CHAPTER ELEVEN

Detection and functional evaluation of arginase-1 isolated from human PMNs and murine MDSC

Stefania Canè, Vincenzo Bronte*
Department of Medicine, Section of Immunology, University of Verona, Verona, Italy
*Corresponding author: e-mail address: vincenzo.bronte@univr.it

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Abstract

Immunotherapy has emerged as a potent alternative for cancer treatment, unfortunately, the clinical benefit remains limited to few patients and immunotherapy resistance due to immunosuppressive tumor microenvironment represents the major reason of such a failure. Arginase-1 is one of the enzymes contributing to the establishment of such immunosuppression. Among the human immune cells, polymorphonuclear cells (PMNs) represent the major source of arginase-1, while myeloid-derived suppressor cells (MDSCs) are the main arginase-1 producing cells in mice. Due to arginase-1 potential impact in dampening the immune response, there is a growing interest in assaying arginase-1 levels and functions. Thus, in this chapter we propose how to evaluate the expression and activity of arginase in human peripheral blood-derived PMNs and in MDSCs isolated from tumor-bearing mice.

1. Introduction

Arginase (ARG) is a manganese metalloenzyme hydrolyzing L-arginine to L-ornithine and urea. It is found throughout the living organisms, in bacteria, yeasts, plants, invertebrates, and vertebrates, and is thought to have appeared first in bacteria (Dzik, 2014). The subsequent transfer of ARG to an eukaryotic cell likely occurred through mitochondria. In fact, most invertebrates, plants, bacteria, and yeasts have only one form of ARG (ARG2) that localizes in mitochondria, while the ureotelic organisms, which metabolize excess nitrogen as urea, express a second cytosolic isoform (ARG1). In humans, up to now, three different isoforms of ARG1 have been identified and produced by mRNA alternative splicing. Isoform 1, *alias* liver ARG1, consists of 322 amino acids (aa) (Dizikes, Grody, Kern, & Cederbaum, 1986) and is localized in the cytosol of hepatocytes where it contributes to the urea cycle and nitrogen metabolism; 330 aa isoform 2, identified in erythroid cells and PMNs (Munder et al., 2005), carrying eight additional amino acids beginning from aa 43, is important for L-arginine homeostasis via the competition with nitric oxide synthase (NOS) for the available L-arginine substrate; 236 aa isoform 3, missing amino acids 204–289, has still an undefined function and tissue distribution (UniProt-P05089).

Crystal structure resolution indicates that active human ARG1 is a 105 kDa homotrimer and each of 35 kDa subunit contains a binuclear Mn(II) located at the bottom of the catalytic cleft. In particular, the metal ions establish hydrogen bonds with histidine and aspartate residues, ensuring protein stability and ARG activation (Ash, 2004). The overall fold of each

subunit belongs to the α/β family, consisting of a parallel, 8 stranded β-sheet flanked on both sides by numerous α-helices. Similarly to rat and mouse arginases, which have alkaline pH optimal activity, human ARG1 has a maximal enzyme velocity in the range of pH 9.0–9.5 with a pK value of 7.8 (Di Costanzo et al., 2005). In non-hepatic tissues, ARG1 reaction is thought to provide a source of ornithine, the biosynthetic precursor of proline and the polyamines required for collagen deposition and cellular proliferation, respectively (Casero, Murray Stewart, & Pegg, 2018), even though these conclusions were never experimentally confirmed. While this is true in general, in immune cells, like neutrophils, a direct role of ARG1 in decreasing L-arginine levels cannot be underestimated. In human neutrophils, ARG1 is stored in tertiary granules and, upon different stimuli (e.g., inteleukin-8 (IL-8), phorbol-12 myristate-13 acetate (PMA), Ca^{2+} ionophore A23187, ionomycin; Barrientos et al., 2013; Jacobsen, Theilgaard-Monch, Christensen, & Borregaard, 2007; Rotondo et al., 2009, 2011), is secreted and participate to the homeostasis of L-arginine in the extracellular milieu. L-arginine is a semi-essential amino acid that is generally provided by protein turnover but, in special cases (e.g., inflammation), needs to be supplemented with the diet. While the release of ARG1 from activated PMNs was shown to dampen T cell proliferation in vitro (Rotondo et al., 2011), increased ARG1 activity is required to sustain microbial clearance in neutrophils, through the depletion of L-arginine in the phagolysosome (Munder et al., 2005). Moreover, alteration of L-arginine levels was shown to contribute to the pathogenesis of Alzheimer's disease (Liu et al., 2014) and diabetes (Romero et al., 2012). Even though administration of L-arginine was though to prevent and or delay the onset of diabetes-associated vascular alterations, several studies, both in mice and humans, found no real benefit on clinical outcomes after prolonged supplementation of L-arginine (Lucas, Fulton, Caldwell, & Romero, 2014). These negative effects could be related to the ability of L-arginine to induce either the expression or activity of ARG1, consequently reducing L-arginine plasma levels, independently from ornithine production.

