and control cells at ratio of 1:1 for detecting apoptosis. T cells were gated using PeCy5-labeled anti-CD3 mAb, and apoptotic cells were stained with FITC-labeled Annexin V. Cells in Q4 represent apoptotic T cells. fMDSCs significantly promoted apoptosis of both IL-2– and anti-CD3/CD28–stimulated T cells. Data in this figure are representative of at least five separate experiments. *p < 0.05 compared with the indicated group, **p < 0.05 compared with all other groups in a graph.
A

B

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**FIGURE 4.** fMDSCs express IDO and the IDO expression level in fMDSCs correlates with infiltration of Foxp3+ Tregs in situ. Paraffin-embedded breast tissues were collected from 50 breast cancer patients for IHC staining of IDO and CD33. IDO was stained using mouse anti-human IDO Ab and HRP-conjugated second Ab. CD33 was stained using rabbit anti-human CD33 Ab and ALP-conjugated second Ab. (A) IDO in cancer tissues mainly expressed in tumor cells and some karyocytes dispersed in stroma (red arrows) with brownish yellow staining in cytoplasm. (B) The coexpression of IDO and CD33 in tumor tissues was determined using double-staining method. The double-positive cells (IDO+fMDSCs, red arrows) were stained red in the cytoplasm (IDO) and blue on the membrane (CD33). IDO+fMDSCs were detected in 87.5% of breast cancer samples. (C) Tregs in tumor tissues were detected by staining Foxp3 using mouse anti-human Foxp3 mAbs and HRP-conjugated goat anti-mouse IgG secondary Ab. Foxp3 was expressed in lymphocytes dispersed in stroma with brownish dark yellow staining in nucleus (red arrows). Original magnification ×400. (D) The correlation between the distribution of IDO+ fMDSCs and Foxp3+ Tregs was compared in situ. The percentages of Foxp3+ Tregs infiltrated in cancer tissues were selected for histology evaluation, and at least 100 cells were counted per field. (1) High frequency of Foxp3+ Tregs was detected in IDO+ tumors at a rate of 69.44%, and significantly higher than that in IDO- tumors (35.71%; p = 0.029). (2) High Tregs was more frequently observed in IDO+ fMDSC positive tumors than in IDO- fMDSC negative tumors (78.57 versus 36.36%; p = 0.002), which implied that fMDSC-mediated apoptosis of T cells required IDO activity. Therefore, fMDSCs inhibited T cell immunity in an IDO-dependent manner in vitro. iMDSCs exert an immunosuppressive effect on T cells via an IDO-dependent manner

To mimic a breast cancer cell–conditioned microenvironment in vitro, CD33+ myeloid progenitors isolated from five healthy donors’ UCB samples were cocultured with MDA-MB-231 breast cancer cells using a Transwell system. After 48 h of coculturing, the proportion of CD45+CD33+CD13+CD14+CD15- cells in CD33+ cells significantly increased 5-fold, from 4.17 ± 2.16% to 19.37 ± 3.88% (p < 0.05; Fig. 6A). We defined these cells as breast cancer cell iMDSCs. We then sorted iMDSCs for functional analysis. The untreated CD33+ myeloid progenitors isolated from healthy UCB were used as negative control. In untreated CD33+ cell population, <5% of cells showed the same iMDSCs’ phenotype, CD45+CD13-CD33-CD14- CD15+ cells. To define whether iMDSCs exhibit a similar immunosuppressive function as fMDSCs isolated breast cancer tissues, we cocultured iMDSCs with T cells as described earlier to detect apoptosis and cytokine production. Although both CD33+ progenitor controls and iMDSCs induced apoptosis of T cells (18.53 ± 2.58 and 38.20 ± 1.70%, respectively; p < 0.05 compared with T cell only), iMDSCs stimulated a...
higher level of apoptosis, compared with CD33+ progenitors (p < 0.05; Fig. 6B). Blocking IDO activity by 1-MT attenuated apoptotic effect of iMDSCs on T cells (p < 0.05; Fig. 6B), but not on T cell apoptosis induced by CD33+ progenitor controls (data not shown). Furthermore, compared with the CD33+ progenitors controls, iMDSCs significantly decreased secretion of IFN-γ and increased production of IL-10 and TGF-β by T cells (p < 0.05; Fig. 6C). Importantly, 1-MT significantly suppressed the inhibitory effect of iMDSCs on IFN-γ and the stimulatory effect on TGF-β and IL-10 (p < 0.05; Fig. 6C). These data suggest that iMDSCs, like fMDSCs, exert an immunosuppressive effect on T cells in an IDO-dependent manner.

