Journal of Immunological Methods 440 (2017) 12–18

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Review

Avoiding phagocytosis-related artifact in myeloid derived suppressor cell T-lymphocyte suppression assays

Ruth J. Davis [a], Christopher Silvin [a], Clint T. Allen [a,b,*]

[a] Tumor Biology Section, Head and Neck Surgery Branch, National Institute on Deafness and Other Communication Disorders, National Institutes of Health, Bethesda, MD, United States
[b] Department of Otolaryngology-Head and Neck Surgery, Johns Hopkins School of Medicine, Baltimore, MD, United States

ARTICLE INFO

Article history:
Received 14 July 2016
Received in revised form 27 September 2016
Accepted 12 November 2016
Available online 14 November 2016

Keywords:
Myeloid-derived suppressor cells
MDSC
T-cell suppression assay
Phagocytosis
Artifact
CD3/28 microbeads

ABSTRACT

Myeloid-derived suppressor cells (MDSCs) have garnered much attention in recent years as a potential target for altering the immunosuppressive tumor microenvironment in a variety of solid tumor types. The ability to accurately assess the immunosuppressive capacity of MDSCs is fundamental to the development of therapeutic approaches aimed at disabling these immunosuppressive functions. In this article we provide evidence that the use of CD3/28 coated microbeads leads to artefactual T-lymphocyte suppression due to sequestration of beads by MDSCs isolated from the spleens of wild-type mice bearing subcutaneous syngeneic, carcinogen-induced oral cavity carcinomas. Mechanisms of this finding may include early MDSC death and acquisition of phagocytic capacity. These artefactual findings were avoided by eliminating the use of microbeads and instead using plate bound CD3/28 antibody as the T-lymphocyte stimulus. We propose model-specific validation of microbead-based MDSC assays, or use of an alternative stimulation approach such as plate bound CD3/28 antibodies.

Contents

1. Introduction

Myeloid-Derived Suppressor Cells (MDSCs) are a heterogeneous population of immunosuppressive myeloid cells that are recruited into solid tumors through chemokine signaling (Gabrilovich and Nagaraj,

* Corresponding author at: National Institutes of Health, Building 10, Room 7N240C, Bethesda, MD 20892, United States.
E-mail address: clint.allen@nih.gov (C.T. Allen).

http://dx.doi.org/10.1016/j.jim.2016.11.006
0022-1759/

2009). Elevated numbers of MDSCs in both the tumor and the periphery are correlated with poor outcomes in a variety of cancer types (Gabrilovich and Nagaraj, 2009; Pak et al., 1995; Weed et al., 2015; Gabitass et al., 2011). MDSCs mediate immunosuppression through a number of mechanisms, including the release of immunosuppressive cytokines and the depletion of nutrients and metabolites required for T-lymphocyte function (Gabrilovich and Nagaraj, 2009; Condamine and Gabrilovich, 2011). Strategies to either block the recruitment or alter the function of MDSCs within the tumor microenvironment (TME) can increase effector T-cell function and improve responses to anti-cancer therapies (Serafini et al., 2006).

To characterize the mechanisms of MDSC-mediated immunosuppression and to evaluate therapies that may reverse this suppression, a reliable assay is required to quantify MDSC function. However, *ex vivo* evaluation of MDSCs is complicated by their poor survival in culture and tendency to differentiate into mature myeloid cells when cultured in the presence of growth factors such as GM-CSF (Youn et al., 2012). A variety of *in vitro* assays have been used to measure the immunosuppressive capacity of MDSCs. Mixed leukocyte assays evaluating the impact of MDSCs on T-lymphocytes stimulated with anti-CD3/anti-CD28 coated microbeads have become popular due to their relative simplicity and the potency of the CD3/28-mediated T-cell stimulation. In these assays, reduced T-cell proliferation or IFNγ production in the presence of MDSCs has been interpreted as an accurate indication of MDSC suppressive function.

However, concerns in both our lab and others have begun to arise as to the physiologic accuracy and potential for artifact in this polystyrene microbead-based assay (Hock and McKenzie, 2013). Here, using splenic MDSCs isolated from mice bearing syngeneic, carcinogen-induced oral cavity carcinomas grown subcutaneously in wild-type mice, we demonstrate artefactual suppression of CD3/28 microbead stimulated T-lymphocyte proliferation by MDSCs due to sequestration of beads away from T-lymphocytes in a mixed leukocyte assay. This effect could not be reversed with inhibitors of known MDSC immunosuppressive mechanisms, and was likely due in part to early phagocytic activity and death of sorted peripheral MDSCs. Reversible and dose-dependent inhibition of T-lymphocyte proliferation by MDSCs was achieved with elimination of polystyrene beads from the assay. We propose model-specific validation of microbead-based MDSC assays, or use of an alternative stimulation approach such as plate bound CD3/28 antibodies.