In mice ARG1 is constitutively expressed in the cytosol of hepatocytes while its expression is induced in myeloid cells (e.g., MDSC and alternatively activated macrophages) by cytokines like interleukin 4 and interleukin 13 (IL-4 and IL-13, respectively) (Munder et al., 1999; Munder, Eichmann, & Modolell, 1998), as well as by transforming growth factor-β (TGF-β) (Boutard et al., 1995) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Jost et al., 2003). The promoter

region of mouse *ARG1* contains elements that control its transcription in response to IL-4, cyclic adenosine monophosphate (c-AMP), TGF-β and lipopolysaccharide (LPS) (Morris, 2002). In alternatively activated macrophages (M2 macrophages), ARG1 expression contributes to wound healing and tissue regeneration processes and in immune defense against multicellular pathogens, parasites and immune suppression and allergic inflammation (Barron et al., 2013; Campbell, Saville, Murray, Cruickshank, & Hardman, 2013; El Kasmi et al., 2008; Gray, Poljakovic, Kepka-Lenhart, & Morris, 2005; Herbert et al., 2010; Modolell, Corraliza, Link, Soler, & Eichmann, 1995; Pauleau et al., 2004; Pesce et al., 2009). In tumor-infiltrating dendritic cells (DCs) and myeloid-derived suppressor cells (MDSCs) ARG1 plays a role in suppression of T cell-mediated response (Liu et al., 2009; Narita et al., 2013) by downregulating the CD3ζ chain of the T cell receptor (TCR), even though a systematic analysis was never been done and controversial results keep the question open (Van de Velde et al., 2017). This mechanism seemed to be relevant for tumor escape in vivo, because injection of the ARG inhibitor N-hydroxy-nor-L-arginine (nor-NOHA) was shown to slow the growth of lung carcinoma in a dose-dependent manner (Rodriguez et al., 2004). Therefore, L-arginine degradation by ARG1 appears to exert a major negative impact on anti-tumor T cell function and, indeed, L-arginine directly modulates T cell metabolism by enhancing cell survival and sustaining their antitumor activity (Geiger et al., 2016). In this manuscript, however, very high concentrations of L-arginine were used in the in vitro assays. To dissect the contribution of myeloid-derived ARG1 to the immune response, conditional myeloid-specific *ARG1* knockout mice were developed (Arg1 flox/flox; Tie2cre) in which myeloid cells are nearly devoid of ARG1 activity, as a measure of urea production (El Kasmi et al., 2008). In these mice, the absence of ARG1 in myeloid compartment, unleashed the therapeutic activity of adoptively transferred, tumor-specific T cells by a mechanism requiring the release of NO by monocyte-derived DCs (Marigo et al., 2016).

On the light of this information, it appears that profound dissimilarities exist in the ARG1 biology between rodents and humans. While in mice ARG1 is a cytosolic enzyme induced in MDSC/M2 macrophages, in humans ARG1 is stored in PMN granules whose exocytosis is induced by different pro-inflammatory stimuli (Rotondo et al., 2011). Moreover, human ARG1 is constitutively expressed by PMNs and its expression is not upregulated by stimuli that affect the mouse analogue (Munder et al., 2005). Thus, in this chapter, we will describe assays optimized to determine the ARG1 level and activity (Corraliza, Campo, Soler, & Modolell, 1994), in human PMNs and mouse MDSCs.

2. Arginase inhibitors

In view of findings suggesting that altered L-arginine homeostasis can contribute to the pathogenesis and exacerbation of several conditions (e.g., cancer, autoimmune diseases, asthma), controlling intracellular and extracellular arginase activity is attractive for disease modulation, both in humans and mice. Targeting ARG is not an easy task. The few known ARG inhibitors suffer from poor structural diversity. Two types of inhibitors are known to bind to ARG (Di Costanzo, Pique, & Christianson, 2007). The first group comprises product analogues to the ligand. Such inhibitors prevent the natural hydrolysis taking place with the natural substrate. The second group comprises transition state analogues like hydroxyarginines, boronic acids, or sulfonamides (Boucher et al., 1994; Di Costanzo, Ilies, Thorn, & Christianson, 2010). Boronic acids, like 2-S-amino-6-boronohexanoic acid (ABH) and S-(2-boronoethyl)-L-cysteine (BEC), together with hydroxyarginine, like nor-N(ω)-hydroxy-L-arginine (nor-NOHA), react to form transition state analogs complexing the Mn^{2+} ions and sequestering them from the catalysis. Unfortunately, none of these inhibitors exhibit sufficient differences in affinity to provide isoform-selective inhibition, both in vitro and in vivo (Morris, 2009; Pudlo, Demougeot, & Girard-Thernier, 2017), beside been toxic in vivo to some extent. Recently, renewed interested in targeting ARG has brought scientists to design novel non-aminoacid-based inhibitors (Mortier et al., 2017) that selectively interact with ARG1, which is the prerequisite for the development of novel ARG1 modulators with therapeutic relevance. Furthermore, a novel inhibitor, CB-1158, was develop and the initial encouraging results in preclinical cancer models (Steggerda et al., 2017) have paved the way to start a phase I clinical trial for treatment of solid tumors. For the matter of this chapter we will evaluate ARG1 dependent effects using nor-NOHA, which has been broadly used in vivo and in vitro (Rotondo et al., 2011).

