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Research paper

Conditioned media from the renal cell carcinoma cell line 786.O drives human blood monocytes to a monocytic myeloid-derived suppressor cell phenotype

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ABSTRACT

Myeloid-derived suppressor cells (MDSCs) are immature myeloid cells critical in mediating immune suppression in cancer patients. To develop an in vitro assay system that functionally mimics the tumor microenvironment, we cultured human monocytes with conditioned media from several cancer cell lines. Conditioned media from five tumor cell lines induced survival and differentiation of monocytes into cells characteristically similar to macrophages and MDSCs. Notably, media from the 786.O renal cell carcinoma line induced monocytes to acquire a monocytic MDSC phenotype characterized by decreased HLA-DR expression, increased nitric oxide production, enhanced proliferation, and ability to suppress autologous $CD3^+$ T cell proliferation. We further demonstrated that these in vitro MDSCs are phenotypically and functionally similar to patient-derived MDSCs. Inhibitors of STAT3, CK2, and GM-CSF resulted in partial reversal of the MDSC phenotype. MDSCs generated in vitro from 786.O tumor conditioned media represent a platform to identify potential therapeutics that inhibit MDSC activities.

1. Introduction

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells that arise from myeloid progenitor cells and accumulate in the blood, lymphoid organs, and tumor tissues under different pathological conditions, including cancer. MDSCs suppress both innate and adaptive immune responses, and the accumulation/expansion of MDSCs is considered one of the important mechanisms of tumor evasion by the immune system [1–3]. Clinical observations from cancer patients have demonstrated that the number of MDSCs in peripheral blood is positively associated with tumor burden and clinical stage in several cancers [4–8]. In humans, MDSCs are most commonly characterized by cell surface expression of $CD11b^+$, $CD33^+$, $HLADR^{-/lo}$, and are further classified into two subsets as either $CD15^+$ polymorphonuclear MDSCs (PMN-MDSCs) or $CD14^+$ monocytic MDSCs (mMDSCs) [9–11]. MDSCs are functionally characterized by their ability to suppress T cell activation and proliferation.

MDSCs suppress anti-tumor immunity by a variety of mechanisms that include depletion of L-arginine mediated by arginase 1 (Arg-1), L-cysteine deprivation, activation and expansion of regulatory T cells (Tregs), and production of suppressive factors including nitric oxide (NO), TGFβ, indoleamine 2,3-dioxygenase (IDO), and reactive oxygen species (ROS) [1,3,10].

A number of tumor-derived factors have been reported to induce MDSCs including (but not limited to) GM-CSF, IL-6, PGE2, TGFβ, VEGF, IL-10, and IL-1b [12–15]. Several groups were able to induce MDSC suppressive cell types in vitro by co-culturing PBMCs with GM-CSF + IL-6 [13] or by co-culturing PBMCs or $CD14^+$ monocytes with many different tumor cell lines [16–19]. A limited number of groups have established suppressive myeloid cell populations using only conditioned media from tumor cell lines. For example, several groups have cultured $CD14^+$ monocytes with conditioned media from tumor cell lines and were able to induce phenotypes similar to tumor associated macrophages (TAM) [20,21], while others cultured PBMCs with tumor conditioned media and induced phenotypes similar to MDSCs [22,23]. Another group developed a simple MDSC differentiation system where they culture $CD14^+$ monocytes with conditioned media from primary

Abbreviations: CK2, casein kinase 2; CM, conditioned media; HNSCC, head and neck squamous cell carcinoma; mMDSCs, monocytic myeloid-derived suppressor cells; PMN, polymorphonuclear; RCC, renal cell carcinoma; ROS, reactive oxygen species; TAM, tumor associated macrophages; Tregs, regulatory T cells

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cell lines developed from glioblastoma patients [24]. Since many different soluble factors, tumor cells and tumor conditioned media have generated myeloid cells with suppressive phenotypes, it is likely multiple pathways for MDSC induction exist.

Interrogating MDSCs and evaluating therapies targeting MDSCs are limited by high cost, and other challenges associated with human tumor tissue acquisition, and by the fact that MDSCs are not present in appreciable numbers in healthy human donors. As an alternative, we sought to develop a reliable and simple method to generate large numbers of tumor conditioned MDSCs in vitro. Many of the methods described to date involve starting with a mixed population of PBMCs or co-culturing $CD14^+$ cells with tumor cells. Both methods require subsequent purifications after co-culture for functional assays (e.g. for T cell suppression assays). Other methods described previously use primary cell lines developed from human patient samples, and are not commercially available. We extended previously published work by co-culturing $CD14^+$ monocytes with conditioned media from easily obtainable tumor cell lines, providing pure myeloid populations that do not require additional purification prior to further characterization or evaluation. Here, we report that conditioned media from the renal tumor cell line 786.O induce $CD14^+$ monocytes to acquire an mMDSC phenotype, and we characterize the proliferation capacity and functionality of these cells.

2. Material and methods

2.1. Cell lines and tumor conditioned media collection

Cell lines were purchased from American Type Culture Collection (ATCC) and all were maintained in RPMI supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% Na pyruvate except for SW-1990 (Leibovitz's L-15 Medium), ES-2 (McCoy's 5A) and Cal27 (DMEM). Conditioned media was collected after 48 h from 50-75% confluent cells, filtered at 0.2 μm and stored at −80 °C [20].

