Methods to Measure MDSC Immune Suppressive Activity *In Vitro* and *In Vivo*

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This unit presents methods to assess the immunosuppressive properties of immunoregulatory cells of myeloid origin, such as myeloid-derived suppressor cells (MDSCs), both *in vitr*o and *in vivo* in mice, as well as in biological samples from cancer patients. These methods could be adapted to test the impact of different suppressive populations on T cell activation, proliferation, and cytotoxic activity; moreover, they could be useful to assess the influence exerted by genetic modifications, chemical inhibitors, and drugs on immune suppressive pathways

Keywords: adoptive cell transfer • chromium release test • flow cytometry • immunomagnetic sorting • immunosuppression • MDSC • thymidine incorporation

How to cite this article:
Solito, S., Pinton, L., De Sanctis, F., Ugel, S., Bronte, V., Mandruzzato, S., & Marigo, I. (2019). Methods to measure of MDSC immune suppressive activity *in vitro* and *in vivo*. *Current Protocols in Immunology*, *124,* e61. doi: 10.1002/cpim.61

INTRODUCTION

MDSCs are immature myeloid cells with potent immune suppressive activity that are characterized by a pathological state of activation (Bronte et al., 2016; Gabrilovich et al., 2007).

Two major subsets of MDSCs have been classified based on their phenotypic and morphological features: monocytic (M)-MDSCs and polymorphonuclear (PMN)-MDSCs. In mice, MDSCs described in the past as $CD11b^+$ and $Gr\text{-}1^+$ cells (Bronte et al., 2000; Dolcetti et al., 2010) are now better identified as $CD11b^+Ly6C^{hi}Ly6G^-$ (M-MDSCs) and $CD11b^+Ly6C^{lo}Ly6G^+$ (PMN-MDSCs) (Bronte et al., 2016; De Sanctis, Bronte, & Ugel, 2016a; Dolcetti et al., 2010; Movahedi et al., 2008; Peranzoni et al., 2010).

In cancer patients, the characterization of MDSCs is more complex, although it is currently accepted that three main subsets of circulating MDSCs exist, i.e., M-MDSCs and PMN-MDSCs, while a more immature population, whose counterpart in the mouse is not known, is defined as early-stage MDSCs (eMDSCs). Each of these subsets have been characterized by different combinations of myeloid markers and contain more than a single phenotype (Bronte et al., 2016; Damuzzo et al., 2015). Moreover, a recent study demonstrated that human MDSCs, under different conditions, can express additional

CURRENT PROTOCOLS
A Wiley Brand
Current Protocols in Immunology e61, Volume 124
Published in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpim.61

markers such as CD38, LOX-1, and PD-L1, highlighting their great plasticity and adding additional layers of complexity to MDSC characterization and targeting (Tcyganov, Mastio, Chen, & Gabrilovich, 2018).

Basic Protocol 1 shows how to isolate MDSCs from spleens of tumor-bearing mice, and Basic Protocol 2 illustrates how to generate MDSCs from mouse bone marrow (BM) samples. MDSCs obtained using these protocols can be assessed for their ability to suppress T cell proliferation in response to antigen-specific activation (Basic Protocol 3) or following nonspecific stimulation (Basic Protocol 4). Basic Protocol 5 shows how to assess T cell cytotoxic effector function in response to antigen-specific activation, and therefore a Support Protocol describing the calculation of results is included. A method to evaluate *in vivo*–induced immune suppression in mice is also presented (Basic Protocol 6).

Basic Protocol 7 indicates how to isolate circulating human $CD14^+$ cells enriched in M-MDSCs. Basic Protocol 8 illustrates how to expand *in vitro* human bone marrow–derived MDSCs (BM-MDSC), and Basic Protocol 9 describes how to test these cells for their suppressive function on T cell proliferation.

PMN-MDSCs share the expression of the markers CD11b, CD15, (or $CD66b^+$) and CD33 with neutrophils, but differ in their buoyant density, since PMN-MDSCs can be isolated in low-density Ficoll-gradient fraction, while neutrophils are present in the high-density fraction. Thus, the addition of a specific marker is warranted to obtain an enrichment of PMN-MDSCs. In this regard, we demonstrated that CD124 is up-regulated both in M-MDSCs and in PMN-MDSCs, although its presence only correlates with an immunosuppressive phenotype in M-MDSCs but not PMN-MDSCs of tumor-bearing patients. However, the isolation of CD124-positive versus -negative cells is technically challenging, since the staining of this antigen has a low intensity and a unimodal expression, thus making the separation of a highly enriched $CD124^+$ population difficult to achieve. More recently, lectin-type oxidized LDL receptor 1 (LOX-1) has been proposed as a marker to distinguish human neutrophils from PMN-MDSCs without the use of a density gradient (Condamine et al., 2016).

