Bone Marrow Derived Dendritic Cells are More Suitable Than Dendritic Cell Line DC2.4 to Study Tumor-Mediated Suppression of DC Maturation Through STAT3 Hyperactivation

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ABSTRACT – Purpose. Tumors can escape immune eradication by harnessing dendritic cell (DC) maturation. However, DC types used as *in vitro* models to study tumor-mediated immunosuppression possess fundamental variability that could influence research outcomes. Therefore, we assessed the behavior of two distinct murine DC models upon exposure to tumor-conditioned medium of B16.F10 melanoma (B16-CM). Methods. Using primary bone-marrow derived dendritic cells (BMDCs) or immortalized DC2.4 cell line, we evaluated the level of signal transducer and activator of transcription 3 (STAT3) phosphorylation by Western blot as a molecular parameter. We also examined the surface expression of co-stimulatory molecules on DCs by flow cytometry as a phenotypic parameter. Results. Our results revealed critical discrepancies between the two models in response to tumor-conditioned medium. While conditioned medium was able to induce STAT3 phosphorylation in BMDCs, it did not significantly induce STAT3 phosphorylation in DC2.4 cell line. Moreover, only in BMDCs, the expression of CD86 and CD40 was remarkably downregulated by B16-CM and was not totally recovered after LPS stimulation. In contrast, DC2.4 cells did not show any signs of harnessed maturation upon exposure to B16-CM. Conclusions. In order to study the effect of tumor-mediated immunosuppression on DC maturation *in vitro* via tumor-induction of STAT3 activation, primary BMDCs are more reliable as a model than DC2.4.

INTRODUCTION

Aberrant maturation of dendritic cells (DCs) is a hall mark for tumor-mediated immunosuppression (1). Upon exposure to tumor microenvironment, DCs encounter tumor-derived factors (TDF) that suppress their maturation through modulation of specific molecular signals. One of these is the signal transducer and activator of transcription 3 (STAT3) signaling pathway. This downstream cytoplasmic protein, STAT3, becomes activated by phosphorylation on a single critical tyrosine (Y^{705}) in response to cytokines and growth factor-receptor stimulation (2). Unlike transient activation in normal cells, STAT3 has been detected in a wide variety of cancer cell lines and primary tumors in a constitutively active form (2). This has been proven to mediate immunosuppression by inducing STAT3 activation in tumor-exposed DCs (3). As a result, those DCs are neither capable of priming naïve T cells nor restoring T cell activity (3). On the contrary, they rather result in T cell tolerance (4), in both laboratory and clinical settings (5, 6).

Two murine DC models have been used in literature in order to study TDF effects on DC maturation and STAT3 activation *in vitro*. The first model is primary DC culture generated from murine bone marrow (BMDCs) (7). This model provides $CD11c^+$ myeloid cells of DC population of 70-75% purity based on the expression of CD11c (7). These DCs have basal expression of MHC-II, low expression of co-stimulatory molecules (CD40, CD80, and CD86), and low expression of macrophage surface markers (CD11b and CD14) (7). The second model is a dendritic cells line (DC2.4) that has been developed by Dr. Kenneth Rock (8). Characterization of this cell line indicates high expression of MHC-I, MHC-II, CD80, CD86, and ICAM-1. Nevertheless, due to the inconsistency in the characteristics of the two models, there is a potential inconsistency between the models with respect to tumor-mediated suppression of DC maturation following STAT3 hyperactivation in DCs.

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In this study, we address this possible discrepancy, by exposing BMDCs and DC2.4 to tumor-conditioned medium from B16.F10 murine melanoma cells (B16-CM) that constitutively express high levels of activated STAT3. Therefore, this could induce STAT3 activation in target DCs.

MATERIALS AND METHODS

Materials

Fetal Bovine Serum (FBS) was obtained from HyClone (Logan, UT). Dulbecco's modified Eagle's medium (DMEM), RPMI-1640, L-glutamine, and gentamicin were purchased from Gibco-BRL (Burlington, ON, Canada). Recombinant murine GM-CSF was purchased from Peprotech (Rockville, IL). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St. Louis, MO). Anti-mouse CD11c, CD86, and CD40 mAbs, and corresponding isotype controls, were purchased from BD Biosciences (Mississauga, ON, Canada). IL-6 ELISA kit was purchased from e-Biosciences (San Diego, CA). Anti-phosphotyrosine (Y^{705}) STAT3 mAb, anti-STAT3 mAb, and anti-actin antibody (I-19) were purchased from Santa Cruz Biotechnology (Stanta Cruz. CA). ECL Plus™ detection kit was purchased from GE Healthcare Life Sciences (Piscataway, NJ). Micro BCA Protein Assay Kit was purchased from Thermo Fisher Scientific Inc. (Rockford, IL).