**Phosphorylation of STAT3 is required for upregulation of IDO expression and IDO-dependent immunosuppressive activity of iMDSCs**

Because IDO mediates the apoptotic effect on T cells by iMDSCs (Fig. 6), we next studied the mechanism of breast cancer cell–induced IDO expression in iMDSCs. The expression of IDO in iMDSCs at mRNA and protein levels was examined using qRT-PCR and Western blot analysis, respectively. Compared with the untreated UCB-derived CD33+ myeloid progenitors, the IDO mRNA level, but not the STAT3 mRNA level, significantly increased in iMDSCs (p = 0.002; Fig. 7A). The IDO protein level also increased in iMDSCs compared with that in CD33+ progenitors control (Fig. 7B).

It has been reported that both STAT1 and STAT3 are involved in the regulation of IDO expression in macrophages and DCs, and activation of STAT1 induced by type I and II IFN is widely accepted as the canonical mechanism (30, 31). Therefore, the expression and phosphorylation of STAT1 and STAT3 in iMDSCs were examined using Western blot analysis. Although the total protein levels of STAT3 and STAT1 in iMDSCs and CD33+ progenitors were the same, the level of p-STAT3 protein, but not p-STAT1 protein, increased significantly in iMDSCs. There was no detectable level of p-STAT3 in untreated CD33+ progenitors, but after coculturing with MDA-MB-231 breast cancer cells for 2 d, the level of p-STAT3 significantly upregulated in iMDSCs (Fig. 7A, 7B). JSI-124, the specific antagonist of STAT3 phosphorylation, downregulated p-STAT3 and IDO proteins in iMDSCs (Fig. 7B). These results indicate that upregulated expression of IDO in iMDSCs is dependent on phosphorylation of STAT3.

Furthermore, compared with iMDSCs treated with IL-2–stimulated T cells, the proliferation rates significantly increased by JSI-124 (36.89 ± 10.3%) and by 1-MT (32.19 ± 5.4%; p < 0.05; Fig. 7C). Similarly, the proliferation rates significantly increased by JSI-124 (30.28 ± 7.5%) and by 1-MT (32.53 ± 7.02%), compared with iMDSCs treated with anti-CD3/CD28 mAb–stimulated T cells (p < 0.05; Fig. 7C). Thus, blocking STAT3 phosphorylation suppressed iMDSC-mediated inhibition of IL-2– or anti-CD3/CD28–stimulated T cell proliferation.

JSI-124 displayed the same potent inhibitory effects as 1-MT on iMDSC-mediated suppression on Th1 polarization, in which JSI-124 significantly attenuated iMDSC-induced inhibition on IFN-γ production and enhancement of IL-10 and TGF-β (p < 0.05; Fig. 7D). These results implied that blocking phosphorylation of STAT3 downregulated IDO expression in iMDSCs, resulting in attenuating iMDSC-mediated immunosuppressive activity on T cells.

