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Hyperactivation of STAT3 Is Involved in Abnormal Differentiation of Dendritic Cells in Cancer

Yulia Nefedova, Mei Huang, Sergei Kusmartsev, Raka Bhattacharya, Pingyan Cheng, Raoul Salup, Richard Jove, and Dmitry Gabrilovich

Abnormal differentiation of myeloid cells is one of the hallmarks of cancer. However, the molecular mechanisms of this process remain elusive. In this study, we investigated the effect of tumor-derived factors on Janus kinase (Jak)/STAT signaling in myeloid cells during their differentiation into dendritic cells. Tumor cell conditioned medium induced activation of Jak2 and STAT3, which was associated with an accumulation of immature myeloid cells. Jak2/STAT3 activity was localized primarily in these myeloid cells, which prevented the differentiation of immature myeloid cells into mature dendritic cells. This differentiation was restored after removal of tumor-derived factors. Inhibition of STAT3 abrogated the negative effects of these factors on myeloid cell differentiation, and overexpression of STAT3 reproduced the effects of tumor-derived factors. Thus, this is a first demonstration that tumor-derived factors may affect myeloid cell differentiation in cancer via constitutive activation of Jak2/STAT3. The Journal of Immunology, 2004, 172: 464–474.

Effective immune response depends on adequate function of bone marrow (BM)–derived APCs. One of the hallmarks of cancer is abnormality in myeloid cell differentiation. This abnormality manifests in two major phenomena: defective differentiation of the most potent APCs—dendritic cells (DCs)—and accumulation of immature myeloid cells (ImCs) (1–13). A decreased presence of mature DCs in tissues impairs the immune system’s ability to induce and maintain an effective anti-tumor immune response (1, 2, 11, 13). Accumulation of immature DCs and immunosuppressive ImCs is responsible for generation of immune tolerance and tumor-associated immune suppression (3, 9, 10, 12, 14). All together, these events contribute greatly toward tumor escape from immune system control and represent one of the important mechanisms that compromise the effectiveness of cancer vaccines. It is established that abnormal differentiation of myeloid cells in cancer is mediated by soluble tumor-derived factors, which include vascular endothelial growth factor (VEGF), M-CSF, GM-CSF, gangliosides, IL-10, etc. (6, 8, 9, 15, 16). However, the molecular mechanisms of this process remain elusive.

The Janus family of tyrosine kinases (JAK) and STAT family of transcription factors are critical components of diverse signal transduction pathways that are actively involved in cellular survival, proliferation, differentiation, and apoptosis. JAKs are constitutively associated with many cytokine and growth factor receptors (reviewed in Ref. 17). The binding of a cytokine to its receptor induces the receptor’s oligomerization that triggers recruitment of JAKs to close proximity of the receptors. This results in activation of JAKs by either auto- or transphosphorylation. The activated JAKs phosphorylate receptors on target tyrosine sites, which generates docking sites for STATs. Subsequently, recruited STATs are phosphorylated by activated JAKs and dimerized, followed by their translocation into the nucleus, where they modulate expression of target genes. Constitutive activation of STAT3 has been implicated in Src-mediated transformation of fibroblasts and hematopoietic cell lines (reviewed in Ref. 18). JAKs and STATs play a critical role in development. Disruption of different members of the JAK family results in perinatal lethality due to neurological deficits (Jak1) (19), defects in erythropoiesis (Jak2) (20), or severe defects in lymphoid development and myelopoiesis (Jak3) (21). Lack of STAT3 results in severe defects in development and early fetal death (22). Thus, the JAK/STAT pathway is critically important for normal cell differentiation. We hypothesized that tumor-derived factors may affect myeloid cell differentiation by affecting the JAK/STAT activity, because many growth factors and cytokines that affect myeloid cell differentiation are known to induce JAK/STAT signaling. In this study, we have tested this hypothesis and, for the first time, have demonstrated that hyperactivation of Jak2/STAT3 induced by tumor-derived factors is responsible for abnormal myeloid cell differentiation in cancer.

Materials and Methods

Animals

Female BALB/c and C57BL/6 mice aged 6–8 wk were obtained from the National Cancer Institute (Frederick, MD). Mice were kept in pathogen-free conditions and handled in accordance with the requirements of the Guideline for Animal Experiments.

Reagents and cell culture

RPMI 1640, DMEM, FBS, and antibiotics were obtained from Life Technologies (Grand Island, NY), recombinant murine GM-CSF, IL-4, and TNF-α from RDI (Flanders, NJ), LPS from Sigma-Aldrich (St. Louis, MO), VEGF from R&D Systems (Minneapolis, MN), low-tox rabbit complement and Lympholyte M from Cedarlane Laboratories (Hornby, Ontario, Canada); The Abs used for flow cytometry, anti-pSTAT3, anti-Cr-1 (anti-Ly-6G), anti-CD11b, anti-CD11c, anti-I-A^d, anti-I-A^k, anti-CD86, anti-Gr-1, anti-CD34, and anti-Sca1, were obtained from BD PharMingen (San Diego, CA); and F4/80 was purchased from Serotec (Raleigh, NC). Anti-Jak1 Ab was obtained from BD Transduction Laboratories (Lexington, KY); anti-Jak2, anti-Tyk2, anti-jb-actin Abs, and A/G Plus-agarose beads from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-Jak3 and anti-phospho-tyrosine Abs (clone 4G10) from Upstate Biotechnology (Lake Placid, NY). Murine NIH-3T3 fibroblasts and the CT26 colon carcinoma cell line were obtained from ATCC (Manassas, VA).