Cell culture

BMDCs primary cultures were generated from bone marrow precursors of C57BL/6 mice femurs and propagated in complete RPMI-1640 in presence of GM-CSF as previously described (7). DC2.4 cell line, obtained from Dr. Kenneth Rock (University of Massachusetts, Worcester, MA), was propagated in complete RPMI-1640 with 10% FBS. B16.F10 cell line was provided by Dr. Mavanur Suresh (University of Alberta, Edmonton, AB), and NIH 3T3 cell line was obtained from American Type Culture Collection (Manassas, VA). Both cell lines were propagated in DMEM with 10% FBS. To generate tumor-conditioned medium from B16.F10 (B16-CM), confluent B16.F10 cells were incubated in fresh serum-free DMEM medium for 24 h. Thereafter, the medium was collected and centrifuged before exposure to DC culture.

DC exposure to B16-CM

Culture media for day-7 BMDCs or DC2.4 cultures were replaced by 50% of B16-CM. FBS was supplemented to 10% final concentration in culture. Untreated DCs and DCs exposed to conditioned medium of NIH 3T3 were used as controls. Thereafter, BMDCs or DC2.4 were stimulated with 100 ng/mL LPS for 6 h to induce DC maturation. Moreover, BMDCs or DC2.4, unexposed to B16-CM but stimulated with LPS, were used as positive controls for maturation studies.

Flow cytometry

After designated treatments, cells were harvested, washed twice with PBS (pH 7.4). Cell suspensions consisting of 2×10^5 cells/100 μL were prepared in FACS buffer (Phosphate-buffered saline [PBS] containing 5% FBS) and incubated with anti-CD11c or anti-CD86 with FITC-conjugated secondary antibodies, or anti-CD40 with Cy-Chrome conjugated secondary antibody. The isotype standard for each antibody was also used to measure background fluorescence intensity. The samples were acquired on a Becton–Dickinson FACSort™ flow cytometer (Franklin Lakes, NJ) and the data was analyzed with CellQuest™ software. At least triplicates of each sample were tested.

Western blot

Cells were collected and washed twice with ice-cold PBS and then lysed in buffer containing 50 mM HEPES (pH 7.4), 5 mM CHAPS, 2 mM Na_3VO_4, 25 mM NaF, 2 mM EGTA, 2% Nonidet P-40, 1:100 Protease Inhibitor Cocktails (Sigma-Aldrich), 0.5 mM DTT and 6.4 mg/mL Phosphatase Substrate (4-Nitrophenyl phosphate) (Sigma-Aldrich). Cell lysates were centrifuged for 20 seconds at 16,000×g. Total protein concentration in the cell extract was determined by micro BCA protein assay. Equal amounts of protein (20 μg) were loaded in 8% SDS-PAGE. Proteins were then transferred into PVDF membrane and were probed with desired antibodies. Membranes were developed using ECL Plus™ detection kit. The experiment was independently performed 4 times.

RESULTS

Effects of B16-CM on BMDC

Our aim was to induce STAT3 activation in DCs by B16-CM exposure. One of the TDFs that is a potent inducer of STAT3 phosphorylation in DCs is IL-6 (6). Therefore, we assessed IL-6 secretion in B16-CM. Our results indicate that IL-6 secretion by B16.F10 follows a time-dependent fashion reaching a plateau level at 24 h (Fig. 1A). Hence, we chose 24 h time point to collect B16-CM and expose it to BMDCs or DC2.4 cultures. Moreover, we detected DC-lineage-specific surface marker (CD11c) by flow cytometry to rule out possible change in lineage differentiation in BMDCs primary culture due to exposure to B16-CM. The results showed no difference in CD11c expression between the B16-CM exposed groups compared to untreated DC population (Fig. 1B). This indicates culture commitment to DC lineage by day 7 in spite of B16-CM exposure.