Next, we investigated the expression of STAT3 protein in situ of 50 cases of paraffin-embedded primary breast cancer tissues. In addition to cancer cells, some karyocytes appeared in a cluster around the cancer nest with myeloid precursor-like histomorphology that showed positive STAT3 staining (Fig. 7E), which suggested that activation and accumulation of STAT3 might occur in iMDSCs. Thus, the total and phosphorylated STAT3 levels in iMDSCs from primary cancer tissues were examined. Compared with peripheral blood–derived CD33+ controls, increase of p-STAT3

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**Table III. The correlation between the distribution of IDO+ fMDSCs in breast cancer tissues and clinicopathological features in 50 patients**

<table>
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<tr>
<th>Clinicopathological Features</th>
<th>Case</th>
<th>Negative</th>
<th>Positive</th>
<th>p</th>
<th>IDO+ fMDSCs in Tumor</th>
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<th>Positive</th>
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<td>0.305</td>
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<td>6</td>
<td>10</td>
<td>0.513</td>
<td>5</td>
<td>11</td>
<td>0.174</td>
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<td>34</td>
<td>8</td>
<td>26</td>
<td>0.513</td>
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<tr>
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<tr>
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<td>24</td>
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<tr>
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<td>3</td>
<td>0.100</td>
<td>4</td>
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<tr>
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<td>4</td>
<td>0.046</td>
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<td></td>
<td></td>
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<tr>
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<td>4</td>
<td>23</td>
<td>0.024</td>
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<td>Her2 +</td>
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</tbody>
</table>

Positive or negative was determined according to the percentages of the positive staining cells in tissues: positive means the percentage of positively staining cells ≥10%; negative means the percentage of positively staining cells <10%.
protein and IDO protein was observed in fMDSCs (Fig. 7E). Therefore, STAT3 phosphorylation-dependent IDO expression may serve as one of the mechanisms underlying fMDSC-mediated immunosuppression of T cells in breast cancer.

**Discussion**

An inflammatory microenvironment plays a pivotal role in tumorigenesis and metastasis (31). In the “immunoediting theory” (32, 33), inflammatory leukocytes with potent immunosuppressive effects, including MDSCs, are recruited from the peripheral blood to the tumor and assembled in situ. MDSCs have been recognized by their roles in the inhibition of innate and adaptive antitumor immunity, and in the direct participation in tumor-associated angiogenesis (25, 34). In this study, we reported that a population of CD45+CD33+CD13+CD14-CD15- cells increased in primary cancer tissues and peripheral blood, which displayed myeloid precursors alike in histological appearance and expressed MDSCs’ surface markers, including CD66b and IL-4R. The proportion of this cell population correlated with clinical stage and lymph node metastasis status in breast cancer patients and exerted potent immunosuppressive activity on T cells, and thus defined them as MDSCs in breast cancer.