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cell line were obtained from American Type Culture Collection (Manassas, VA), and the murine C3 fibrosarcoma cell line was kindly provided by Dr. W. Kast (Loyola University of Chicago, Maywood, IL). Neutralizing anti-VEGF and anti-M-CSF Abs were obtained from R&D Systems, and G-CSF and anti-IL-10 Abs were obtained from RDI. Cells were grown in DMEM supplemented with 10% FBS and antibiotics. To generate conditioned medium (CM), cells were kept in medium with reduced (3%) serum concentration for 48 h. After that time, supernatants were collected, filtered, and used in experiments.

Generation of DCs in vitro

BM cells were obtained from the femurs and tibias of mice and then enriched for hematopoietic progenitor cells (HPCs) by depletion of lineage-negative cells using an Ab mixture against T cells (TIB-210 and TIB-207), B cells (B220), macrophages and DCs (TIB-120), granulocytes (Gr-1), and red cells (Ter-119) as described earlier (23). HPCs were cultured in RPMI 1640 supplemented with 10% FBS, 20 ng/mL GM-CSF, 10 ng/mL IL-4, and 50 μM 2-ME (complete medium). Half of the medium was replaced every 2 days. To stimulate DC maturation, 1 μg/mL LPS was added to the cell culture on day 5 of culture with subsequent 48-h incubation. To study the effect of tumor-derived factors on HPC differentiation, DCs were generated in the presence of 30% (v/v) control CM (from 3T3 fibroblasts) or tumor CM (from C3 or CT26 tumor cell lines).

Cell proliferation assay

HPC-enriched BM cells (5 × 10^5) were resuspended in 1 mL of PBS containing 5% FBS. CFSE (Molecular Probes, Eugene, OR) dye was added to a final concentration of 5 μM, and cells were incubated at room temperature for 5 min. Cells were washed four times with 10 mL of PBS with 5% FBS, resuspended in complete medium supplemented with control or tumor-cell (TC) CM, and cultured for 3, 5, or 7 days. Cells were then collected and stained with Gr-1-allophycocyanin and CD11b-PE Abs. CFSE dye distribution was analyzed on FL-1 channel in gated Gr-1-positive population as determined by flow cytometry.

Cell isolation

CT26 colon carcinoma was established in BALB/c mice by s.c. inoculation of 3 × 10^5 tumor cells into the shaved right flank, and C3 sarcoma in C57BL/6 mice by s.c. injection of 5 × 10^5 tumor cells. Control mice were injected with 0.1 mL of PBS. Mice were sacrificed 3 wk after tumor inoculation. A single-cell suspension was prepared from spleens, and Gr-1-positive cells were isolated using MiniMACS magnetic beads according to the manufacturer’s protocol (Miltenyi Biotec, Auburn, CA). The purity of the Gr-1-positive population was 95% as determined by flow cytometry.

Immunoprecipitation and Western blotting

Cells were collected, washed twice with ice-cold PBS, and lysed in the following buffer: 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, 2 mM Na_2VO_4, 25 mM NaF, and 1:100 protease inhibitor mixture (Sigma-Aldrich). An equal amount of protein (500 μg) was incubated with anti-Jak1, -Jak2, -Jak3, or -Tyk2 Abs for 1.5 h at 4°C followed by addition of protein A/G Plus-agarose beads and incubation for an additional 1.5 h at the same conditions. The protein/Ab/beads mix was then washed three times with lysis buffer, resuspended in Laemmli SDS sample buffer, and denatured at 95°C for 5 min. Samples were resolved in 8% SDS-PAGE followed by transfer to polyvinylidene difluoride membrane. After blocking, the membrane was probed with phospho-tyrosine mouse mAb and then reprobed with Abs against Jak1, Jak2, Jak3, or Tyk2. Membrane was developed using ECL detection kit. Alternatively, in some experiments, cells were lysed in buffer containing 10 mM NaCl, 30 mM HEPES (pH 7.5), 5 mM MgCl_2, 25 mM NaF, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 2 mM Na_2VO_4, 10 μg/mL aprotinin, 10 μg/mL soybean trypsin inhibitor, 25 μg/mL leupeptin, 2 mM PMSF, and 6.4 mg/mL Sigma 104 phosphatase substrate (Sigma, Aldrich). Equal amounts of proteins were loaded in an 8% SDS-PAGE gel followed by a transfer onto a nitrocellulose membrane. The blot was probed with anti-phospho-Jak2 or anti-phospho-STAT3 Abs. To confirm equal loading, the membrane was stripped and reprobed with Abs against nonphosphorylated proteins and β-actin.

EMSA

Nuclear extracts were prepared in hypotonic buffer containing 20 mM HEPES (pH 7.9), 420 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20% glycerol, 20 mM NaF, 1 mM Na_2VO_4, 1 mM Na_3P_2O_7, 1 mM DTT, 0.5 mM PMSF, 0.1 μM aprotinin, 1 μM leupeptin, and 1 μM antipain. Extracts were normalized for total protein, and 5 μg of protein was incubated with the 32P-labeled hSIE probe, 5′-AGCTTACCTCCCCGTTAATCCCTA-3′, for STAT1 and STAT3, and MGFc probe, 5′-AGATTTCGGAATACTCGAG-3′ for STAT5, as previously described (24–26). Protein-DNA complexes were resolved by nondenaturing PAGE and detected by autoradiography.