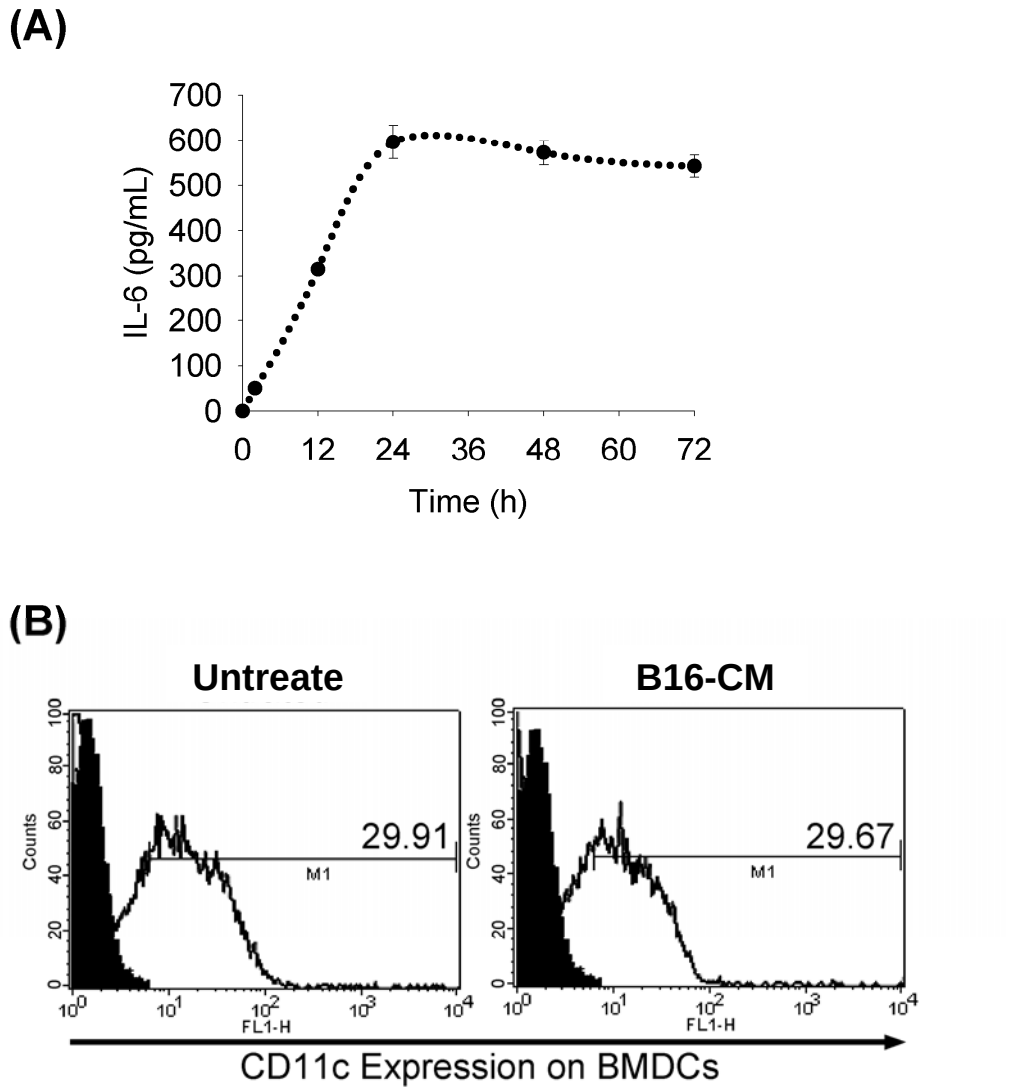

Figure.1. Characterization of B16-CM and its influence on BMDC differentiation. (A) B16.F10 secretion of IL-6 over time determined by ELISA. Results represent mean of IL-6 level at each time point ± SD for triplicates of 4 independent experiments. (B) Flow cytometry histograms of CD11c expression (unfilled histogram) on untreated BMDCs (left panel) or B16-CM exposed BMDCs (right panel). Mean fluorescence intensity is shown for M1 population. M1 gate region indicates the inclusion area for CD11c expression where in-gate cells are considered positive for marker expression. Isotype control (filled histogram) represents non-specific binding.

