Research Article

An In vitro Model System to Generate Breast Cancer MDSCs and Study Immune Cell Interactions in Immunocompetent C57bl/6 Mice

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Abstract

In cancer, immune dysfunction and immunosuppression contributes to the failure of cancer therapies and cancer-related mortality. Myeloid derived suppressor cells (MDSCs) are a potently immunosuppressive population of cells which contribute to dysfunction and immunosuppression. In breast cancer, MDSC levels are clinically relevant and correlate with disease outcome and response to treatment. In this study, the E0771 breast cancer adenocarcinoma cell line was used to induce MDSCs in vitro to recapitulate the in vivo induction of MDSC in immunocompetent C57BL/6 mice. In vivo, approximately 25% of splenocytes derived from the E0771 breast cancer model are phenotypically (CD11b+ GR-1+) and functionally MDSCs with the level of induction dependent on tumor location and burden. Approximately 70% of the cells differentiated in vitro from bone marrow precursors were phenotypically MDSCs and found to suppress the proliferation of responder immune cells. In this study, we describe a parallel in vivo and in vitro model system of MDSC induction utilizing the E0771 breast cancer cell line. The development of this model system in immunocompetent mice is a useful new method to investigate mechanistic questions of MDSC development and MDSC-immune interactions in breast cancer.

ABBREVIATIONS

MDSC: Myeloid Derived Suppressor Cell; MΦ- Macrophage; GEM: Genetically Engineered Mic; H&E: Hematoxalin and Eosin; DC: Dendritic Cell; TCM- Tumor Conditioned Media

INTRODUCTION

Metastatic breast cancer is associated with poor prognosis due, in large part to the immune dysfunction caused by myeloid derived suppressor cells (MDSCs) [1,2]. In particular, anti-cancer vaccines and immunotherapies that depend on the generation of an anti-tumor immune response, fail to be effective due to a dysregulated immune response. Given that much research is aimed at the re-education of the immune system to target and kill tumor cells, there is a significant need to better understand the interactions between tumors and immune cells. The depletion or targeting of MDSCs is considered to be a key component in developing effective cancer immunotherapies [3-5]. MDSC levels can be linked to tumor and metastatic burden and can have prognostic importance due to its use as a biomarker of disease [6-10]. Although MDSC levels impact disease in breast cancer, the factors and immune signaling pathways that contribute to MDSC development in breast cancer and across different cancers require further study [11-13]. Many cancer studies utilize xenograft modeling where human tumors are given to immunocompromised mice [14,15]. However, these model systems fail to allow detailed studies of immune responses in cancer. In order to evaluate the factors involved in MDSC effects and immune dysfunction in cancer, immunocompetent models are critical. Previous work has primarily used the 4T1 breast cancer cell line in immunocompetent Balb/c mice. However, C57BL/6 mice are a more commonly employed system for

immune studies and large number of genetically engineered mice (e.g., knock-outs, knock-ins, etc.) available on this background [14,16]. Until recently, the 4T1 model was the primary model used to investigate MDSCs in breast cancer since C57BL/6 strains are relatively resistant to breast cancer development and existing C57BL/6 cancer models are often plagued with lengthy and sporadic tumor development [14,17]. An attractive alternative is to use a cell line developed on the C57BL/6 background, such as the E0771 mammary carcinoma cell line [18]. Although first identified in 1952, there is a revitalized interest within the last several years to utilizing this model in cancer immune studies [18,19]. In particular, recent studies evaluating immune-based therapies has led to a renewed use of the E0771 model system [20-23]. For example, a study showed that blocking PD-1 immunosuppression enhanced cell therapy in part by the reduction of MDSCs [23]. However this model and others lack the capability of studying large numbers of MDSC in vitro. To investigate MDSC interaction with an intact immune system an in vitro model was developed using the E0771 mammary carcinoma cells on the C57BL/6 background.

MATERIALS AND METHODS

Mice and the E0771 tumor line

C57BL/6 (H-2b) mice (4-8 week old) and C57BL/6-Tg (Tcra2D2, Tcrb2D2) 1Kuch/J were purchased from Jackson Laboratories or bred in-house. Mice were housed five per cage, maintained on a 12 hour light/dark cycle, under specific pathogen-free conditions and were housed and cared for according to the institutional guidelines of the Ohio State University’s Institute for Animal Care and Use Committee. E0771 cells were kindly provided by Dr. Fengzhi Li, Roswell Park Cancer Institute, USA, and maintained at 37°C in 5% CO2 in RPMI 1640 (Invitrogen) supplemented with 5% DBS, 25 mM HEPES, 2 mM L-glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin, 50 µM 2-mercaptoethanol.

Histology

Mice were humanely sacrificed; tissues were removed whole and immediately placed in neutral buffered 10% formaldehyde (formalin) solution and fixed for 24 hours. Following fixation, spleens were sectioned, embedded in paraffin and 3-4 µm sections were cut and mounted on glass slides. Slides were stained with routine hematoxilin and eosin staining and evaluated by light microscopy using an Olympus BX41 microscope by a board certified veterinary pathologist.