Cell infection

Retroviral vectors carrying a constitutively active form of STAT3 pMSCV-STAT3C-EGFP and dominant-negative form of STAT3 pMSCV-STAT3D-EGFP were a gift from Dr. D. Link (Washington University, St. Louis, MO). They have been described in detail elsewhere (27). These vectors were used for transient transfection of 293T cells. To generate retrovirus, 293T cells were simultaneously cotransfected with packaging plasmid pCLE-Eco using GenePORTER reagent (Gene Therapy Systems, San Diego, CA). Supernatant containing retrovirus was collected 48 h after transfection. HPCs isolated from murine BM underwent two rounds of infection with STAT3C, STAT3D, or control viruses as described earlier, followed by incubation in the presence of control or tumor CM. Cell phenotype was analyzed by flow cytometry in the gated population of EGFP-FL1-positive cells.

Allogeneic MLR

Cells generated from HPCs were used as stimulators in allogeneic MLR. Lymph node (LN) cells from allogeneic mice were used as responders. Cells were mixed in different ratios and incubated in triplicate in 96-well plates for 4 days. One microcurie of [3H]thymidine (Amersham, Arlington Heights, IL) was added per well 18 h before cell harvest. [3H]Thymidine incorporation was measured using a liquid scintillation counter.

Colony formation assay

Gr-1-positive cells were isolated from cell cultures by magnetic bead separation. Cells were plated in triplicate in a six-well plate at 100,000 cells per well in semisolid 1% methylcellulose medium supplemented with recombinant cytokines that support the optimal growth of CFU-GM, CFU-G, and CFU-M colonies (MethoCult GF M3534, StemCell Technologies, Vancouver, British Columbia, Canada). The number of colonies was evaluated and counted under microscope on day 10.

Analysis of cell proliferation in vivo

Control and tumor-bearing mice were injected i.p. with 1 mg of BrdU solution. Mice were euthanized 24 and 48 h later. Splenocytes were collected and stained with allophycocyanin-conjugated anti-Gr-1 and PE-conjugated anti-CD11b Abs. Cells were then permeabilized and stained with FITC-conjugated anti-BrdU and 7-amino actinomycin D (7-AAD). All reagents and the protocol were obtained from BD PharMingen (BrdU flow kit). The results were analyzed using four-color flow cytometry on FACSCalibur.

Statistics

All in vitro experiments were repeated at least three times to ensure the reproducibility of the results. Statistical analysis was performed using JMP software (SAS Institute, Cary, NC).

Results

Increased activity of Jak2/STAT3 pathway induced by tumor-derived factors during DC differentiation in vitro

To evaluate the possible effects of tumor-derived factors on members of the JAK family, HPC-enriched BM cells were differentiated into DCs by a 5-day incubation with GM-CSF and IL-4 in the presence of control or TC CM. The presence of CM from CT26 or C3 tumor cells resulted in substantial hyperphosphorylation of Jak2 (Fig. 1A). However, they did not affect other members of the JAK family (data not shown). The STAT family of transcription factors is the main downstream target for Jak2. We analyzed DNA-binding activity of STAT1, STAT3, and STAT5 in cells cultured for 5 days in complete medium in the presence of control CM or TC CM. Tumor-derived factors significantly increased DNA binding of STAT3 homodimers (Fig. 1B), but not STAT1 or STAT5 (B and data not shown).
Tumor cells produce a number of factors able to affect differentiation of DCs: GM-CSF, M-CSF, IL-10, IL-6, and VEGF. Direct stimulation of STAT3 activity in hemopoietic cells has been previously demonstrated for all of them, with the exception of VEGF. VEGF is produced by most tumors and directly affects DC differentiation (15, 28, 29). To study whether this tumor-derived factor also can contribute to the described effects of TC CM, HPCs were cultured for 5 days in the presence of control CM with or without tumor CM. HPCs were then collected and used for Western blotting with anti-phospho-Jak2 Ab. Membrane was reprobed with Jak2 and β-actin Abs. Of note, the appearance of the second band representing the phosphorylated portion of the protein in the samples of cells treated with TC CM after probing with Jak2 Ab. Three experiments with similar results were performed. B. Nuclear extracts were prepared and used in EMSA. Three experiments with the same results were performed. C. HPCs were cultured for 5 days in complete medium in the presence of 3T3 CM with (lane 2) or without (lane 1) 100 ng/ml murine rVEGF. EMSA with STAT3-specific probe was performed. Src-transformed NIH-3T3 fibroblasts were used as a positive control (lane C). D. HPCs were cultured with GM-CSF, IL-4, and CM as described above. Abs were added on day 0 and on day 3 with fresh medium. Cells were collected on day 5 and analyzed by EMSA. Lane 1, Cells cultured with control CM and 500 ng/ml goat IgG. Lanes 2–6, Cells cultured with CT26 CM and goat IgG (lane 2), neutralizing anti-M-CSF Ab (500 ng/ml) (lane 3), neutralizing anti-G-CSF Ab (500 ng/ml) (lane 4), neutralizing anti-VEGF Ab (100 ng/ml) (lane 5), and neutralizing anti-IL-10 Ab (100 ng/ml) (lane 6). Intensity of bands was analyzed using phosphor imager. Fold increase over control level (cells cultured with control CM and goat IgG) is shown. E. HPCs were isolated from BM of BALB/c mice and cultured in complete medium in the presence of control CM (lane 1) or CM from tumor C3 cells (2) for 1, 2, 3, 4, and 5 days. Cells were collected, and STAT3 activity was evaluated by EMSA. Lane C represents positive control. Similar results were obtained when HPCs were isolated from C57BL/6 mice (data not shown). Two experiments with the same results were performed.
without VEGF. Addition of VEGF increased STAT3 DNA-binding activity (Fig. 1C). We compared the relative contribution of several known tumor-derived factors for their ability to cause increased STAT3 activity. HPCs were cultured for 5 days with TC CM and neutralizing Abs against several specific tumor-derived factors: M-CSF, VEGF, and IL-10. As a control, we used goat IgG and neutralizing anti-G-CSF Ab. G-CSF is known to induce STAT3; however, it is not produced by the tumor cells used in our study (data not shown). Anti-M-CSF Ab dramatically decreased the effect of TC CM on STAT3 activity, whereas the anti-VEGF or anti-IL-10 Abs had rather modest effects (Fig. 1D). As expected, anti-G-CSF Ab did not affect TC CM-inducible STAT3 activation. Thus, it appears that, during DC differentiation in vitro, different tumor-derived factors specifically hyperactivate STAT3.

