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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.

Keywords

STAT3; ACUTE MYELOID LEUKEMIA; DENDRITIC CELLS

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Author Contribution:

M.T.B. performed all the studies as part of his qualification of doctorate of philosophy, A.M. performed the statistical analyses, S.N.S. performed the FISH analyses, L.A.F. collected patient information and managed the database, H.M. assisted in the ImageStream analyses, E.S.W. contributed to the care of the patients, K.P.L. contributed to the conduct of the study, H.B. contributed to the conduct of the study, M.W. was Dr. Brady's PhD mentor, oversaw the conduct of the study, contributed to the care of the patients and to the manuscript preparation

All authors reviewed and approved the final manuscript.

Conflict of Interest

The authors have no conflict of interest.

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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 shRNA_{mir} 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.

MATERIALS AND METHODS

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). 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.

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), 2mM L-glutamine, 100IU/mL penicillin, 100µg/mL streptomycin (Pen/Strep/Glut), 80ng/mL recombinant human (rh) granulocyte-macrophage colony stimulating factor (GM-CSF) and 20ng/mL rh interleukin (IL)-4 for 6 days. During the last 48 hours of culture 5ng/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 10ng/mL phorbol myristate acetate (PMA) (Sigma, St. Louis, MO) for 5 days. TNF- α was added during the last 48 hours of culture.

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 μ g/mL. Infection was carried out at 25°C for 1 hour, while undergoing centrifugation at $1000 \times g$ in 6 well plates. Due to low transduction efficiencies in cryopreserved samples, blasts were infected twice daily for the first three days of differentiation.

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.

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, Boston, MA).

Immunophenotype

To study the DC immunophenotype the following antibodies were purchased from BioLegend (San Diego, 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).

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 hour with 1mg/mL 10,000 MW Cascade Blue®-dextran. Cells were washed three times with cold phosphate-buffered saline, 1% FBS, 1mM 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).

Flourescent 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.

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.

CTL Assay

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

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 hours 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.

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.

RESULTS

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 Figure 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%. Figure 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, $p < 0.01$ (Figure 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).

A key feature of immature DCs is endocytosis and subsequent processing of environmental antigens. To determine whether this endocytic activity was altered by STAT3 reduction, we analyzed the uptake of Cascade Blue® dextran by shRNAmir-transduced immature AML-DC. Flow cytometric analysis of GFP-positive, CD11c-positive DCs demonstrated no significant difference in the percentage of cells with dextran or the amount of dextran molecules per cell between the non-silence and STAT3 shRNAmir transduced AML-DCs (Figure 1C). Internalization of the dextran molecules was confirmed by ImageStream cytometry (Figure 1D).

The leukemic origin of FACS sorted CD11c-positive and GFP-positive AML-DCs was verified in two samples by FISH, using unique chromosomal aberrations (Supplemental Figures 2A, B).

Pharmacological inhibition of STAT3 activity to enhance AML-DC immunogenicity

Specific reduction of total STAT3 protein with shRNAmirs led to enhanced AML-DC immunogenicity. To determine whether pharmacologically reduced STAT3 activity in AML blasts would provide the same result, we measured the effects of four broad range inhibitors known to lower STAT3 activity (AG490, ATO, JSI-124 and NSC-74859).

Differentiating blasts proved to be very sensitive to inhibitors and either did not differentiate or underwent apoptosis when treated early in culture. Therefore, we treated DCs during the final 24 hours of maturation with inhibitor concentrations that provided acceptable viability. STAT3 phosphorylation in AML-DCs was measured by western blot (Figure 2A). AG490 was most effective at reducing phosphorylated STAT3 levels, followed by ATO and JSI-124. NSC-74859 did not significantly reduce phosphorylated STAT3 at the indicated concentrations; increasing the concentration resulted in poor viability.

The inhibitors were next tested for their ability to improve AML-DC stimulation of allogeneic T-cells. When the inhibitors were added 24 hours prior to harvest, greater viability and enhanced MLR response were observed compared to 48 or 6 hour treatments. The increase in T-cell stimulation was not statistically different from untreated controls when using AG490, JSI-124 or NSC-74859; however treatment of AML-DCs with ATO significantly increased T-cell stimulation. Figure 2B illustrates a representative AML-DC sample treated with the inhibitors showing MLR response as a percentage of the untreated control.