3. Evaluating ARG1 in activated and resting PMN: Immunofluorescence (IF) analysis

An immune-based method for the detection of ARG1 in purified PMNs, isolated from peripheral blood, is described below. Combining the specificity of antibody's binding to ARG1 with the high resolution capacity of confocal microscopy, it is possible to reveal the cellular distribution of ARG1 and changes occurring during PMN priming.

3.1 Equipment

1. BD vacutainer®, K_2EDTA
2. 15 and 50 mL polypropylene conical tubes (BD, Falcon)
3. Centrifuges
4. FACS Canto flow cytometer (BD Biosciences)
5. FlowJo Software (TreeStar)
6. 12 × 75–mm polypropylene tubes (BD, Falcon)
7. Cover glass, 13 mm in diameter, 1 mm thick (VWR)
8. SuperFrost Plus, glass slides, Thermo Fisher
9. 24-well plate (BD, Falcon)
10. Transfer pipette, 3.5 mL (Sarstedt)
11. Serological pipettes (5, 10, 25 mL, Sarstedt)
12. Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems)

3.2 Material

1. Arginine-free RPMI (Biological Industries, Kibbutz Beit Haemek, Israel), supplemented with 10% FBS (Fetal bovine serum; Gibco), 10 U/mL penicillin and streptomycin and 0.01 M Hepes
2. Ficoll-Paque, 1.077 g/mL (GE Healthcare)
3. 4% Dextran (Mr 450,000–650,000; 31392, Sigma-Aldrich).
4. Dulbecco's phosphate-buffered saline (DPBS without Ca^{2+} and Mg^{2+}; Lonza BioWhittaker, BE17-515Q)
5. 0.2% NaCl solution, 0.22 μm filtered
6. 1.2% NaCl solution, 0.22 μm filtered
7. Ionomycin (I0634, Sigma-Aldrich)
8. IL-8 (130-108-979, Miltenyi Biotec).
9. 36.5-38% formaldehyde solution (F8775, Sigma-Aldrich)
10. Tris Buffered Saline (TBS)
11. Tween-20 (Sigma-Aldrich)
12. Triton X-100 (Sigma-Aldrich)
13. Normal goat serum (S-1000, Vector Laboratories)
14. Primary antibodies: Goat anti-human MPO (R&D, AF3667), rabbit anti-human H3 R2 + R8 + R17 citrullinated (Abcam, ab5103), mouse anti-human ARG1 (monoclonal antibody from homemade hybridoma, conjugated to Alexa Fluor 647)
15. Secondary antibodies: donkey anti-goat Alexa Fluor 488 (A-11055, Thermo Fisher) and goat anti-rabbit Alexa Fluor 546 (A-11035, Thermo Fisher)

16. 4′,6-diamidino-2-phenylindole dihydrochlorid (DAPI) (10236276001, Sigma-Aldrich)

Note: all experiments must be performed in accordance with local institutional ethical guidelines.

Note: collect blood within 10–15 min of the experiment to ensure optimal yield and resting PMNs. Avoid to purify PMNs from buffy coat. Cells tend to have an activated and already degranulate phenotype.

Note: avoid cold temperature, PMNs tends to become activated. Try to work at RT (20–22 °C) and to maintain the reagents at 37 °C before use.