Next, we tried to determine the stage of HPC differentiation that is affected by tumor-derived factors. HPCs isolated from BM of BALB/c or C57BL/6 mice were cultured with control CM or TC CM added when cultures were initiated (day 0). Cells were collected for analysis every day starting from day 1. Tumor-derived factors did not affect Jak1, Jak3, and Tyk2 activity at any time point during cell differentiation (data not shown). We also were not able to detect differences in Jak2 and STAT3 activity during the first 3 days of HPC culture (Fig. 1E). Starvation of cells with subsequent stimulation with TC CM did not change these results (data not shown). By day 4, STAT3 activity in the cells cultured with control CM significantly decreased. That decrease was also observed on day 5 of culture. However, cells cultured in the presence of tumor-derived factors maintained high STAT3 activity (Fig. 1E).

ImCs are major contributors to increased STAT3 activity induced by tumor-derived factors

Our data showed that tumor-derived factors prevented down-regulation of STAT3 activity during DC differentiation. Cells generated from HPC with GM-CSF and IL-4 include several populations of myeloid cells: DCs, macrophages, and ImCs. It is possible that tumor-derived factors may affect Jak2/STAT3 activity differently in these cells. Previously published data have demonstrated that tumor growth in vivo is associated with accumulation of ImCs defined as cells expressing both Gr-1 and CD11b surface markers (3, 12, 14, 16). These cells are present in small numbers in tumor-free mice and differentiate into mature myeloid cells in the presence of appropriate growth factors (30). We asked whether tumor-derived factors might have a similar effect in vitro. HPCs were cultured in complete medium supplemented with control CM or TC CM from day 0. Cells were collected every day during the 5-day culture. The accumulation of Gr-1+CD11b+ ImCs was observed 24 h after start of the culture in the presence of TC CM (Fig. 2A). The total number of cells remained unchanged at that time.
The proportion of ImCs gradually increased while cells continued to be exposed to tumor-derived factors. This was associated with an increase in the total number of cells. By day 5, the total number of cells generated in the presence of TC CM was 1.5-fold higher than in the presence of control CM ($p < 0.05$). In the presence of TC CM, 57% of all cells were ImCs, whereas only 26% of cells cultured in the presence of control CM had the same phenotype ($p < 0.05$) (Fig. 2). The proportion of ImCs generated without the presence of any CM was similar to that generated in the presence of control CM (data not shown). If TC CM was added on day 3, no effect on ImC accumulation was observed (Fig. 2B). Extension of incubation with GM-CSF and IL-4 for 2 more days did not affect these results (data not shown).

Thus, it appears that there is a close correlation between the accumulation of ImCs and activation of the Jak2/STAT3 pathway in the population of myeloid cells in the presence of tumor-derived factors.

Next, we asked what was the level of Jak2/STAT3 activity in ImCs. HPCs were differentiated in vitro with GM-CSF and IL-4 in the presence of control or TC CM. After 4 days of culture, cells were collected, and Gr-1$^{+}$ cells were isolated using magnetic beads. Flow cytometry showed that 93–95% of isolated Gr-1$^{+}$ cells had a Gr-1$^{+}$CD11b$^{+}$ phenotype. After depletion, the fraction of Gr-1$^{+}$ cells contained $5\%$ of Gr-1$^{+}$ cells. Activation of Jak2/STAT3 pathway was evaluated separately in Gr-1$^{+}$ (ImCs) and Gr-1$^{-}$ cell fractions by Western blot and EMSA. Tumor-derived factors induced phosphorylation of Jak2 and STAT3 in both populations of cells. The level of STAT3 phosphorylation was substantially higher in ImCs generated in the presence of TC CM than in the cells generated in the presence of control CM (Fig. 2C). It was substantially lower in Gr-1$^{-}$ cells (Fig. 2C). Background levels of STAT3 DNA-binding activity was low in both Gr-1$^{+}$ (ImCs) and Gr-1$^{-}$ cell fractions by Western blot and EMSA. Tumor-derived factors induced phosphorylation of Jak2 and STAT3 in both populations of cells. The level of STAT3 phosphorylation was substantially higher in ImCs generated in the presence of TC CM than in the cells generated in the presence of control CM (Fig. 2C). It was substantially lower in Gr-1$^{-}$ cells (Fig. 2C). Background levels of STAT3 DNA-binding activity was low in both Gr-1$^{+}$ and Gr-1$^{-}$ cells generated in the presence of control CM. This was consistent with our data from the bulk cell population. Tumor-derived factors induced STAT3 activation in both Gr-1$^{+}$ and Gr-1$^{-}$ cells. However, it was dramatically higher in ImCs than in Gr-1$^{-}$ cells (Fig. 2D).
To confirm our finding in vivo, we used the CT26 colon carcinoma mouse model. Three weeks after tumor inoculation, the proportion of ImCs in spleens increased to 35–40% compared with 2–3% in spleens of tumor-free mice. At that time, tumor-bearing and control mice were sacrificed, and Gr-1+ cells were isolated from spleens. Activation of STAT3 was evaluated by EMSA. As shown in Fig. 2E, the level of STAT3 DNA-binding activity was substantially higher in Gr-1+ ImCs isolated from tumor-bearing mice than from control mice. These data indicate that ImCs generated in the presence of tumor-derived factors in vitro or isolated from tumor-bearing mice had a substantially higher level of STAT3 activity than control ImCs. To confirm the hypothesis that tumor-derived factors can activate STAT3 directly in these cells, we isolated Gr-1+ cells from the spleens of naive mice and cultured them with control or TC CM for 24 h. After that time, cells were collected, and phosphorylation of STAT3 was evaluated by Western blot. Consistent with the data obtained in vitro, ImCs incubated with control CM showed a low level of pSTAT3. TC CM substantially increased phosphorylation of STAT3 (Fig. 2F).