No increases were observed in the proportion of cells expressing, or in the densities, of the markers CD80, CD86, CD40, CD11c, CD83 or HLA-DR in AML-DCs treated with AG490 or JSI-124 (Figure 2C). Treatment of DCs with ATO increased, in six of nine AML samples, the percentage of cells with a more mature phenotype. A representative sample in Figure 3A demonstrates the dose-dependent enhancement in the mature phenotype recovered after ATO treatment.

ATO enhances AML-DC immunogenicity

To further explore the treatment benefits of ATO, AML-DC derived from nine patient samples and AML-DC derived from the MUTZ-3 and KG-1 cell lines were treated with ATO for 24 hours and the MLR response was measured using allogeneic T-cells (Figure 3B). ATO treatment produced an average increase in T-cell stimulation of 30% ($p<0.01$) with increases seen in eight of nine patient derived AML-DCs and in both MUTZ-3 and KG-1 AML-DCs. Experiments were repeated for each sample at least 3 times using $1\mu\text{M}$ and/or $2\mu\text{M}$ doses of ATO. MLR response was increased by a statistically significant margin in ATO-treated AML-DCs compared to untreated controls, but not between $1\mu\text{M}$ and $2\mu\text{M}$ concentrations ($p<0.01$ and $p=0.51$, respectively).

We asked whether the response to ATO is unique to AML-DCs or whether it could be detected in normal cells. For this, the effect of ATO on maturation of normal hematopoietic cells was determined using mononuclear cells isolated from three umbilical CB samples. Treatment with $1\mu\text{M}$ ATO for 24 hours increased the MLR response on average 14% ($p<0.01$), whereas treatment with $2\mu\text{M}$ ATO led to lower MLR responses (Figure 3C).

We next analyzed ATO-treated CB-DCs for changes in immunophenotype. No changes in the percent of cells expressing DC markers or the density of DC markers per cell were observed in the two of the three samples. One sample treated with ATO revealed a modest increase in the percentage of cells expressing CD80 and CD86; however this increase was not accompanied by an increase in the percentage of cells expressing the mature DC marker CD83 as was observed in the ATO-treated AML-DCs (data not shown).

Supernatants from untreated and ATO-treated AML-DCs were analyzed to determine the effect of ATO on IL-12 expression. The amount of IL-12p40 secretion varied among samples, however four of six AML-DCs demonstrated increased IL-12p40 secretion, up to 4.5 fold, upon treatment with $1\mu\text{M}$ ATO (Figure 4A). To confirm that IL-12p40 contributed to or even accounted for the increased MLR response by ATO, 1.3ng/mL of IL-12 (the approximate amount of increased IL-12p40 secretion from ATO-treated AML-DCs) was added to an MLR co-culture of AML-DCs and allogeneic T-cells. MLR response was compared between IL-12p40-treated and ATO-treated AML-DC derived from the same sample. A similar increase in T-cell response was detected between IL-12p40-treated (20%) and ATO-treated (19.6%) AML-DC (Figure 4B). In contrast to AML-DC, CB-DC responded to ATO treatment with only a modest increase of 43% of IL-12p40 production (data not shown).

Finally, we asked whether ATO-treated AML-DCs could increase the leukemia specific cytotoxic potential of autologous CD8 lymphocytes. One sample for which both diagnostic AML blasts and corresponding autologous remission mononuclear cells was available. CD3 T-cells obtained in remission were incubated with control or ATO-treated AML-DCs for 7 days. CD8 T-cells isolated from these cultures were then used as effectors in co-culture with CFSE-labeled unmodified blast targets. The percentage of dead and apoptotic blasts was measured 18 hours later by flow cytometry. CD8 T-cells stimulated by ATO-treated AML-DCs demonstrated increased cytotoxicity toward autologous AML blasts (average 2.1 fold) compared to CD8 T-cells stimulated by untreated AML-DCs (Supplemental Figure 3). Due to limited autologous diagnostic and remission patient samples this assay could not be repeated with additional samples.