3.3 Protocol

3.3.1 Isolation of PMNs from peripheral blood of healthy donors

1. Collect blood in K_2EDTA vacutainer tubes, dilute 1/2 with DPBS, and carefully layer onto a density gradient Ficoll-Paque. Centrifuge at $800 \times g$ for 20 min at 24 °C, without brake.
2. Aspirate the top layer containing PBMC, keep momentarily aside, on ice. Erythrocytes (RBC) and PMNs sediment at the bottom of the tube, gently remove the excess of Ficoll-Paque and add DPBS and a volume of 4% dextran (both kept at 37 °C) to reach 1% final, gently invert the tube to mix and allow to sit at RT for 20 min.
3. Collect the upper PMN-rich layer avoiding the RBC, spin down and lyse the residual RBC by resuspending the pellet in 3 mL of 0.2% NaCl for 2½ min and then in 7 mL of warm 1.2% NaCl (both kept at 37 °C) to restore isotonicity. Centrifuge at $450 \times g$ at 24 °C for 5 min.
4. Discard supernatant and wash pellet with 15 mL warm of PBS (kept at 37 °C) and centrifuge again at $450 \times g$ at 24 °C to get rid of any remaining lysing solution. The pellet obtained at this point is enriched in PMNs.
5. Check the cell purity by FACS analysis on forward/side scatter parameters, and viability by trypan blue dye exclusion. Generally, PMNs yielded purity and viability is >95%. Dilute PMNs in warm L-arginine free RPMI to a concentration of 5×10^6 PMNs/mL.

Note: higher concentration of PMNs can result in spontaneous activation and degranulation.

3.3.2 Immunofluorescence analysis of ARG1 in activated PMNs

1. Prepare a 24-well cell culture plate by inserting sterile 13 mm round glass cover slip in each well.
2. Plate 2×105 cells in 300 µL L-arginine free RPMI containing 10% FBS in each well and incubate at 37 °C for 1 h for adherence.

3. Meanwhile, prepare 20 μM ionomycin in L-arginine free RPMI containing 10% FBS and add 100 μL per well (final concentration, 5 μM). Alternatively, add 100 μL of 80 nM of IL-8 (final concentration, 20 nM). Incubate the cells from 30 min up to 4 h at 37 °C, 5% CO2.
4. Fix cells with 4% paraformaldehyde solution (final concentration in the well) for 15 min at RT.
5. Carefully remove the supernatant and wash three times with PBS. Permeabilize the cells by adding 300 μL of 0.5% Triton X-100 in TBS and incubate for 1 h at RT.
6. Remove the liquid and add blocking buffer (20% normal goat serum in 0.1% Triton X-100). Let stand for 2 h at RT.
7. Without washing add primary antibodies diluted in PBS. The following antibodies are routinely used: anti-MPO (1:200, stock 0.2 mg/mL), anti-ARG-1 (1:1000, stock 1.5 mg/mL), anti-H3 citrullinated R2 + R8 + R17 (1:500, stock 0.8 mg/mL). Incubate O/N at 4 °C.
8. The following day, remove the primary antibodies and wash three times with 0.05% Tween-20 in TBS.
9. Dilute the secondary antibodies in blocking buffer as follows: anti-rabbit AF546 (1:2000, stock 2 mg/mL), anti-goat AF488 (1:2000, stock 2 mg/mL). Incubate for 1 h at RT.
10. Wash with 0.05% Tween-20 as before. Wash once with TBS only.
11. Stain DNA with 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/mL) for 10 min, RT then wash twice with distilled water.
12. Set a 10 μL drop of ProLong Antifade reagent (Thermo Fisher) onto a glass slide and mount the cover slip. For microscopic analysis with immersion lenses let the mounting media to dry for at least 1 h. Store at 4 °C.
13. Acquire images at the Leica TCS SP5 confocal microscope.

4. Intracellular determination of ARG1 levels in PMN by flow activated cell sorting (FACS)

PMNs are a heterogeneous population of cells expressing ARG1. FACS analysis, by combining antibodies directed to different cellular surface markers, represents a sensitive and semi-quantitative method to evaluate total intracellular levels of ARG1 in PMN subtypes.

4.1 Equipment

1. BD vacutainer®, K2EDTA
2. 15 and 50 mL polypropylene conical tubes (BD, Falcon)

3. Centrifuges
4. FACS Canto flow cytometer (BD Biosciences)
5. FlowJo Software (TreeStar)
6. 12 × 75–mm polypropylene tubes (BD, Falcon)
7. Transfer pipette, 3.5 mL (Sarstedt)
8. Serological pipettes (5, 10, 25 mL, Sarstedt)

4.2 Material

1. Cytofix/Cytoperm kit (BD Biosciences)
2. Human FcR blocking reagent (Miltenyi Biotec, 130-059-901)
3. Staining buffer: DPBS + 0.5% w/v bovine serum albumin (BSA, A4503, Sigma-Aldrich)
4. Anti-CD16 (clone 3G8)-FITC, anti-CD15 (clone HI98)-PE, anti-CD66b (G10F5)-BV421 are from BD Biosciences, anti-ARG1 (clone 1.10 from homemade hybridoma, conjugated with Alexa FLuor-647, Thermo Fisher)

4.3 Protocol

1. Isolate PMNs as in Section 3.3.1 and incubate the cells with Fc-receptor blocking reagent diluted 1/25 at 4 °C for 10 min.
2. Add the mixture of antibodies (CD16/CD66b or CD15) and incubate at 4 °C for 20 min. The dose of each antibody depends on the lot number and must be evaluated before.
3. Wash the cells with staining buffer and proceed to fixed and permeabilized using the BD Cytofix/Cytoperm kit according to the manufacturer's instructions.
4. At the end of the incubation time, wash the cells extensively with permeabilization buffer diluted following BD protocol.
5. Add the antibody clone 1.10 AF-647 to anti-ARG1 and incubate 1 h at 4 °C. The amount of antibody should be predetermined using PMNs and the PBMC fraction as negative control.
6. Wash three times with permeabilization buffer and finally resupsend the cells in staining buffer.
7. Acquire directly to the flow cytometry.