Characteristics of ImCs generated in the presence of tumor-derived factors

Because the Jak2/STAT3 pathway is important for cell growth, we hypothesized that tumor-derived factors constitutively activate Jak2/STAT3 in ImCs and, therefore, maintain a high proliferation rate in these cells rather than allowing them to differentiate. To test this hypothesis, HPCs were isolated from BM and labeled with the fluorescent dye CFSE. Intensity of this dye gradually decreased with every cell division, allowing for detection of cell proliferation by flow cytometry. Cells were cultured for 3, 5, or 7 days in the presence of control or TC CM, and then collected, labeled with Gr-1 and CD11b Abs, and analyzed by flow cytometry. C3 or CT26 CM significantly increased the population of Gr-1+CD11b+ ImCs (Fig. 3A, upper right quadrant). Those cells were gated, and CFSE fluorescence distribution was analyzed as shown in Fig. 3B. Gates were set as follows: M1, nonproliferative cells; M2, intermediates, and M3, highly proliferative Gr-1+CD11b+ cells. As expected, tumor-derived factors induced proliferation of ImCs. After 3 days of culture, 38% of ImCs generated in the presence of control CM remained nonproliferative. This proportion was significantly smaller in ImCs generated in the presence of C3 and CT26 CM (Fig. 3C). After 5 days of culture, the proportion of nonproliferative cells dropped to 6 and 10% in the presence of C3 and CT26 CM, respectively, whereas it was unchanged in control cultures (36%) (Fig. 3, B and C). Similar data were observed at day 7 of culture (Fig. 3C).

To measure the apoptosis of ImCs, cells were collected at different time points (1–5 days) after the start of the cultures, labeled with anti-CD11b-FITC and anti-Gr-1-allophycocyanin Abs in combination with annexin V and 7-AAD staining, and then analyzed by four-color flow cytometry. No differences in the levels of apoptosis in ImCs generated in the presence of control CM or TC CM were found (data not shown). To characterize these cells further, Gr-1+ generated after the 5-day culture with TC CM were isolated and used for three-color flow cytometry with Abs against markers of HPCs and stem cells: c-kit, Sca1, and CD34. Less than 1% of ImCs expressed these molecules (data not shown). Furthermore, <1% of ImCs were able to form colonies in a colony formation assay (data not shown).

Our in vitro data indicated that a high level of STAT3 in ImCs generated in the presence of TC CM was associated with increased cell proliferation. To confirm this observation in vivo, we measured cell proliferation of ImCs using immunofluorescent staining of incorporated bromodeoxyuridine (BrdU) and flow cytometry. BrdU (an analog of the DNA precursor thymidine) is incorporated into newly synthesized DNA by cells entering and progressing through the S phase of the cell cycle. The incorporated BrdU is stained with specific anti-BrdU fluorescent Abs. In combination with the staining with a dye that binds to total DNA (7-AAD), this permits the enumeration and characterization of cells that are actively synthesizing DNA. Control and CT-26 tumor-bearing mice were injected i.p. with BrdU and analyzed 24 and 48 h later. Splenocytes were collected and labeled with anti-Gr-1, anti-CD11b, and anti-BrdU Abs and 7-AAD. DNA synthesis was measured within the population of Gr-1+CD11b+ ImCs. ImCs from tumor-bearing mice had a ~3-fold higher proportion of cells in the S phase of the cell cycle than ImCs from control mice (Fig. 3D). This indicates that ImCs from tumor-bearing mice have a substantially higher proliferation rate than ImCs from control mice, which is consistent with our in vitro data.