DISCUSSION

It is well documented that STAT3-related pathways are involved in differentiation, but it is unclear whether STAT3 is involved in the initiation and maintenance of differentiation or in

the activation and maturation stage of DC development. Further complicating the role of STAT3 in differentiation is the fact that guidelines to characterize differentiated DC are not standardized, leaving the definition of a mature DC somewhat ambiguous. For this reason, one of the key measures for determining the extent of DC activation and maturation is through MLR response. Several groups [5, 6] have inhibited STAT3 signaling in murine and cell line models and used both DC markers and MLR response as a measure of DC immunogenicity. While STAT3 inhibition appears to increase immunogenicity, the exact roles of STAT3 and when it comes into play are still unknown.

Our research shows that the level of STAT3 phosphorylation is increased in AML-DCs compared to undifferentiated blasts, leading to the possibility, that constitutive STAT3 activation may allow blasts to differentiate, but failure of its down-regulation may inhibit the final maturation steps necessary for efficient T cell stimulation. This is in agreement with our finding that the ability of AML blasts to differentiate to DCs, but not the ability of AML-DCs to stimulate allogeneic T-cells, was dependent of the level of phosphorylated STAT3.

Specifically targeting STAT3 protein using shRNAmir in AML blasts undergoing differentiation resulted in an increased ability of AML-DCs to stimulate allogeneic T-cells. Reducing STAT3 protein levels had no effect on the endocytic properties in immature AML-DCs and supports the notion that STAT3 regulation is important for events related to maturation. Based on observations made in murine models we did, however, fail to observe significant corresponding changes in the immunophenotype [1, 6]. Due to the limited number of transduced AML-DCs, we were not able to measure secreted cytokine levels. The shRNAmir experiments demonstrated that reduced STAT3 protein in AML-DCs led to enhanced MLR response; they did not, however, determine whether simply reducing the activation of STAT3, such as by inhibiting its tyrosine phosphorylation, would be equally effective. Moreover, the shRNAmir experiments could not be used to determine the time point during which STAT3 inhibition was most important. In order to answer these questions, we treated AML blasts with four pharmaceutical compounds known to modulate STAT3 activity at different time points during differentiation.

Prolonged exposure to the inhibitors during differentiation was generally toxic to blasts. When inhibitors were added during the last 24 hours of differentiation (the maturation phase), viability was retained and DC morphology did not appreciably deviate from controls. Interestingly, only ATO consistently provided increased MLR responses. There are several possible explanations as to why the other inhibitors were ineffective at enhancing DC immunogenicity. The first is that the inhibitors were too toxic to be used at the concentrations required to fully inhibit STAT3 activity without affecting viability. This is likely for JSI-124 and NSC-74859 which were highly toxic, but unlikely for AG490, since neither 5 μ M nor 10 μ M doses consistently changed the MLR response or immunophenotype, yet both were more effective than ATO at reducing phosphorylated STAT3 levels. A second possibility is that AG490 and ATO are not specific inhibitors of STAT3 and the inhibition of other pathways may affect differentiation differently. AG490 for example has a 1000 fold higher inhibition constant toward EGFR than it does for JAK proteins [7, 12], while ATO is known to regulate a number of tyrosine kinases. A third explanation may be related to the prolonged effects of the inhibitors on the AML-DCs after they were removed prior to the 72 hour T-cell co-culture. The other small molecule inhibitors may have transient effects and lack the prolonged effect of ATO.

To further evaluate whether the inhibitors were capable of enhancing AML-DC maturation, we studied the immunophenotype of the treated cells. ATO-treatment led to increased expression of CD80 and CD86 co-stimulatory markers, the mature DC marker CD83 and the

class II HLA-DR protein. The up-regulation of these proteins is indicative of more mature DCs and is in agreement with the notion that ATO ultimately acts as a maturing agent. A recent study supporting our ATO data demonstrated that STAT3 inhibition with AG490 during the final stages of maturation of murine macrophages led to modest increases in antigen-specific T-cell stimulation [1]. Although we observed similar data using ATO, we did not observe a similar effect of AG490, which may have very different effects on murine macrophages compared to human leukemia-derived DCs.