All the *in vitro* protocols were optimized for microcultures in order to reduce the number of cells to be used.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional ethics committee and must be executed in accordance with governing laws, directives, and guidelines.

NOTE: Patients must provide their informed consent and Institutional ethics committees must approve all experiments with human samples.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique must be used accordingly.

NOTE: All incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise noted.

BASIC PROTOCOL 1

ISOLATION OF MYELOID CELL SUBSETS FOR MEASUREMENT OF IMMUNOSUPPRESSIVE ACTIVITY

This protocol is optimized to isolate MDSCs from the spleens of tumor-bearing mice, preserving their functional activity in order to be used for both *in vitro* and *in vivo* functional assays. It allows the high- purity separation of PMN-MDSCs and M-MDSCs by FACS sorting, as required for the accurate immunosuppressive assays described here

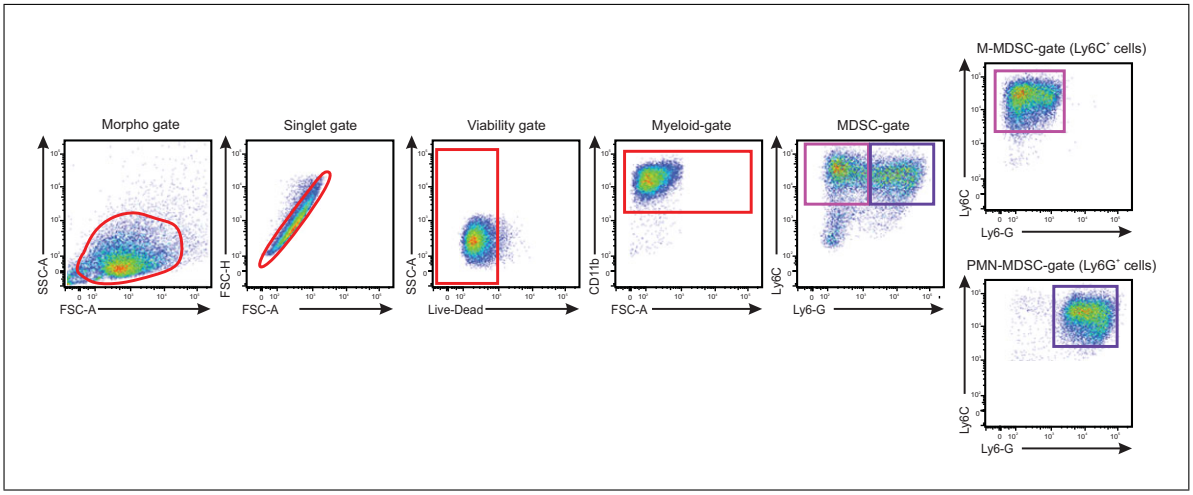

Figure 1 Gating strategy to isolate mouse MDSC using flow cytometric sorting. Cell suspension from tissues (spleen, blood, or tumor) of tumor-bearing mice or *in vitro*–differentiated BM cells were stained with the following mix: anti-Ly6C, anti-ly6G, anti-CD11b and live–dead probe. The figure illustrates isolation of the two main MDSC subsets derived by sequential steps: a morpho gate, a viability gate, a myeloid gate, and an MDSC gate. The procedure makes it possible to isolate M-MDSCs and PMN-MDSCs with a purity of ~80% to 90%.

and the molecular analysis protocols. In Figure 1, the gating strategy and an example of separation are presented.

For tumor models in which low percentages of $CD11b^+$ cells accumulate in the spleen, the purity of MDSCs obtained could be lower; titration of antibodies and reagents may help in obtaining better results. Moreover, a pre-enrichment step through immunomagnetic sorting using specific CD11b microbeads could be coupled with this protocol according to the manufacturer's instructions (see mouse and human CD11b MicroBeads, Miltenyi). This might improve the results, but it might also affect the viability and performance of the sorted cells.