We then asked whether the effect of tumor-derived factors on ImCs was reversible. HPCs were cultured in complete medium in the presence of CM from C3 tumor cells for 5 days. On day 5, Gr-1+ cells were isolated, washed, and cultured for another 5 days in complete medium in the presence of CM from control 3T3 (N) or C3 tumor (T) cells. LPS (1 μg/ml) was added during the last
FIGURE 5. Tumor-derived factors affect DC differentiation. A, HPCs were isolated from BM of BALB/c mice and cultured for 7 days in complete medium with control (1), C3 (2), or CT26 (3) CM. LPS was added for the last 24 h of culture. Cells were collected on days 5, 6, or 7, and the CD11c+ population was isolated using magnetic beads. STAT3 binding activity was evaluated by EMSA in CD11c+ cells isolated on days 5 and 7. B, Splenocytes were collected from control tumor-free and CT-26 tumor-bearing mice (3 wk after tumor inoculation). Cells were labeled with aliphosphocyanin-conjugated anti-CD11c Ab and FITC-conjugated anti-IAα Ab. This was followed by fixation with 2% paraformaldehyde and permeabilization with 90% methanol followed by staining with PE-conjugated anti-pSTAT3 (Tyr705) Ab. Protocol and Abs were obtained from BD PharMingen. Gates were set using isotype control Abs (not shown). Mean fluorescence intensity (MFI) for STAT3 and isotype control IgG was measured within the population of CD11c+IAα+ DCs. A typical result of one of three performed experiments is shown. C, Cells were culture as described above in A. Cells were collected on day 6 of culture and labeled with CD11c-allophycocyanin and IAα-PE Ab. The MHC class II expression of HPCs was analyzed by flow cytometry in the gated CD11c+ cell population. A typical result of three performed experiments is shown. CD11c+ cells were isolated on day 6 of culture, using magnetic bead separation, and mixed with LN cells from C57BL/6 mice. Cells were cultured for 4 more days (3). [H]Thymidine was added for the last 16 h of culture. Average ± SE of two performed experiments is shown. D, Cells were cultured for 10 days. Medium with cytokines and control or TC CM was replaced on days 2, 5, and 8. On day 9, 5 μg/ml LPS was added in all wells. Cells were collected on day 10 labeled with anti-CD11c-allophycocyanin, anti IAα-PE, and anti-CD40-FITC or anti-B7-2-FITC Abs. CD11c+ cells were gated, and expression of CD40 and B7-2 was analyzed within the population of CD11c+ cells. MFI, Mean fluorescence intensity. Two experiments with the same results were performed. In addition, CD11c+ cells were isolated and cultured with LN cells from C57BL/6 mice. Cell proliferation was evaluated in triplicate as described above. Please note that the level of cell proliferation in D is substantially higher than that in C, because no LPS stimulation was used in the experiments shown in C. (Figure continues)
24 h of incubation to activate DCs. Cells were then collected, and their phenotype was analyzed by flow cytometry. We used a combination of different markers specific for mature DCs. As shown in Fig. 4, withdrawal of tumor-derived factors significantly improved the ability of ImCs to differentiate into DCs. The proportion of CD11c^+IA^+ DCs increased ~2-fold, and the proportion of CD11c^+CD86^+ or CD11c^+CD40^+ mature DCs increased 2.5- to 3-fold after TC CM was removed.

**Effect of tumor-derived factors on STAT3 activity in DCs**

Our data presented above demonstrated that increased STAT3 activity in myeloid cells induced by tumor-derived factors was largely due to the accumulation of Gr-1^+ ImCs. However, it was not clear whether DCs generated in the presence of tumor-derived factors had increased STAT3 activity. To clarify this issue, we investigated the level of STAT3 activity in immature and mature DCs. HPCs were grown for 5 days with GM-CSF and IL-4 in the presence of control or TC CM. To obtain relatively immature DCs, one-half of the cells was collected, and CD11c^+ cells were isolated using magnetic beads. The other half of the cells was cultured for an additional 24 h with GM-CSF and IL-4 followed by a 24-h incubation in the presence of 5 μg/ml LPS and isolation of CD11c^+ cells. This protocol provides for the generation of mature DCs. As shown in Fig. 5A, tumor-derived factors maintained increased STAT3 binding activity in CD11c^+ cells (immature DCs). CD11c^+ cells collected after activation with LPS for the last 24 h of culture (mature activated DCs) had a very low level of STAT3 DNA binding activity regardless of the presence of TC CM (Fig. 5A). Similar results were obtained when DC maturation was stimulated by TNF-α (data not shown).

To test whether our in vitro findings of increased STAT3 activity in DCs are reproducible in vivo, we labeled splenocytes from control and CT-26 tumor-bearing mice with anti-CD11c and anti-IA^+ Abs followed by intracellular staining with FITC-conjugated anti-pSTAT3 Abs, using a recently established protocol from BD PharMingen. Consistent with previously reported observations, tumor-bearing mice had a substantially reduced proportion of CD11c^+IA^+ DCs than control mice (Fig. 5B, top panel). However, when the level of pSTAT3 was analyzed within this cell population, it was found to be substantially higher in tumor-bearing than in control mice (Fig. 5B).

To evaluate whether increased STAT3 activity influenced immature DC function, we used the allogeneic MLR, the hallmark of DC activity. HPCs isolated from BALB/c mice were grown for 6 days with control or TC CM. CD11c^+ cells were isolated from the culture and used as stimulators. LN cells obtained from C57BL/6 mice were used as responders. As shown in Fig. 5C, the presence of tumor-derived factors during DC differentiation significantly decreased their ability to stimulate T cell proliferation. Decreased response in MLR was probably related to the insufficient expression of DC surface markers, particularly MHC class II. IA^+ fluorescence intensity in CD11c^+ generated in the presence of TC CM was only one-half of that in control DCs (Fig. 5C). Similar effects were observed on the expression of B7-2 (CD86) (data not shown). We asked whether activation of DCs with LPS or TNF-α might reverse this effect. Similar experiments were performed with CD11c^+ cells isolated after a 7-day culture and stimulation with LPS. CD11c^+ cells generated in the presence of tumor-derived factors had decreased expression of costimulatory molecules and a decreased ability to stimulate allogeneic MLR when compared with CD11c^+ generated in the presence of control CM. The differences were similar to those observed in nonactivated DCs (data not shown).