Dendritic cell secreted IL-12 is important for T cell activation and induction of Th1 responses [13]. As such we measured IL-12 protein levels in the supernatants of ATO-treated and untreated AML-DCs. Treatment of AML-DCs with 1 μ M ATO increased IL-12p40 secretion but not IL-12p70 in four of six samples tested. We asked whether this increase in IL-12 secretion could account for the increased MLR response. The addition of IL-12 to untreated AML-DCs in MLR co-culture provided a 20% increase in T-cell response; similar to ATO's effect. This concordance suggests that ATO directly or indirectly enhances MLR response through the promotion of IL-12 secretion. Interestingly, increased IL-12 secretion has been linked to a STAT3-regulated event. Jobin et al. showed that murine bone marrow-derived DCs, induced with lipopolysaccharides had an accumulation of IL-12p40 mRNA and increased protein secretion and further showed that a constitutively activated form of STAT3 blocked this activity [14]. In the same system, STAT3 inhibition by AG490 increased IL-12p40 protein secretion by 64%, indicating the importance of STAT3 in IL-12 regulation. These data further suggest that the effect of ATO is mediated, in part, through down-regulation of STAT3 activity.

Finally, only 20 of 58 samples differentiated to DC. A majority of these cells did not survive in culture, a common problem when working with cryopreserved leukemia samples. We selected 10 of the 20 samples, which represented a population of enriched viable AML-DCs and allowed us to avoid sorting viable DCs, a process that may have altered STAT3 activation. Interestingly, the majority of these samples contained FMS-related tyrosine kinase 3 (*FLT3*)-internal tandem duplications (ITD), explaining the availability of large number of cryopreserved cells needed for studies as described here. A previous study suggested that *FLT3*-ITD mutations impeded AML blast differentiation; however this study used FLT3-ligand as a differentiating agent, which is likely to yield different results than GM-CSF and IL-4 differentiated cells [15]. FLT3 activates STAT5; however no difference in STAT5 expression was detected in the STAT3 knock-downs suggesting that the effect was independent of STAT5 activity. Future trials will need to consider the fact that not all AML blasts can differentiate into AML-DC.

CTL responses could only occur if T-cells recognize an antigen presented on the surface of the AML-DC. Due to a limited number of samples we were able to test induction of CTL response in only one sample; however the results provide a proof-of-principle that CTL responses can occur with AML-DC and that ATO augments this effect.

In summary, AML-DCs targeted with STAT3 specific shRNAmir have increased MLR response, clearly demonstrating a key role for STAT3 in AML-DC differentiation. Treatment of AML-DCs with ATO, a compound possessing STAT3 inhibitory properties, enhances immunogenicity through enhanced DC maturation, increased expression of co-stimulatory molecules and increased secretion IL-12p40, all events that can be related to STAT3 signaling

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Effects of STAT3 knock-down on phenotype and function in patient sample-derived AML-DCs

(A) Representative sample demonstrating STAT3 knock-down in sorted cells. (B) Allo-MLR of four sorted non-silence and STAT3 shRNA samples, * $p < 0.05$. (C) Flow cytometry plots showing dextran and CD11c-positive DCs within the GFP gated population. (D) ImageStream depicting intracellular localization of dextran within an AML-DC. Bright field GFP and Cascade Blue® Dextran images were captured for a single shRNA-transduced AML-DC. No differences were observed between non-silence and STAT3 shRNA-transduced cells.

2A

2B

2C

Figure 2. Inhibitors' effect on P-STAT3, MLR and phenotype

(A) Western blot of a representative AML-DC incubated with inhibitors for 24 hours. (B) MLR response was measured in inhibitor-treated AML-DCs. T-cell cpm were averaged and normalized against controls to determine percent change, * $p < 0.05$. (C) Flow cytometry plots showing change in phenotype of AML-DC treated with inhibitors for 24 hours.

3A

3B

Figure 3C

Figure 3. Treatment with ATO enhances phenotype and immunogenicity

(A) Flow cytometry plot showing dose-dependent changes in phenotype in ATO treated AML-DCs. An increased proportion of cells expressed CD83 in addition to the co-stimulatory molecules CD80 and CD86. (B) MLR showing increased T-cell stimulation

after ATO treatment in 8/9 AML samples and 2/2 cell lines * $p < 0.05$. (C) 1 μ M but not 2 μ M ATO treatment enhances CB-DC stimulation of T-cells.

4A

4B

Figure 4. ATO enhances IL12p40 secretion in of AML-DCs

(A) Luminex analysis of 24 hour DC supernatants reveals increased IL-12p40 secretion in ATO-treated AML-DCs compared to untreated controls. (B) IL-12 and ATO treatment of

AML-DCs increase MLR response by a similar margin, 20% and 19.6%. Treatment with AG490 or JSI-124 did not significantly increase response.