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Down-regulation of signal transducer and activator of transcription 3 improves human acute myeloid leukemia-derived dendritic cell function

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ABSTRACT

Signal transducer and activator of transcription (STAT) 3 inhibits dendritic cell (DC) differentiation and is constitutively activated in blasts of approximately half of AML patients. We investigated the correlation between STAT3 activity, DC maturation and the ability to stimulate T-cells in primary acute myeloid leukemia (AML)-derived DCs. STAT3 knock-down by shRNAmir increased the ability of AML-DCs to stimulate T-cells. Treatment of AML-DC with arsenic trioxide, but not AG490, JSI-124 or NSC-74859, led to a more mature phenotype and enhanced T-cell stimulation, while having minimal effect on normal DC. We conclude that AML-DCs have improved immunogenicity after reducing STAT3.

1. Introduction

Signal transducer and activator of transcription (STAT) 3 has recently emerged as a potential negative regulator of immune function. Cheng et al. [1] showed that the activation state of STAT3 in murine antigen presenting cells (APCs) was critical in directing the outcome of antigen-specific T-cell responses. Reduced STAT3 activation led to T-cell priming and activation, while STAT3 activation in APCs led to impaired antigen-specific T-cell responses. Since this finding, several groups have described different roles for STAT3 as an immune regulator [2–5]. One group, for example, showed that immature murine myeloid cells in the presence of STAT3-activating tumor-derived factors did not differentiate into mature dendritic cells (DCs); instead they retained an immature myeloid phenotype [6]. The same group went on to show that removal of the tumor-derived factors allowed full differentiation and that pharmacologic inhibition of Janus Activated Kinase (JAK)/STAT signaling by JSI-124 abrogated the effects of the tumor-derived factors.

Few of these studies have specifically identified STAT3 as the sole protein behind APC impairment. A clear interpretation of the role of STAT3 and APC function from previously published murine models is also obscured by the fact that STAT3-activating tumor-derived factors used in these studies activate several pathways. In addition, previously used JAK/STAT inhibitors do not specifically target STAT3 signaling and the inhibition of additional non-target pathways may have led to improved APC differentiation.

In this work we specifically identify STAT3 as one of the key regulators of cytokine-induced DC differentiation in acute myeloid leukemia (AML) blasts. We show that reducing STAT3 protein with shRNAmir during differentiation leads to more immunogenic DCs. In addition, we have comparatively evaluated four broad range inhibitors capable of reducing STAT3 phosphorylation, for the ability to enhance AML-DC immunogenicity: AG490, a tyrosine kinase inhibitor with activity against JAK2, epithelial growth factor receptor (EGFR) and mitogen-activated protein kinase [7]; arsenic trioxide (ATO), a protein tyrosine kinase inhibitor [8]; JSI-124, a semi-selective JAK2/STAT3 inhibitor [9] and NSC-74859, a phospho-tyrosine mimic [10]. Treatment of AML-DCs during maturation with ATO, but not the remaining inhibitors, enhanced immunogenicity.

2. Materials and methods

2.1. Cell lines and primary cells

The human AML cell lines HEL, KG-1 and MUTZ-3 were purchased from DSMZ (The German Collection of Microorganisms and Cell Cultures). The HEK293T cell line, used for lentiviral packaging, was purchased from Open Biosystems (Pittsburg, PA).

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Cryopreserved and fresh low density fraction bone marrow samples from newly diagnosed (no acute promyelocytic leukemia) AML patients, containing >75% blasts with more than 10^9 cells, and cord blood (CB) mononuclear cells were obtained from the Institute's Hematopoietic Procurement Facility following informed consent and approval by the Roswell Park Cancer Institute Scientific Review Committee and Institutional Review Board.

2.2. Generation of DCs

Primary AML and CB cells were cultured at 1.5×10^6 cells/mL in RPMI 1640 containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin (Pen/Strep/Glut), 80 ng/mL recombinant human (rh) granulocyte-macrophage colony stimulating factor (GM-CSF) and 20 ng/mL rh interleukin (IL)-4 for 6 days. During the last 48 h of culture 5 ng/mL rh tumor necrosis factor (TNF)-α was added to induce maturation. Cytokines were purchased from R&D Systems (Minneapolis, MN); all other supplies were purchased from Invitrogen (Grand Island, NY) unless otherwise specified.

KG-1 and MUTZ-3 cell lines were cultured at 1.5×10^6 cells/mL in Isocove's DMEM and MEM-α, respectively, containing 20% FBS, Pen/Strep/Glut and 10 ng/mL phorbol myristate acetate (PMA) (Sigma, St. Louis, MO) for 5 days. TNF-α was added during the last 48 h of culture.

2.3. STAT3 knock-down

The Expression Arrest™ pGIPZ lentiviral shRNAmir system was purchased from Open Biosystems. Lentiviral packaging plasmids (Didier Trono Laboratory, Cambridge, MA) pCMV-dR8.74 and pMD2.G and non-silence shRNAmir or STAT3 shRNAmir pGIPZ plasmids were transfected into HEK293T packaging cells using Lipofectamine™ 2000. Two days following transfection, supernatant containing lentivirus was filtered and polybrene (Sigma) was added to a final concentration of 8 μg/mL. Infection was carried out at 25 °C for 1 h, while undergoing centrifugation at 1000 × *g* in 6 well plates. Due to low transduction efficiencies in cryopreserved samples, blasts were infected twice daily for the first three days of differentiation.

2.4. STAT3 inhibitors

AG490, ATO and JSI-124 were purchased from Sigma. NSC-74859 was purchased from Calbiochem (Billerica, MA). All four inhibitors were tested for the ability to reduce STAT3 phosphorylation and enhance AML-DC immunogenicity. Only ATO was tested for the ability to enhance CB-DC immunogenicity.