STAT3 activation profile in DCs exposed to B16-CM

Intracellular levels of phosphorylated STAT3 (p-stat3) were determined in BMDCs and DC2.4 by Western blot. Upon exposure to B16-CM, intracellular p-stat3 level remarkably increased in BMDCs when compared to untreated BMDCs, which only showed basal p-stat3 level (Fig. 2A). In addition, conditioned medium of NIH 3T3 did not induce p-stat3 levels in DCs (data not shown). Moreover, when LPS was used to stimulate BMDCs, p-stat3 was highly expressed with or without B16-CM exposure. On the other hand, Western blot analysis indicated elevated basal level of p-stat3 in untreated DC2.4 culture compared to untreated BMDCs. Although this was slightly induced by LPS, B16-CM remarkably reduced p-stat3 levels in the presence or absence of LPS (Fig. 2A). In all groups, no remarkable difference in total stat3 expression was noticed.

Effect of B16-CM on DC maturation

Flow cytometry was employed to detect the surface maturation markers CD86 and CD40 on BMDCs and DC2.4 upon exposure to B16-CM. In BMDCs, both CD86 and CD40 expressions were reduced after B16-CM exposure compared to untreated control. LPS was able to induce BMDCs maturation as evidenced by high expression of CD86 and CD40. However, pretreatment with B16-CM reduced the ability of LPS to induce BMDC maturation (Fig. 2B). In contrast, exposure of DC2.4 to B16-CM did not reduce maturation markers expression (Fig. 2B). On the contrary, B16-CM seems to further induce CD86 and CD40 expression in DC2.4 cells in comparison to LPS.

DISCUSSION

Tumor-derived factors that activate STAT3 in DCs, such as IL-6, lead to the inhibition of their phenotypic and functional maturation (6, 9) In addition, compelling evidence shows that STAT3 hyperactivation in DCs prime them to secrete anti-inflammatory cytokines, such as IL-10, that act in an autocrine and paracrine fashion to contribute in the deleterious effect on DC maturation (9). However, murine DC models to study and manipulate this effect carry potential discrepancies at least *in vitro*. This is because of the fundamental disparity between murine DC models used in these studies.

BMDCs are primary cells that have not been genetically modified. On the contrary, DC2.4 cells were infected with retrovirus encoding *myc* and *raf* genes in order to immortalize the cell line (8). Since STAT3 is required for the expression of *myc* in myeloid monocytes (10), this explains, at least in part, the high constitutive level of p-stat3 in untreated DC2.4 lysate. Moreover, unlike the scenario in BMDCs, failure of B16-CM to further induce p-stat3 in DC2.4 confirms the independence of STAT3 hyperactivation in DC2.4 from exogenous inducers such as cytokines or growth factors. The reason for the reduction seen in p-stat3 after DC2.4 exposure to B16-CM is unclear to us. Nevertheless, mere detection of p-STAT3 upregulation does not conclusively reflect DC maturation status. As we and others have shown, STAT3 is also activated in mature DCs upon stimulation by LPS. Therefore, we evaluated DC-maturation surface markers in order to assess the influence of STAT3 activation by B16-CM on DC maturation.

Our flow cytometry studies demonstrated distinct phenotypic maturation profiles between BMDCs and DC2.4 cell line. Untreated DC2.4 expresses higher levels of CD86 and CD40 expression than BMDCs, indicating a more mature status. As mentioned earlier, the fact that DC2.4 cells were infected with retrovirus encoding *myc* and *raf* for immortalization may contributed to the induced maturation (8). The use of retrovirus vectors in that process could trigger inflammatory signals in DCs as a result of viral component recognition by TLR-3 or TLR-7 in DC endosome. Therefore, it is not surprising to see reduction in DC maturation in response to B16-CM exposure only with BMDCs. Furthermore, it was only with BMDCs that the ability of B16-CM to reduce LPS stimulation was noticed, while DC2.4 exposure to B16-CM neither reduced basal expression of CD86 and CD40 nor mitigated LPS stimulation. This is consistent with the previous observation from Western blot analysis since STAT3 should provoke an anti-inflammatory state and render DCs immature.