Note: we recommend performing the staining protocol in 96-round well plate to avoid losing cells.

Note: do not let the stained PMNs standing for longer time before flow cytometry.

5. Determination of ARG1 activity from PMN-derived supernatant

The enzyme activity is determined by an end-point colorimetric method to evaluate the ornithine production, using L-arginine as a substrate. Beside evaluating the overall level of ARG-1 by commercial ELISA it is imperative to determine the activity of the protein, since it is an enzyme with defined biochemical properties (e.g., Vmax, Kd).

5.1 Equipment

1. BD vacutainer®, K_2EDTA
2. 15 and 50 mL polypropylene conical tubes (BD, Falcon)
3. Centrifuges
4. Transfer pipette, 3.5 mL (Sarstedt)
5. Cell culture treated petri dishes (Corning)
6. Serological pipettes (5, 10, 25 mL, Sarstedt)
7. BCA assay kit (Thermo Fisher)
8. Spectra Max microplate reader (Molecular Device)
9. Heating block
10. 2 mL Eppendorf tubes
11. pH-meter

5.2 Materials

1. Reagents for PMN isolation and activation as indicted in Section 3.3.1.
2. L-Arginine (A5131, Sigma-Aldrich)
3. L-Ornithine (O2375, Sigma-Aldrich)
4. $MnCl_2$ (244589, Sigma-Aldrich)
5. Ninhydrin (N4876, Sigma-Aldrich)
6. Acetic acid (45754, Sigma-Aldrich)
7. Tris/HCl and NaCl (Sigma-Aldrich)
8. Carbonate-bicarbonate buffer (C3041, Sigma-Aldrich)

5.3 Protocol

1. Determine the protein content in the supernatant by BCA assay.
2. Take 100 μg of total protein and bring to 100 μL volume with a carbonate solution, pH 10.

3. Adjust the pH to 9.5 then add 100 µL of 75 mM $MnCl_2$. Incubated 20 min at 55 °C.
4. Add 50 µL of 140 mM L-arginine and incubate for 2 h up to O/N at 37 °C.
5. Stop the reaction by adding 850 µL of acetic acid and add 300 µL of ninhydrin solution.
6. Incubate at 100 °C for 30 min. Remove from the heating block and let stand for 10 min avoiding light exposure.
7. Spin the samples at 14000 rpm for 5 min, take 250 µL of the supernatant and plate in a 96 well plate (flat bottom). Read at 492 nm.
8. Calculate L-ornithine concentration using a standard curve with serial dilutions ranging from 2.0 to 0.5 mmol/L. Reagent stock solutions to use are: 100 mmol/L L-arginine (fresh every day), 2 mmol/L L-ornithine (stable 1 month at 4 °C), 25 g/L ninhydrin in 10 mol/L acetic acid/ 2.4 mol/L phosphoric acid (stable for several months), 10 mmol/L $MnCl_2$ (stable for 1 month).

6. Evaluation of human PMN-derived ARG1 dependent suppressions of T cell proliferation and determination of ARG1 activity

Immune suppression exerted by degranulating PMNs on activated T cells can be measured in terms of inhibition of T cell proliferation due to the ability to induce proliferative arrest of actively dividing cells. Even though, in the protocol we recommend employ PBMCs, the use of purified T cells might help in avoiding potential interference from other cell subsets present among PBMCs.

6.1 Equipment

1. BD vacutainer®, K_2EDTA
2. 15 and 50 mL polypropylene conical tubes (BD, Falcon)
3. Centrifuges
4. FACS Canto flow cytometer (BD Biosciences)
5. FlowJo 7.6.5 Software (TreeStar)
6. 12 × 75-mm polypropylene tubes (BD, Falcon)
7. TruCount tubes (BD Biosciences)
8. Transfer pipette, 3.5 mL (Sarstedt)
9. Cell culture treated petri dishes (Corning)
10. Serological pipettes (5, 10, 25 mL, Sarstedt)
11. 96-well flat-bottom plate, cell culture treated
12. Incubator 8% CO_2