It was possible that the presence of CM could simply delay differentiation of DCs. To address this question, we cultured HPCs in the presence of control and TC CM (CT-26 tumor) for 9 days. Cells were stimulated with LPS for an additional 24 h and then
analyzed. The presence of TC CM significantly decreased the proportion of CD11c+ DCs. Our data indicated that it was not because of induction of apoptosis but because of accumulation of CD11c- cells, primarily Gr-1+CD11b+ ImCs (Fig. 5D and data not shown). However, when CD11c+ DCs were analyzed, the expression of molecules critically important for T cell activation (CD40; B7-2) was ~2-fold lower in DCs generated in the presence of TC CM than those generated in the presence of control CM (Fig. 5D). Sorted CD11c+ DCs were used as stimulators in allogeneic MLR.

CD11c+ cells generated in the presence of TC CM had a substantially decreased ability to stimulate allogeneic T cells than CD11c+ cells from cultures with control CM (Fig. 5D). These data indicate that the observed effects of TC CM on DCs were not caused by a simple delay in cell differentiation.

Thus, tumor-derived factors maintain activated STAT3 in DCs. This was associated with decreased expression of MHC class II and costimulatory molecules as well as a decreased ability to stimulate T cells.

FIGURE 6. The Jak2/STAT3 pathway is involved in the accumulation of ImCs and defective DC differentiation in cancer. A and B. HPCs were infected twice with MSCV-STAT3D-EGFP or MSCV-EGFP retrovirus and cultured in the presence of control CM (3T3) or TC CM (C3). Cells were collected and labeled with Gr1-allophycocyanin and CD11b-PE Abs on day 5 of culture (A) and with CD11c-allophycocyanin/IAα-PE and CD11c-allophycocyanin/CD86-PE Abs on day 7 of culture (B). FL1-EGFP-positive cells were gated, and 10,000 gated events were acquired. Three experiments were performed. C and D. Overexpression of STAT3 in HPCs reproduces the effect of TC CM on DC differentiation. HPCs were infected twice with MSCV-STAT3C-EGFP or MSCV-EGFP retrovirus and cultured in the presence of 3T3 CM for 5 or 7 days. Flow cytometry analysis was performed as described above. C. Dot plots depict the ImC population in FL1-EGFP-gated cells. D. Expression of DC markers on day 7 of culture. Average ± SE of two performed experiments is shown.
Increased STAT3 activity is directly involved in accumulation of ImCs and decreased DC differentiation in the presence of tumor-derived factors

To inhibit STAT3 activity, we used the dominant-negative STAT3D protein encoded in the pMSCV-STAT3D-EGFP retroviral vector. STAT3D bears mutations in the DNA-binding domain and is unable to bind DNA. Inhibition of STAT3 was confirmed by evaluating the level of pSTAT3 in transduced EGFP-positive cells using flow cytometry (data not shown). BM HPCs were infected with STAT3D or control virus on day 0 and day 1 and then cultured with GM-CSF and IL-4 in the presence of control or TC CM for an additional 4 days (5 days total). Cells were then labeled with Gr-1-allophycocyanin and CD11b-PE Abs, and the EGFP-positive population was analyzed by flow cytometry. The proportion of EGFP-positive cells was 10–12% after infection with control virus and 6–7% after infection with STAT3D virus. Expression of STAT3D completely abrogated the effect of tumor-derived factors on the production of ImCs (Fig. 6A). Importantly, it did not affect the accumulation of ImCs in the presence of control CM. To evaluate the effect of TC CM on DC differentiation, HPCs were cultured for 7 days. LPS was added 24 h before cell harvesting. As shown in Fig. 4B, blocking of STAT3 activity in HPCs cultured in the presence of tumor-derived factors restored their ability to differentiate into mature CD11c+/Gr-1−/CD86+ DCs (Fig. 6B).

To confirm the critical role of STAT3 in observed abnormalities in myeloid cell differentiation, activated STAT3 was overexpressed in HPCs. HPCs were isolated from BM of mice and infected with a retrovirus construct containing the constitutively active mutant form of STAT3, STAT3C. Cells were collected on day 4 to evaluate the proportion of ImCs and on day 7 of culture (last 24 h with LPS) to evaluate DC differentiation. As shown in Fig. 6C, constitutive activation of STAT3 in HPCs resulted in accumulation of Gr-1+CD11b+ ImCs. As mentioned above, similar data were obtained when HPCs were exposed to tumor-derived factors. STAT3C-transduced HPCs were also significantly impaired in their ability to differentiate into DCs (Fig. 6D). Thus, constitutive activation of STAT3 in HPCs completely reproduces the effect of tumor-derived factors on myeloid cell differentiation.

Discussion

Our data demonstrate for the first time that tumor-derived factors induced activation of STAT3 in ImCs may result in abnormal differentiation of myeloid cells.