MDSCs are often characterized as Lin-CD33+CD11b+HLA-DR- in humans, which can be divided into two subtypes: polymorphonuclear granulocytic MDSCs (PMN-MDSCs) and monocytic MDSCs (MO-MDSCs) (29). As myeloid lineage markers,
iMDSCs exert immunosuppressive effect on T cells via an IDO-dependent manner. iMDSCs were generated by coculturing UCB-derived CD33+ cells with breast cancer cell line MDA-MB-231 cells in a Transwell system at a ratio of 1:5. (A) Cancer cell–treated, UCB-derived CD33+ progenitors were analyzed using flow cytometry. Untreated UCB-derived CD33+ cells were used as the negative control. iMDSCs were gated by CD33+CD13+CD14-CD15- in the CD45+ subpopulation. Representative flow cytometry images showed that iMDSCs increased ∼5-fold after 2-d coculture. (1–3) Isotype control, untreated CD33+ controls, and MDA-MB-231–treated CD33+ iMDSCs. (B) The sorted iMDSCs were cocultured with healthy donors’ T cells at ratio of 1:1 with or without 1-MT. The apoptosis of T cells was detected using Annexin V staining method. A total of 500 IU/ml IL-2–stimulated T cells were used as T cell control. T cells were gated using anti-CD3 mAb as described in Fig. 3, and apoptotic cells were stained with FITC-labeled Annexin V. iMDSCs significantly promoted apoptosis of IL-2–stimulated T cells, which was blocked by the IDO inhibitor 1-MT. (1–5) Isotype control, T cells control, CD33+ controls stimulated T cells, iMDSCs stimulated T cells and iMDSC–stimulated T cells with 1-MT. (6) Summary of (1–5). (C) iMDSCs were cocultured with healthy donors’ T cells at ratio of 1:1 with or without 1-MT. Cytokines secreted by T cells was detected as described in Fig. 3. iMDSCs significantly decreased the secretion of IFN-γ, but increased the secretion of IL-10 and TGF-β by IL-2–activated T cells, which was completely reversed by 1-MT. Data in this figure are representative of at least five separate experiments. *p < 0.05.
Phosphorylation of STAT3 is required for upregulated IDO expression and IDO-dependent immunosuppressive activity of iMDSCs. (A) Cancer cell–treated UCB-derived CD33+ progenitors were sorted using flow cytometry to isolate iMDSCs. Untreated UCB-derived CD33+ progenitors were used as the negative control. The mRNA levels of IDO and STAT3 were quantified using qRT-PCR. The primers for IDO, STAT3, and β-actin are listed in Table I. The relative mRNA levels were calculated using β-actin normalized Ct (ΔΔCt). IDO mRNA, but not STAT3 mRNA, significantly increased in iMDSCs compared with the CD33+ cells control. (B) UCB-derived CD33+ progenitors were cocultured with breast cancer cell line MDA-MB-231 in the presence or absence of a specific STAT3 antagonist JSI-124. iMDSCs were sorted using flow cytometry. Untreated UCB-derived CD33+ progenitors were used as the negative control. The expression and the phosphorylation status of IDO and STAT3 were analyzed by Western blot analysis. The expression of IDO and p-STAT3 is upregulated in iMDSCs compared with CD33+ progenitors, and IDO expression was fully eliminated after blocking STAT3 activity by specific STAT3 antagonist JSI-124. (C and D) iMDSCs generated from cancer cell–treated, UCB-derived CD33+ progenitors in (Figure legend continues)
CD11b and CD14 express during late granulocytic and monocytic differentiation, and are used as the markers of phenotypes of PMN-MDSCs and MO-MDSCs in this study, respectively. We found that CD33+CD11b+CD14+CD15− cells significantly increased in patients’ blood compared with healthy donors’ blood, which is consistent with CD33+HLA-DR− cells but not with CD11b+HLA-DR+ cells and CD14+HLA-DR− cells. Considering that CD33 expresses earlier and more extensively in myeloid lineage than the above two markers, we proposed that CD45+CD33+CD13+CD14+CD15− cells might represent a unique MDSC subpopulation different from PMN-MDSCs and MO-MDSCs in breast cancer.

MDSCs are recruited by multiple proinflammatory molecules secreted by cancer or stroma cells to the tumor sites where they exploit a plethora of redundant mechanisms to inhibit Ag-specific and nonspecific immune responses, including depleting required nutrients for lymphocytes, generating oxidative stress, interfering with lymphocyte trafficking and viability, and activating and expanding the Treg population (15, 29). Among them, ARG1 has been widely accepted as one of the most important immunosuppressive mechanisms of MDSCs in mouse and human because it consumes essential nutrients (l-arginine and l-cysteine) for proliferation and activation of T cells in the microenvironment (29). The deprivation of the earlier amino acids induces proliferative arrest of T cells via GCN2 pathway and causes resistance to TCR-mediated T cell activation for downregulation of the ζ-chain. However, it has been reported that ARG1 might distribute differently among mouse and human MDSCs, which is constitutively expressed in human granulocytes but not in monocytes (35). In our studies, the IDO protein, rather than ARG1 protein, significantly increased in fMDSCs.