6.2 Materials

1. Anti-CD3 (5 μg/mL), clone OKT3, obtained after expansion and purification of a commercially available hybridoma
2. Anti-CD28 (5 μg/mL), clone CD28.2 (BioLegend, 302923)
3. Dulbecco's phosphate-buffered saline (DPBS without Ca and Mg; LonzaBioWhittaker, BE17-515Q)
4. CellTrace TM Violet Cell Proliferation Kit (Thermo Fisher)
5. Fetal bovine serum (FBS; Gibco). Note: FBS used for functional assay should be tested to guarantee that immunosuppression is detectable and not overcome by an excessive T cell proliferation
6. Human FcR blocking reagent (Miltenyi Biotec, 130-059-901)
7. L-Arginine (A5131, Sigma-Aldrich)
8. IMDM medium (12440053, Gibco)
9. Arginine-free RPMI (Biological Industries, Kibbutz Beit Haemek, Israel), supplemented with 10% FBS (Fetal bovine serum; Gibco), 10 U/mL penicillin and streptomycin and 0.01 M Hepes.
10. Ficoll-Paque, 1.077 g/mL (GE Healthcare).
11. 4% Dextran (Mr 450,000-650,000; 31392, Sigma-Aldrich).
12. 0.2% NaCl solution, 0.22 μm filtered.
13. 1.2% NaCl solution, 0.22 μm filtered.
14. Ionomycin (I0634, Sigma-Aldrich).
15. IL-8 (130-108-979, Miltenyi Biotec).
16. nor-NOHA (acetate) (10006861, Cayman Chemical).
17. Human serum (H3667, Sigma-Aldrich).
18. Anti-CD3, clone UCHT1 (Beckman Coulter), anti-CD4, clone SK3 (BD Biosciences) and anti-CD8, clone SK1 (BD Biosciences)
19. Staining buffer: DPBS + 0.5% w/v bovine serum albumin (BSA, A4503, Sigma-Aldrich)

6.3 Protocol

1. Prepare coating buffer with anti-CD3 (5 μg/mL final concentration in DPBS) to fill 96 well flat bottom microtiter plates with 200 μL/well using a multichannel pipettor. Fill an equal number of wells with 200 μL/well of DPBS without antibodies, for background proliferation measurement.
2. Incubate the plate overnight at 4 °C or alternatively at 37 °C for 1 h.
3. Follow the detailed protocol highlighted in Section 3.3.1 to isolate PMNs and resuspend them at 5×10^6 cells/ml in L-arginine free media.

4. Transfer PMNs in a petri dish and stimulate them with ionomycin (5 μM) or IL-8 (20 nM) for 30 min at RT.
5. Gently aspirate the media with transfer pipette. Spin for 10 min at 450 × *g* at 24 °C. PMNs and any remaining cells/debris will be pelleted at the bottom of the tube, leaving a cell-free supernatant.
6. Meanwhile, take the PBMC layer isolated in Section 3.3.1, transfer into a 50 mL conical tube and wash three times with ice cold DPBS containing 1% human serum.
7. Dilute an aliquot of PBMC suspension in trypan blue dye solution and evaluate the viable cell concentration.
8. Adjust the concentration of PBMCs to 2×10^7 cells/ml in DPBS and the concentration of CellTrace to 2 × in DPBS (final concentration ranging from 0.5 to 2 μM); quickly mix equal volumes of PBMCs and CellTrace and incubate for 5 min at 37 °C. Quench the reaction by adding 1/5 of the total volume of FBS, centrifuge 5 min at 300 × *g*, 4 °C. Check the incorporation of CellTrace by flow cytometry.
9. Plate CellTrace$^+$ PBMCs in a 24-well plate at 3×10^6 cell/well for at least 1 h at 37 °C in IMDM medium containing 10% FBS.
10. Harvest PBMCs, count them by trypan blue dye assay, adjust their concentration to 1×10^6 cells/mL in either supernatant from PMNs treated or left untreated. Add 150 μM L-arginine to all the conditions and plate in triplicate, 200 μL/well, in 96-well plates coated with anti-CD3 antibody extensively washed with DPBS to remove unbound anti-CD3 antibody. In some condition add nor-NOHA, an arginase inhibitor, at the final concentration of either 100 or 300 μM. Add 1 μL/well of soluble anti-CD28 (5 μg/mL) and incubate at 37 °C, 8% CO_2 for 4 days.
11. At day 4, pool triplicates and wash samples once with sorting and staining buffer, centrifuge the suspension 6 min at 300 × *g*, 4 °C, and discard the supernatant.
12. Block nonspecific binding with 25 μL FcR blocking reagent for 10 min at 4 °C.
13. Stain cells with anti-CD3 clone UCHT1, anti-CD4 clone SK3 and anti-CD8 clone SK1 for 20 min at 4 °C. Adjust the volume of staining mix to 100 μL with staining buffer.
14. Wash samples once with staining buffer, centrifuge the suspension for 5 min at 300 × *g*, 4 °C, and discard the supernatant.
15. Resuspend samples in 250 μL DPBS, transfer the mix in TruCount tubes, gently vortex, and proceed with flow cytometric acquisition and analysis.