2.2. Monocyte differentiation

Monocytes were enriched from leukopack (Astarte Biologics, Bothell, WA)-derived PBMCs by negative selection using the Human Monocyte Enrichment kit without CD16 Depletion (StemCell Technologies) according to the manufacturer's instructions. Monocytes were seeded at 5×10^4 per well in a TC-treated 96-well plate (for proliferation assays), or 5×10^5 per well in a TC-treated 24-well plate (for flow cytometry). Macrophages were differentiated in X-Vivo 15 (Lonza) 10% FBS in the presence of 50 ng/mL rhCSF-1. On day 6, M1 macrophages were polarized in X-Vivo 15 (no FBS) plus 50 ng/mL rhIFN-γ, then further polarized on day 7 by adding LPS to a final concentration of 1 ng/mL overnight. Starting on day 6, M2 macrophages were polarized for 48 hours in X-vivo 15 (with 10% FBS) plus 50 ng/mL rhIL-4. Cytokine-generated MDSCs were derived from monocytes by culturing in X-Vivo 15 10% FBS with rhGM-CSF and rhIL-6, both at 10 ng/mL, for 8 days [13]. Tumor conditioned cells were generated by culturing monocytes in tumor conditioned media from the indicated cell line for 6–8 days [20].

2.3. Cytokine quantitation assays

Conditioned media were collected, filtered, aliquoted and stored at −80 °C prior to analysis. Cytokines (GM-CSF, IL-1β, IL-4, IL-6, IL-10, IL-12p70, IL-13, CSF-1, TNFα, VEGF) and chemokines (CCL11, CCL26, CCL17, CXCL10, CCL3, CXCL8, CCL2, CCL22, CCL13) were quantified using Meso Scale Discovery (MSD) multiplexed electrochemiluminescent immunoassay system and SECTOR Imager 2400, according to the manufacturer's instructions (Gaithersburg, MD).

Table 1
Tumor cell lines used for conditioned media generation.

Cell line	Tumor type	Percent monocyte viability day 6 after culture with CM
786.O	Renal cell carcinoma	82.2 ± 7.9
SW-1990	Pancreatic	< 25
AsPC-1	Pancreatic	43.3 ± 17.7
DLD-1	Colorectal carcinoma	73.8 ± 8.6
ES-2	Ovarian	< 25
CAL-27	HNSCC	81.8 ± 4.6
U118	Brain	86.8 ± 5.4
U87	Brain	86.8 ± 4.7
rhCSF-1	Control	80.6 ± 8.0

CM, conditioned media; HNSCC, head and neck squamous cell carcinoma.

2.4. Nitric oxide and arginase activity

Nitric oxide production was measured in collected conditioned media samples using QuantiChrom Nitric Oxide Assay kit (D2NO-100, Bioassay Systems, Hayward, CA) according to the manufacturer's instructions. Briefly, deproteinated samples were mixed with provided working reagent and incubated at 60 °C for 10 min for the reduction of nitrate to nitrite. After centrifugation, supernatants were transferred to 96-well plates and the absorbance at 540 nm was recorded. Total nitrite concentration was calculated from the standard curve formed by serial dilution of nitrite standards. Arginase activity was measured using QuantiChrom Arginase Assay Kit (DARG-100, Bioassay Systems, Hayward, CA) according to the manufacturer's instructions. Briefly, cells were washed with cold PBS and cell pellets were lysed for 10 min in 1000 mL of 10 mM Tris-HCl (pH 7.4) containing protease inhibitors (complete EDTA-free cocktail tablets, Roche) and 0.4% Triton X-100. Cell lysates were centrifuged and harvested supernatants were mixed with the substrate buffer and reacted with L-arginine to produce urea, which was then incubated with urea reagent. Absorbance at 430 nm was recorded and calculated into arginase activity. One unit of arginase converts 1 μmol of L-arginine to ornithine and urea per minute.

2.5. Isolation of MDSCs from cancer patients

Cancer blood samples were purchased from Conversant Healthcare Systems (Huntsville, AL) where all samples were collected, processed, and distributed in full ethical and regulatory compliance with the sites from which human samples were collected. All participating patients gave written informed consent. Circulating human MDSCs were isolated from whole blood of patients with a history of pathologically-confirmed ovarian carcinoma or renal cell carcinoma. Patients did not receive any immune-modulating therapies for at least two weeks prior to blood collection. Myeloid lineage cells were recovered from whole blood of patients or healthy donors using Whole Blood CD33 MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions. From this population, CD33 high expressing cells were further isolated using the LS column (Miltenyi). CD33 high expressing cells were seeded at 5×10^4 per well in a TC-treated 96-well plate (proliferation), or 5×10^5 per well in a TC-treated 24-well plate (flow cytometry), in X-Vivo 15 + 10% FBS.

2.6. T cell suppression assay

Autologous T cells were isolated from peripheral blood mononuclear cells by positive selection using the Human CD3 Positive Selection Kit (StemCell Technologies) according to the manufacturer's instructions. T cells were stained with a 5 μM solution of CellTrace Violet Proliferation Dye (ThermoFisher) in PBS for 20 min at 37 °C. After quenching with 2x volume FBS, T cells were washed and brought up in Xvivo 15 + 10% FBS. T cells were stimulated with CD3/CD28