IDO is a rate-limiting enzyme of tryptophan catabolism along the kynurenine pathway. In addition to its known function to prevent infection of intracellular pathogens, IDO has been regarded as a predominant endogenous suppressive factor of host immune system during pregnancy, transplantation, and tumorigenesis (20, 36). Many researchers demonstrated that IDO acts as a major regulatory mechanism by which inhibitory DCs induced immune tolerance in tumor-bearing animals and cancer patients (37). IDO expression results in localized depletion of tryptophan and production of kynurenine-based metabolites in the microenvironment, which leads to inhibition of T cell proliferation and induction of T cell apoptosis via the GCN2 pathway (38). Our previous work demonstrated that IDO inhibited immune surveillance and promoted immune tolerance by suppressing TCR-mediated activation of T cells via blocking phosphorylation of Vav1 and multiple downstream kinases (ERK, MAPK, and IκB), as well as inducing amplification of Tregs (27, 28). IDO and ARG1 share similar mechanisms of immunosuppression on T cells, but information about the role of IDO in MDSCs is limited. Recently, it has been reported that ARG1 and IDO act simultaneously to alter the functions of intratumoral CD8+ T cells in plasmacytoid DCs in prostate cancer, which suggests that immunosuppressive programs might be shared across different myeloid cells in cancer (39). Thus, it is possible that MDSCs can exert immunosuppressive functions on T cells via an alternative IDO-dependent mechanism in breast cancer.

We and others previously demonstrated that IDO is expressed on some myeloid-like cells in the stroma surrounding breast cancer nests (24, 40). In this study, we further investigated the expression of IDO in fMDSCs and found the proportion of IDO+ MDSCs significantly correlated with increased infiltration of Foxp3+ Tregs in situ and detectable lymph node metastasis in patients. We enriched fMDSCs from primary cancer tissues using CD33 MicroBeads and demonstrated that IDO expression was upregulated exclusively in fMDSCs recruited to the tumor site, but not CD33+ controls isolated from patients’ peripheral blood. In addition, inhibition of IDO enzyme activity by 1-MT significantly attenuated fMDSC-mediated suppression on T cell proliferation and Th1 polarization, and promotion of T cell apoptosis, which suggested that the immunosuppressive activity of fMDSCs was IDO dependent.

Moreover, differently from cytokine production, MDSC-induced apoptosis is not fully recovered by the use of IDO inhibitors compared with the T cells control. To elucidate the proapoptotic effects of MDSCs, we set up a control group composed of T cells cocultured with patients’ PB-derived CD33+ cells for further comparison. We found that IDO inhibitor 1-MT efficiently inhibited the increased apoptosis of T cells induced by fMDSCs, but both groups (T cells treated with CD33+ controls and T cells treated with fMDSCs and 1-MT) displayed higher apoptosis than T cells control. The reason why MDSC-induced apoptosis is not fully recovered by the IDO inhibitor in our experiment may be that: 1) the concentration of 1-MT used in our study is not high enough to completely block IDO activity; and 2) the alternative apoptosis pathway, such as Fas/Fas ligand (FasL) ligation-dependent apoptosis, occurred in CD33+-treated T cells. It has been reported that both freshly isolated monocytes and neutrophils constitutively express FasL on the cell surface, which is responsible for spontaneous apoptosis during cell culture, and overexpression of FasL by myeloid cells is observed in some pathological circumstances (41). Considering that activated T cells always express a high level of Fas receptors, it is possible that the CD33+ T cell–T cell coculture system might provide an opportunity of Fas/FasL ligation, and thus induce apoptosis of T cells. However, further investigation will be conducted to elucidate the underlying mechanisms.

To define whether upregulation of IDO is a constitutive or an inducible mechanism for MDSC-mediated immunosuppression, we established a noncontact coculture system including normal myeloid progenitors and breast cancer cells to induce MDSCs. iMDSCs possessed potent immunosuppression on T cells as fMDSCs did. IDO expression was induced in iMDSCs, which mediated immunosuppressive activity of the iMDSCs. Our results implied that IDO was induced in both fMDSCs and iMDSCs by cancer cells in a contact-independent manner and played a pivotal role in developing and maintaining MDSC-mediated immunosuppressive functions on T cells.