Although the phenomenon of abnormal myeloid cell differentiation and its significance in cancer is well established, the molecular mechanisms of this event have remained unknown. It is known that tumors exert their effect on myeloid cell differentiation via various soluble factors. Therefore, to evaluate the effect of tumor-derived factors on myeloid cells, we used an in vitro system where DCs were differentiated from HPC with defined cytokines. Under normal conditions, this system allows for differentiation of functionally potent DCs (1). In the presence of CM from tumor cells, differentiation of DCs was significantly impaired (31). In present study, a 5-day culture with tumor-derived factors resulted in activation of only one JAK family member, Jak2. JAKs by themselves are not absolute determinants of the specificity in cytokine signaling, because different cytokines may activate the same JAKs. However, previous studies have shown that Jak2 is primarily activated by IFN-γ, IL-13, IL-3, IL-5, GM-CSF, growth hormone, erythropoietin, and thrombopoietin (reviewed in Ref. 32). As in the case of JAKs, there is a certain level of specificity in STAT involvement in cytokine signaling. For instance, IL-2, IL-10, and G-CSF are strong activators of STAT3. However, this specificity is not absolute, because the same STAT family member is activated by several (sometimes many) cytokines, and one cytokine may activate several STATs (32). Tumors produce a number of growth factors and cytokines able to activate the Jak2/STAT3 pathway. Some of them (GM-CSF, M-CSF, IL-10, and VEGF) were implicated in abnormal differentiation of myeloid cells and DCs in particular (6, 9, 15, 16, 28). In contrast to GM-CSF, M-CSF, or IL-10, the effect of VEGF on STAT activation was not well established. In a previously published study, VEGF induced tyrosine phosphorylation and nuclear translocation of STAT1 and STAT6 in endothelial cells. It also stimulated STAT3 tyrosine phosphorylation, but nuclear translocation did not occur (33). In our experiments, VEGF induced nuclear translocation of STAT3 in myeloid cells. Interestingly, it did not affect STAT1. It is likely that activation of STAT3 by VEGF is dependent on cell type. VEGF may exert its effect via VEGFR1 (Flt-1), known to be expressed on HPCs and myeloid cells (34–36). However, it appears that VEGF does not play the major role in STAT3 activation by tumor-derived factors. Experiments with neutralizing Abs suggest that M-CSF may play a more prominent role in this process. It is not surprising, because earlier studies have demonstrated the important role of M-CSF in tumor-mediated defects in DC differentiation (6, 15). It is likely that constitutive activation of STAT3 in myeloid cells is a result of the combined effect of several different tumor-derived factors, and the contribution of each particular factor is dependent on the level of its production by a particular tumor.

Our data demonstrated that activation of Jak2/STAT3 in myeloid cells in the presence of tumor-derived factors was closely associated with the accumulation of ImCs. These cells have a phenotype of Gr-1+CD11b+ and their production was found to be dramatically increased in every type of cancer tested (12, 14). These myeloid cells have a high immunosuppressive potential and are considered one of the major factors responsible for tumor-associated immune suppression. However, ImCs with the same phenotype are also part of the normal process of myeloid cell differentiation. They are present in normal BM and spleen and in culture during DC differentiation from HPCs. A recent study has demonstrated that ImCs differentiate in vivo into mature granulocyte, macrophages, or DCs (30). Our data have shown that ImCs generated in the presence of tumor-derived factors or isolated from tumor-bearing mice have a much higher level of Jak2 and STAT3 activity than their control counterparts. Removal of ImCs from the population of myeloid cells generated in the presence of tumor-derived factors significantly reduced STAT3 activity. Thus, it appears that accumulation of ImCs with high STAT3 activity was largely responsible for the observed increased level of STAT3 in the total population of myeloid cells generated in the presence of TC CM.

ImCs generated in the presence of tumor-derived factors and in tumor-bearing mice had a significantly higher proliferative potential than control ImCs. This was not surprising, because increased STAT3 activity promotes cell proliferation. Our data demonstrated that the continuous presence of tumor-derived factors is necessary for preventing differentiation of DCs. Removal of those factors allows ImCs to quickly differentiate into fully mature DCs. These data are consistent with previously published work demonstrating improvement of DC differentiation after tumor resection (4).

Tumor-derived factors induced accumulation of ImCs and reduced production of DCs. However, a substantial proportion of DCs could still be obtained under those experimental conditions. Some DCs, albeit in a decreased number, can be found in tissues from tumor-bearing mice and cancer patients. We asked what happens with the level of STAT3 and functional activity of those cells. To identify DCs, we used CD11c, a marker relatively specific for
these cells. CD11c+ cells isolated from cultures with tumor-derived factors had substantially higher STAT3 activity than control CD11c+ DCs. CD11c+ DCs generated in the presence of tumor-derived factors had a significantly lower ability to stimulate allo-
genetic T cells and expression of MHC class II and costimulatory molecules. These data indicate that CD11c+ DCs obtained in the presence of tumor-derived factors have a higher level of STAT3 activity and are functionally impaired. Activation of these cells with either LPS or TNF-α inhibited STAT3 DNA binding activity but was not able to improve their function. This may explain a well-established fact that DCs that still can be found in tumor tissues are lacking markers of activated cells and have impaired functional activity. To establish a possible causative relationship between STAT3 activation and abnormal myeloid cell differentiation in cancer, we inhibited STAT3 using a dominant-negative STAT3 vector or overexpressed STAT3 in HPC using a constitutive activated Jak2/STAT3 in tumor-derived factors may inhibit DC differentiation and increase production of ImCs via constitutive activation of Jak2/STAT3 in tumor-derived factors. Increased production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. Nat. Med. 2:1096.