2. Materials and methods

2.1. Murine tumor model

The murine oral cancer (MOC) model is a carcinogen-induced model of oral cavity cancer that is transplantable into fully immunocompetent C57BL/6 (B6) mice (Judd et al., 2012). MOC1 cells were provided by Dr. R. Uppaluri (Washington University School of Medicine). MOC cells were cultured as previously described (Cash et al., 2015). All animal experiments were approved by the NIDCD Animal Care and Use Committee (ASP #1364-14). To generate syngeneic tumor-bearing mice, 4×10^6 MOC1 cells were injected subcutaneously in matrigel into the flank of WT C57BL/6 (B6) mice. Tumors were engrafted and allowed to reach at least 500 mm^3 before MDSC isolation.

2.2. Cell sorting

Splenic single cell suspensions were generated from WT B6 or MOC1 tumor-bearing mice through mechanical dissociation and RBC lysis (Biolegend). To isolate responder T-cells, WT B6 splenocytes were stained and sorted on an autoMACS magnetic sorter (Miltenyi Biotec) using the pan T-cell negative selection kit from Miltenyi (#130-095-130) per the manufacturer's instructions. For MDSC isolation, splenic single cell suspensions were stained with the anti-Ly6G microbead kit from Miltenyi (#130-092-332) per the manufacturer's instructions and isolated on an autoMACS magnetic sorter.

2.3. Flow cytometry

Cell surface staining was performed using fluorophore conjugated anti-mouse CD4 clone GK1.5, CD8 clone 53-6.7, Gr1 clone RB6-8c5, and CD11b clone M1/70 antibodies from Biolegend. Dead cells were excluded *via* 7AAD negativity. Data was acquired on a FACSCanto using FACSDiva software (BD Biosciences) and analyzed on FlowJo software vX10.07r2.

2.4. T-cell proliferation assay

WT B6 T-cells were labelled with 5 μM carboxyfluorescein succinimidyl ester (CFSE, Sigma Aldrich) as previously described (Quah et al., 2007). 8×10^4 CSFE-labelled T-lymphocytes were stimulated with a 1:1 ratio of anti-CD3/anti-CD28 coated dynabeads (ThermoFisher) in round-bottom 96-well plates in the presence of MDSCs as indicated for 3–4 days. For plate-bound CD3/28 stimulation, 5 μg/mL each of anti-CD3 (clone 145-2C11, eBioscience) and anti-CD28 (clone 37.51, eBioscience) was diluted in PBS and coated onto flat-bottom 96-well plates (Corning) overnight at 4 °C. CFSE labelled T-cells were co-cultured with the indicated ratios of MDSCs for 4 h, then added to the prepared CD3/28 coated plate (wells were washed with PBS twice to remove unbound antibody prior to adding cells). Where indicated, MDSCs and T-lymphocytes were exposed to 300 μM of nor-NOHA (arginase inhibitor) or L-NMMA (iNOS inhibitor) for 4 h before T-lymphocyte stimulation with either CD3/28 microbeads or plate bound antibody. After 3 days in culture, T-cell CFSE peak distribution was quantified by flow cytometry. T-cells and MDSCs were cultured in complete media (RPMI 1640 supplemented with 10% FCS, 1.5% HEPES, 1% glutamine, 1% nonessential amino acids, 1% sodium pyruvate, 1% Pen/Strep, 0.1% gentamycin, 50 μM beta-mercaptoethanol). T-lymphocyte proliferation was quantified as the average number of divisions for all cells in the culture (division index) using FlowJo software. Percept inhibition of proliferation was calculated using the following:

$$([\text{Prolif}_{\text{test}} - \text{Prolif}_{\text{Unstim}}] / [\text{Prolif}_{\text{max}} - \text{Prolif}_{\text{unstim}}])$$

2.5. In vitro cell viability

Viability of MDSCs was measured by staining with acridine orange and propidium iodide (AOPI solution, Nexcelcom) and quantified on a Cellometer Auto 2000 fluorescent imager.

2.6. Phagocytosis assay

Sorted MDSCs were exposed to pHrodo *E. coli* BioParticles (ThermoFisher P35366) at 100 μg/1×10^5 cells in live cell imaging solution (ThermoFisher #A14291DJ) for 90 min. Conjugated cell surface antibodies were added as indicated for 30 min, cells were extensively washed, and cells were analyzed by flow cytometry.

2.7. Cellular photography

Photomicrographs were obtained on an Olympus IX70 inverted light microscope and an Olympus DP72 camera using CellSens Standard 1.5 software. No images were manipulated with editing software.

2.8. Statistical analysis

Tests of significance between pairs of data are reported as p-values, derived using a student's *t*-test with a two-tailed distribution with significance set to $p < 0.05$. Comparison of mean values to zero was done