The presence or absence of JSI-124 were cocultured with healthy donors’ T cells at ratio of 1:1 with or without 1-MT. T cells stimulated by 500 IU/ml of IL-2, and anti-CD3/CD28 mAbs at bead/cell ratio of 1:1 were used as the negative controls. Proliferation was detected as described in Fig. 3C. JSI-124 and 1-MT significantly reversed fMDSC- but not untreated UCB-derived, CD33+-mediated inhibition of IL-2 and anti-CD3/CD28 mAb–induced T cell amplification. Cytokines secreted by T cells were detected as described in Fig. 3D. JSI-124 significantly attenuated fMDSC-mediated inhibition on IFN-γ and promoter on IL-10 and TGF-β as 1-MT did. p < 0.05. (E) Paraffin-embedded breast cancer tissues were collected from 50 patients for staining of STAT3. (1, 2) STAT3+ myeloid precursor and karyocytes in primary cancer tissues. Original magnification ×200 (1); ×400 amplification (2). (3) fMDSCs were isolated from fresh cancer tissues to detect the expression and phosphorylation level of STAT3 using Western blot analysis with indicated Abs. CD33+ cells in patients’ blood were used as a negative control. STAT3+ karyocytes in stroma and increased p-STAT3 and IDO protein levels in isolated fMDSCs were identified.
The posttranscriptional regulation of IDO is complex and cell-type specific. The Jak-STAT pathway, as well as MAPK and noncanonical NF-κB signaling pathways, have been reported to modulate IDO expression in response to a variety of stimuli (42). In macrophages and DCs, STAT1 activation–dependent transcription of IDO is a predominant molecular event that occurs in response to type I or type II IFN stimulation (43). In addition, recent studies demonstrated that posttranslational modification of STAT3 by phosphorylation and/or acetylation increases expression of IDO in DCs, which modulates their functions in immunosuppression on T and NK cells (30, 44–46). However, the molecular mechanism involved in the upregulation of IDO expression in MDSCs is still unclear. In this study, we found decreasing secretion of IFN-γ in iMDSCs as compared with untreated CD33+ myeloid progenitors, suggesting that IDO expression in iMDSCs is upregulated by an alternative pathway, rather than the classic IFN-γ–dependent pathway (Supplemental Table I). To further distinguish the two potential mechanisms, we examined the expression and phosphorylation of STAT1 and STAT3, which are known to regulate the expression of IDO in DCs and macrophages. We showed that neither STAT1 nor STAT3 protein levels changed in iMDSCs, compared with those in CD33+ progenitors. However, the level of p-STAT3, but not p-STAT1, significantly increased in iMDSCs, compared with CD33+ progenitors. STAT3 phosphorylation-dependent IDO expression was one of the mechanisms of breast cancer–derived MDSC-mediated immunosuppression on T cells.

STAT3 is a well-known signaling protein during myeloid lineage development and differentiation, and activation of STAT3 results in crucial DC and macrophage defects and MDSC population expansion (29, 46). STAT3–induced proinflammatory proteins, S100A8 and S100A9, inhibit DC differentiation, recruit MDSCs to the tumor site, and promote the suppressive activity of MDSCs (47, 48). In addition, STAT3 has been found to regulate differentiation of myeloid progenitors to functional MDSCs and induce the expansion of MDSCs via upregulating C/EBPβ protein (29, 49), but STAT3–dependent regulation of IDO expression remains unclear. To our knowledge, we first reported the role of STAT3 in IDO expression and IDO-dependent, MDSC-mediated immunosuppression on T cells. Future studies will be performed to evaluate the therapeutic significance of IDO-specific inhibitors in attenuating MDSC-mediated immunosuppression and preventing disease progression in breast cancer patients.

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Disclosures
The authors have no financial conflicts of interest.

References