MDSC development in vivo

Approximately 0.5x10^6 E0771 mammary adenocarcinoma cells in 100 µL PBS were injected into the 4th mammary fat pad of C57BL/6 mice, subcutaneously into axillary peri-mammary tissue or intravenously [19,21]. Tumors were allowed to grow for approximately 3 weeks, mice were humanely sacrificed and the spleen and bone marrow was removed and used for experiments. Red blood cells were lysed and cells washed with PBS for either flow cytometry or subsequent purification. MDSCs were purified using the MDSC isolation kit per the manufacturer’s instructions (Miltenyi Biotec Cat. # 130-094-538). Populations of purified MDSCs were rested and activated overnight with LPS (1 µg/ml, 055:B5, Sigma-Aldrich) and INF-γ (100 U/ml) as previously described [24].

Flow cytometry

In vivo and in vitro derived MDSCs were labeled and evaluated by three-color flow cytometry using a combination of the following directly conjugated fluorescent FITC and/or PE antibodies to CD11b and GR-1 with appropriate isotype controls in PBS with 1% FBS (FACS buffer) (BD Bioscience, eBiosciences or Miltenyi Biotec). Cells were stained with fluorochrome-labeled antibodies or isotype controls for 20 min in the dark at 4°C, washed twice with FACS buffer and re-suspended in 200 µl FACS buffer for flow cytometric analysis.

MDSC differentiation in vitro

Bone marrow (BM) cells were collected from C57BL/6 mice femurs, tibias and/or humeri. Red blood cells were lysed and the remaining cells were cultured with RPMI 1640 (Invitrogen) supplemented with 10% FBS, 25 mM HEPES, 2 mM L-glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin, 50 µM 2-mercaptoethanol and 200 U/ml recombinant murine GM-CSF (R&D Systems) for 4 days at a density of 2x10^6 cells/ml ± 1x10^6 cells/5 ml E0771 carcinoma cells or ± 20% carcinoma tumor conditioned media (TCM). Cells were stimulated with LPS (1 µg/ml, 055:B5, Sigma-Aldrich), and INF-γ (100 U/ml) during culture on day 3. Day 4 harvested cells were considered MDSCs or myeloid progenitors (MPCs).

Functional immunosuppressive assays: T cell proliferation assay

In vitro differentiated MDSCs or myeloid progenitors were cultured with responder spleen cells from CD4+ antigen-specific T cell receptor transgenic mice specific for the myeloid oligodendrocyte glycoprotein (MOG 35-55) peptide. To assess T cell proliferation, co-cultures were stimulated with anti-CD3 (BD Bioscience) or peptide (MOG35-55,Bio Matic). To assess the effects of MDSC/myeloid cell activation, co-cultures were stimulated with LPS from Escherichia coli (055:B5, Sigma-Aldrich) for 96 hours, pulsed with H3 thymidine (Perkin Elmer Life Sciences or MP Biomedicals) in the last 18 hours, harvested and counted. Data is expressed as counts per million (cpm) ± SEM [25].

Statistical analysis

Data are represented as mean ± standard error method or fold change. Statistical significance was determined using a Student’s t-test significance level (p-value) < 0.05. All analyses were performed using Excel and/or GraphPad Prism software (La Jolla, CA).

RESULTS AND DISCUSSION

Histology of tumors derived from the E0771 cells demonstrated aggressive features

The E0771 cell line is a mammary adenocarcinoma line that produces extensive metastatic disease and was originally isolated by Drs. Sugiura and Stock [18,19]. Importantly, this mammary adenocarcinoma induces disease in immunocompetent mice on the C57BL/6 background [19,21]. To confirm that the E0771
cell line is invasive and assess the ability of this tumor to induce MDSCs in vivo, C57BL/6 mice were injected with 0.5x10^6 cells and tumors were allowed to grow for approximately 3 weeks. After 3 weeks, mice were sacrificed, tumors dissected, paraffin embedded and 3 µm sections cut for routine hematoxalin and eosin (H&E) staining for histological examination. Tumor cells had significant criteria of malignancy including anisocytosis, anisokaryosis and a moderate to high number of mitotic figures (Figure 1). Additionally, Figure 1 illustrates the presence of tumor cells within the local vasculature providing a means for distant metastasis. Taken together, these data demonstrate that the E0771 mammary adenocarcinoma showed histological features consistent with an aggressive and malignant phenotype.