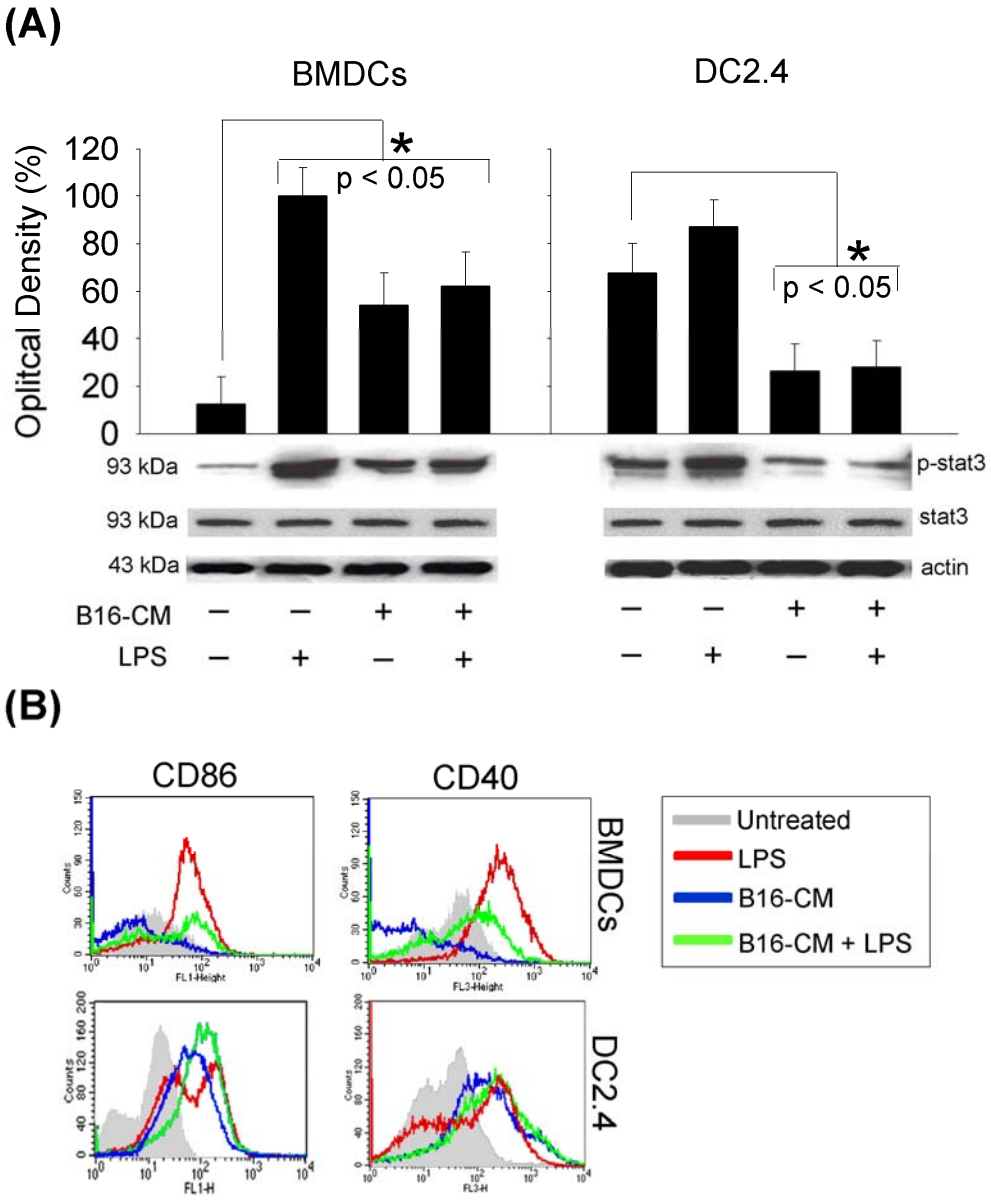

Figure.2: Effects of B16-CM on STAT3 activation and DC maturation. (A) Detection of p-stat3 level in BMDCs (left panel) and DC2.4 (right panel) by Western blot. Data represent the mean of 4 independent experiments ± SD. Optical intensity of p-stat3 band was quantified and normalized to actin protein band using ImageJ software (W. Rasband (2005) National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij). This software accounts for band density, width, thickness, and background. Test-to-loading control ratio was calculated and presented as bar graphs. **(B)** Flow cytometry histograms of CD86 and CD40 expression by BMDCs and DC2.4. Acquisition was performed at least twice and normalized to 10^4 events per run per sample.

However, further studies are needed to shed the light on the molecular reasons of the noticed inconsistency between the two models.

Taken together, these findings preclude DC2.4 cell line as an *in vitro* model to study cancer-mediated immunosuppression. Nevertheless, DC2.4 poses to be an attractive model for antigen-presentation studies because it is easier to propagate and store, and provides more pure population of DCs compared to BMDCs.

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