7. Summary

Several data indicate that ARG1, secreted by activated PMNs or expressed by murine MDSC, by altering L-arginine homeostasis can modulate the immune response toward cancer. At the same time a new reinvigorating interest on understanding the molecular mechanisms driving human PMNs and murine MDSC differentiation has recently emerged due to the contribution of these cells in the pathogenesis of diseases, like systemic lupus erythematosus, rheumatoid arthritis, diabetes and cancer, among others. With this chapter we describe protocols for the detection and functional evaluation of ARG1 in both human PMNs, isolated from peripheral blood of healthy donors, and mouse MDSCs with the aim of defining the basic experimental approaches necessary to evaluate immune-derived ARG1.

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Appendix

Determination of ARG1 levels and activity in mouse-derived myeloid cells

Isolate and purify MDSCs from the spleen of tumor-bearing mice following the instructions present in Basic Protocol 1 recently published by our group (Solito et al., 2019). Alternatively, bone-marrow derived MDSCs (BM-MDSCs) can be generated using Basic Protocol 2 (Solito et al., 2019). As control for MDSC-derived ARG-dependent suppression, we recommend to use cells obtained from conditional myeloid *Arg1*-deficient mice (e.g., ARG1fl/fl;Tie-2Cre mice).

1. ARG-dependent suppressive activity of myeloid cells

   Once obtained, proceed to evaluate ARG-dependent suppressive ability in MDSCs and BM-MDSCs following the Basic Protocol 3 (Solito et al., 2019) with minor modifications:

   1. At point 5 of Basic Protocol 3, MDSCs should be co-cultured with T lymphocytes in the presence of ARG inhibitor, nor-NOHA, previously titrated.

2. In case TCR transgenic mice, developed to recognize a specific antigenic peptide, are not available, it is possible to perform the assay using Basic Protocol 2 (Dolcetti, Peranzoni, & Bronte, 2010).

2. Evaluation of ARG1 protein levels in the pellet of myeloid cells

2.1 Equipment

1. 1.5 mL tubes
2. Minicentrifuge
3. SDS-PAGE running system and wet transfer apparatus
4. Heating block
5. Microplate reader
6. Bio-Rad ChemiDoc

2.2 Material

1. lysis buffer: 50 mM HEPES, 150 mM NaCl, 5 mM EDTA, 0.5% Triton, cocktail protease inhibitors, 1 mM NaOV4, 2 mM PMSF
2. cOmplete™ Protease Inhibitor Cocktail (11836145001, Sigma-Aldrich)
3. Tris-buffer saline (TBS) and Tween-20 (Sigma-Aldrich)
4. Laemmli buffer (161-0747, Bio-Rad)
5. NuPAGE, 10% bis-tris protein gel (NP0302BOX, Thermo Fisher)
6. 20X MOPS buffer (Thermo Fisher)
7. PVDF membrane 0.2 μm (88520, Thermo Fisher)
8. Transfer buffer: 25 mM Tris-HCl (pH 7.6), 192 mM glycine, 20% methanol (Sigma-Aldrich)
9. Non-fat dry milk (1706404, Bio-Rad).
10. Bovine serum albumin (BSA), (A2153, Sigma-Aldrich)
11. Primary antibodies: goat anti-mouse ARG1 (SAB2500101, Sigma-Aldrich); rabbit anti-mouse β actin (ab8227, Abcam)
12. Secondary antibodies: sheep anti-mouse-HRP (NA931, GE Healthcare); donkey anti-rabbit-HRP (NA934, GE Healthcare)
13. SuperSignal West Pico Chemiluminescent substrate (34580, Thermo Fisher)
14. BCA protein assay kit (23225, Thermo Fisher)

2.3 Protocol

1. Collect the cells (MDSCs, BM-MDSCs or M2 macrophages), wash them twice in cold PBS.
2. Disrupt 5×10^5 pelleted cells on ice in lysis buffer. Incubate on ice for 15 min. Vortex every 5 min, maximum speed.