**Site of tumor injection and size of the breast tumors determines the levels of CD11b‘GR-1‘ MDSC**

In order to determine whether tumor injection site influences the accumulation of MDSCs, tumor cells were injected subcutaneously into the mammary fat pad or subcutaneously into axillary peri-mammary tissue and intravenously. When tumors were injected in the mammary fat pad (Figure 2A) the percentage of CD11b‘GR-1‘ cells obtained from the tumor baring spleen was increased to 26% compared to 4% from PBS injected controls. There was an average increase of 16% of CD11b‘GR-1‘ cells when E0771 cells were injected in the axillary region and an increase of 9% of CD11b‘GR-1‘ cells within splenocytes when E0771 cells were injected intravenously (Figure 2A). When the tumor burden was subsequently decreased (i.e. ulceration with tumor loss), the average MDSC levels decreased to levels similar to control, PBS-injected levels (7%, data not shown). We also confirmed that CD11b‘GR-1‘ cells were increased 14% in the bone marrow of tumor-bearing mice compared to control mice (data not shown). Taken together, these results suggest that the site of injection and relative degree of tumor burden is associated with the accumulation of MDSCs in the E0771 mammary carcinoma model. These results are consistent with previous studies demonstrating that increasing tumor burden leads to elevated levels of MDSCs [6,7].

**CD11b‘ GR-1‘ MDSCs suppress the proliferation of responder immune cells**

Since MDSCs are identified by phenotype and their ability to suppress immune responses, we next evaluated the suppressive abilities of splenic CD11b‘GR-1‘ cells from E0771-bearing and control mice. CD11b‘GR-1‘ cells from mice injected with E0771 adenocarcinoma cells were significantly more suppressive (3.57 x10^5 CPM) than CD11b‘GR-1‘ cells isolated from the PBS-injected mice (1.38 x 10^5 CPM, p<0.05) with a polygonal stimulus such as anti-CD3 (Figure 2B). This data demonstrates that splenic CD11b‘GR-1‘ cells from mice bearing E0771 induced mammary adenocarcinomas were suppressive MDSCs. Therefore, the in vivo portion of the model system recapitulates the clinical features of MDSCs in other well-studied cancers [14].

**Differentiation of CD11b‘GR-1‘ cells in an in vitro tumor microenvironment model system**

The ability to differentiate MDSCs in a controlled and biologically relevant model system in vitro is critical to unraveling the key signaling events governing the impact of MDSCs in cancer. To begin to model the in vivo MDSC induction by E0771 cells, an in vitro model system using mouse bone marrow was adapted from a recently described human model of MDSC induction from peripheral blood mononuclear cells (PBMCs) [12]. Importantly, our model system utilized the in vitro application of factors from a tumor microenvironment, rather than cytokine cocktails commonly used in other studies for MDSC differentiation [26,27]. We believe that this model system provides a unique advantage to investigating tumor-MDSC interactions in that it more reliably models the factors involved during MDSC induction in vivo.

The tumor microenvironment model system was created by first seeding E0771 adenocarcinoma cells into cell culture dishes, allowing them to adhere, followed by co-culturing freshly isolated bone marrow cells stimulated to differentiate in the presence of GM-CSF. Given that differentiation of dendritic cells (DCs) with GM-CSF occurs by day 6-7, we expected that the highest levels of MDSC induction would occur at days 3-4 [28]. As expected, the percentage of CD11b‘GR-1‘ cells is increased on day 3 (73.1%), peaks at day 4 (80.5%) and then decreases slightly thereafter (77.7% on day 5). This data shows that peak induction of CD11b‘GR-1‘ cells occurs at day 4 in the in vitro tumor model system.

Application of this simulated tumor microenvironment model could be modified to promote the differentiation of MDSC subsets. For example, G-CSF could be used rather than GM-CSF to promote the induction of granulocytic MDSCs [29-31]. Ongoing studies are investigating modifications of the model system to promote granulocytic versus monocytic MDSC subsets.

**In vitro differentiated MDSC reduced the proliferation of responder immune cells**

We next confirmed that day 4 CD11b‘GR-1‘ cells differentiated within the in vitro tumor microenvironment model system had suppressive abilities similar to splenic MDSCs from the in vivo model. Day 4 differentiated cells from control and tumor microenvironment were co-cultured with responder immune cells and response to polyclonal and antigen-specific stimulation was assessed in the in vitro model. Our data show that day 4 cells
differentiated within the tumor microenvironment model system significantly decreased the proliferation of responder immune cells (Figure 3). This suppression was evident either when bone marrow cells were differentiated with E0771 or with tumor conditioned media from E0771 cells, although direct contact with E0771 appeared to slightly increase the suppressive abilities of day 4 differentiated bone marrow cells (Figure 3). Additionally, day 4 bone marrow cells from tumor microenvironment were suppressive both on stimulated cells (i.e. anti-CD3 and antigen) and also on resting responder immune cells. Taken together, this data strongly suggest that day 4 cells differentiated in vitro within a mammary adenocarcinoma tumor microenvironment are highly suppressive MDSCs.

CONCLUSION

This study demonstrates that E0771 mammary carcinoma cells induce large numbers of MDSCs both in vivo and in vitro with the in vivo model demonstrating that MDSC induction is dependent upon tumor location and burden. The in vitro model described within this manuscript utilizes the breast cancer tumor microenvironment to recapitulate the tumor microenvironment found in vivo and model breast cancer MDSCs. Given that large number of genetically engineered mouse models are available on the C57BL/6 background (e.g. >700 from Jackson laboratories), the E0771 mammary adenocarcinoma model is a useful model to investigate MDSCs and important pathways in the immune responses against breast cancer.

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