2.5. Western blot

Western blots were conducted as previously described [6]. ImageQuant analysis software (BioRad, Hercules, CA) was used to quantify protein bands. The following antibodies were used for western blotting: total STAT3, STAT5A/B, Actin, (Santa Cruz Biotechnology, Dallas, TX) and phosphorylated STAT3 (Cell Signaling Technology, Boson, MA).

2.6. Immunophenotype

To study the DC immunophenotype the following antibodies were purchased from BioLegend (San Digo, CA) unless otherwise specified: (Alexa-647-conjugated) mouse IgG Isotype control, CD11c, CD80, HLA-DR, (phycoerythrin, PE)-conjugated mouse IgG isotype CD83, CD86, CD40, (fluorescein isothiocyanate, FITC)-conjugated mouse IgG isotype and CD54. A PE-conjugated anti-CD3 antibody (Miltenyi Biotech, Auburn, CA) was used to determine T-cell purity. Cells were run on a FACSCalibur, collected data were analyzed using WinList 6.0 software (BD Biosciences, San Jose, CA).

2.7. Endocytosis assay

Non-silenced or STAT3 shRNAmir transduced AML cells were cultured for 6 days in rhGM-CSF and rhIL-4. The cells were cultured at 37 °C for 1 h with 1 mg/mL 10,000 MW Cascade Blue®-dextran. Cells were washed three times with cold phosphate-buffered saline, 1% FBS, 1 mM ethylenediaminetetraacetic acid. Following washing, cells were labeled with Alexa-647 CD11c antibody. Cells were analyzed on a LSRII flow cytometer (BD Biosciences) for green fluorescent protein (GFP), CD11c-Alexa 647, and Cascade Blue®-dextran. To verify localization of the dextran molecules cells were analyzed on an ImageStream cytometer (Amnis, Seattle, WA).

2.8. Fluorescent in situ hybridization (FISH)

To verify the leukemic origin of DCs, one sample with trisomy 8 and one with a del(5q) were analyzed following differentiation by FISH as previously described by us [11]. For more information please see supplemental materials.

2.9. Allogeneic (allo) mixed lymphocyte reaction (MLR) assay

AML-DCs or CB-DCs were used as stimulators in an allo-MLR assay as previously described by us [11]. For more information please see supplemental materials.

2.10. CTL assay

CTL assays were conducted as previously published by us [11]. For more information please see supplemental materials.

2.11. Cytokine analysis

AML-DCs and CB-DCs were washed twice with media before the addition of fresh media containing 10% FBS and Pen/Strep/Glut. Supernatants were collected 24 and 48 h later, centrifuged to remove cellular debris and stored at −80 °C until analysis. A Luminex kit purchased from Millipore (Billerica, MA) was used to analyze the supernatants for cytokine levels.

2.12. Statistical analysis

shRNAmir data were analyzed for averages and standard deviations using Microsoft Excel. Statistical significance was determined using the paired, two-tailed Student's *t* test. Results were considered to be statistically significant for *p* values <0.05.

To determine the effect of ATO dose on the MLR response (counts per minute, CPM) in AML-DCs and CB-DCs, the association was modeled as a mixed effects model treating dose levels as fixed effects and patient identifier as a random effect. The mixed effects model accounts for within-patient correlation in the count per minute outcome measurements. Descriptive statistics such as frequencies and relative frequencies were computed for all categorical variables. Numeric variables were summarized using simple descriptive statistics such as the mean, standard deviation, range, etc. A variety of graphical techniques were used to display data. Given the exploratory nature of this study, all *p* values <0.05 were considered statistically significant. No adjustments were made to account for the effects of multiple testing.

3. Results

3.1. shRNAmir knock-down of STAT3 in differentiating AML blasts improves AML-DC immunogenicity

As shown in the supplemental material, AML-DC can be differentiated from AML blasts (Supplementary Tables 1 and 2 and Supplementary Fig. 1). Initial experiments indicated that AML-DCs derived from blasts with low STAT3 activity had increased T-cell stimulatory function. To determine whether this function of AML-DCs could be improved by specifically reducing STAT3 expression, samples from four AML patients were recovered from cryopreserved stock and transduced with lentiviral shRNAmirs while undergoing differentiation with GM-CSF and IL-4. Infection rates ranged from 5% to 20% of the total cell population. The transduced cells were isolated by flow cytometric sorting of GFP expression mediated by the non-silence and STAT3 shRNAmir lentivirus.

Western blot revealed STAT3 protein levels were specifically reduced in each of the four samples with an average reduction of 50%. Fig. 1A depicts a representative example showing that the STAT3 shRNAmirs had no effect on total STAT5 protein. These results indicate specificity of the shRNAmirs and also exclude the possibility that STAT5 inhibition enhanced DC function. To determine whether the reduced STAT3 protein levels increased immunogenicity, allogeneic T-cell stimulation was quantified by MLR. Cells transduced with STAT3 shRNAmir produced an average increase in T-cell proliferation of 32% compared to non-silence shRNAmir controls, $p < 0.01$ (Fig. 1B).

To determine whether reduced STAT3 protein levels would alter the DC phenotype, shRNAmir-transduced cells were analyzed by flow cytometry. A small increase in the percentage of cells expressing the co-stimulatory proteins CD80 and CD86, the DC marker CD11c and the major histocompatibility class II protein, HLA-DR, were observed in two of four samples after STAT3 knockdown. No significant differences were observed in the marker density or the percentage of cells expressing DC markers in the remaining samples (data not shown).