3. Spin at 14000 rpm for 10 min at 4 °C, transfer the supernatant containing proteins into a new tube and quantify the protein content. Take 20 μg add 1 volume of Laemmli's buffer and carry out the denaturation 5 min at 100 °C.
4. Proteins are separated on a 10% bis-tris acrylamide gel in MOPS buffer and then transferred on a PVDF membrane (Millipore).
5. The membrane was saturated for 1 h at RT in TBS/0.05% Tween-20 (TBST) supplemented with 5% non-fat milk (blocking solution).
6. Hybridizations of primary antibody is carried out as follows: goat anti-ARG1 (1:500, stock 0.5 mg/mL) ON 4 °C in blocking solution; rabbit anti-actin antibody (1:3000, stock 0.3 mg/mL) for 1 h at RT in TBS-T/3% BSA.
7. After washing with TBST, hybridizations with the HRP-conjugated secondary antibodies sheep anti-mouse IgG (1:5000) and donkey anti-rabbit IgG (1:5000) both diluted in blocking buffer are performed for 1 h at RT. Detection of the proteins is carry out using SuperSignal West Pico Chemiluminescent following manufacturer's instructions.

3. Determination of ARG1 levels in mouse plasma, in tissue culture supernatant and in cell homogenate

3.1 Equipment

1. Microplate reader
2. Multichannel pipettes
3. Eppendorf tubes
4. 37 °C incubator
5. Sonicator
6. Minicentrifuge

3.2 Material

1. Mouse ARG1 ELISA kit (LS-F6864, LSBio).
2. Deionized or distilled water.
3. Dulbecco's phosphate-buffered saline (DPBS without Ca^{2+} and Mg^{2+}; Lonza BioWhittaker, BE17-515Q).
4. Lysis buffer: 50 mM Tris-HCl, 150 mM NaCl. Add at the time of lysis cocktail of protease inhibitors and PMSF (final concentration 2 mM).
5. cOmplete™ Protease Inhibitor Cocktail (11836145001, Sigma-Aldrich).

3.3 Protocol

1. Collect and pellet the cells by centrifugation, $450 \times g$ at 4 °C for 10 min and remove the supernatant. Wash the cells three times with DPBS then resuspend in lysis buffer. Lyse the cells by ultrasonication. Centrifuge at $1500 \times g$ at 4 °C for 10 min to remove cellular debris. Collect the supernatant and following manufacture's instruction assay ARG1 levels.
2. Collect plasma in heparin-containing tubes. Centrifuge the samples for 15 min at $1000 \times g$ at 4 °C. Collect the supernatant for assaying following manufacture's instruction.
3. Remove cell culture supernatants and centrifuge the samples for 20 min at $1000 \times g$ to remove cells and debris. Transfer the supernatant into a new tube and proceed to assay following the manufacture's instruction.

4. Quantify ARG activity in cells

4.1 Equipment

1. 24-well tissue culture plates
2. Platform rocker
3. 96-well plate
4. Microplate reader
5. Heating block
6. ELISA plate (15041, Thermo Fisher)
7. 96-well plate flat (353072, Falcon)

4.2 Material

1. Cultured cells (e.g., MDSC)
2. Dulbecco's phosphate-buffered saline (DPBS without Ca^{2+} and Mg^{2+}; Lonza BioWhittaker, BE17-515Q)
3. Lysis buffer: 0.001% Triton X-100 (Sigma-Aldrich), protease inhibitor cocktail and 2 mM PMSF. Prepare fresh each time.
4. Arginase activation solution: prepare a 10 mM $MnCl_2$, 50 mM Tris-HCl, pH 7.5 solution. Store indefinitely at room temperature.
5. Arginase substrate solution: make up a 0.5 M L-arginine, pH 9.7 solution. Store indefinitely at room temperature
6. Urea standard solution: prepare a 100 mg/mL urea solution. Store for 1 year at room temperature. Prepare the standard starting from a concentration of 100 mg/mL up to 5 mg/mL
7. Quantichrom urea assay kit (DIUR-500, Bioassay Systems)

4.3 Protocol

1. To 5×10^5 cells plated in 24-well plate add lysis buffer (100 μL) per well and gently rock the plate for 15 min at 4 °C. Transfer into a new tube. Spin at 14000 rpm 10 min at 4 °C to remove debris.
2. Transfer 50 μL of each lysate to a 96-well flat plate. Add 50 μL of arginase activation solution to each well and incubate 10 min at 55 °C in a heating block.
3. Transfer 25 μL of each activated lysate to a new 96-well flat plate. Add 25 μL arginase substrate solution to each well and incubate from 1 h up to 24 h at 37 °C.
4. Prepare the urea standard and a blank. Mix of 1 vol of lysis buffer, 1 vol of arginase activation solution, and 2 vol arginase substrate solution as a diluent to match the content of the experimental samples.
5. Add 5 μL of each reacted sample, the serially diluted urea standard, and the blank solution control to an ELISA plate. Mix together the Quantichrom urea assay kit reagents following manufacturer's instructions, then add 200 μL per well to the ELISA plate.
6. Incubate 2–20 min at room temperature in the dark and quantify urea by reading the absorbance at 520 nm.

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