This protocol could be applied to separate myeloid subsets in lymphoid organs other than spleen (i.e., bone marrow and lymph nodes) or from the tumor mass; however, an organ might present some peculiarities due to its structure (for example, it is necessary to digest the tumor to obtain a single-cell suspension) or function (for example, lymph nodes contain lower amounts of myeloid cells), which might limit either purity or viability of the recovered cell fractions.

This protocol has been used to successfully isolate different fractions of MDSCs from C26GM and 4T1 tumor-bearing BALB/c mice, and from MCA203 and MN-MCA1 tumor-bearing C57BL/6 mice. The protocol can be used to separate splenocytes of mice bearing tumors of different type and histology, provided that they induce an expansion of MDSCs. High purity is less likely to be achieved with low percentages of MDSCs. The same protocol has also been used to separate MDSC subsets obtained from bone marrow-derived MDSCs, *in vitro*–generated, as previously reported (Marigo et al., 2010) and as described in Protocol 2.

Previously, we published (Peranzoni et al., 2010; also see previous version of this unit; doi: 10.1002/0471142735.im1417s91) a separation protocol that described how to obtain three different subsets of myeloid cells from the spleens of tumor-bearing mice, by immunomagnetic sorting. A commercial Miltenyi kit is now available for this separation based on Gr-1 marker expression, which allows the separation of PMN-MDSCs as Gr-1^{high} cells (corresponding to $Ly6G^{high}$ cells) and M-MDSCs as Gr-1^{dim} $Ly6G^-$ containing different proportions of Ly-$6C^{high}$ and Ly-$6C^{low}$ cells dependent on the tumor models used, and a fraction enriched in macrophage precursors within the Gr-1^{low}Ly-$6Cl^{ow}$

Ly-6G$^-$CD11b$^+$. This protocol is still valid in cases in which a FACS sorter is not available and when highly pure populations are not required.

Materials

Spleens from BALB/c or C57BL/6 tumor-bearing mice (mice purchased from Jackson Laboratories) or *in vitro*-generated MDSCs (see Basic Protocol 2)
RPMI containing 3% FBS (see recipe)
Red cell ACK lysis buffer (Lonza)
Dulbecco's phosphate-buffered saline (DPBS; Lonza BioWhittaker, cat. no. BE17-515Q), cold
Fc-receptor (FcR) blocking reagent (clone 2.4G2, ThermoFisher)
Sorting buffer (see recipe) fixable viability stain
Fixable Viability Dye (ThermoFisher)
Anti mouse-CD11b (ThermoFisher)
Anti mouse-Ly6G (ThermoFisher)
Anti mouse-Ly6C (ThermoFisher)
Fetal bovine serum (FBS), heat inactivated
RPMI containing 10% FBS (see recipe)

10-mm culture dish
2-ml syringe
15- and 50-ml conical tubes
Centrifuge
100-μm nylon-mesh cell strainer (BD Biosciences)
MoFlo Astrios cell sorter (Beckman Coulter)
LSRII flow cytometer (BD Biosciences)
FlowJo 7.6.5 Software (TreeStar)

Additional reagents and equipment to harvest spleens from mice (see Reeves & Reeves, 1992) and to determine cell number using trypan blue dye exclusion (Strober, 2001)

NOTE: All antibodies need to be titrated. When working with more than one spleen and different amounts of cells adapt reagent volume accordingly.

Process spleen(s)

1. Collect spleens of tumor-bearing mice (Reeves & Reeves, 1992) in a small volume of RPMI containing 3% FBS under sterile conditions.

   Take care to clean spleen from surrounding fibrous tissue.

2. Place spleen in a 10-mm culture dish with a small volume of RPMI containing 3% FBS and gently disaggregate using the plunger of a 2-ml syringe.

   If more than one spleen has to be collected to perform more tests, spleens can be stored in a small volume of RPMI containing 3% FBS in a 50-ml conical tube on ice.

3. Add 5 ml of RPMI containing 3% FBS to cells and, pipetting gently, collect the cells in a 50-ml conical tube. Repeat steps 2 and 3 until the complete disaggregation of the spleen has been achieved.

4. Centrifuge the suspension 6 min at 300 $\times g$, 4°C, and discard the supernatant.

   The supernatant may be discarded by decantation or aspiration.

5. Lyse red blood cells by adding a volume of 5 ml (for each spleen) of red cell ACK lysis buffer to the pelleted cells, and incubate at room temperature for 4 min.

6. Add a volume of RPMI containing 3% FBS equal to at least five times the volume of lysis buffer added, and mix by gently pipetting up and down.

7. Centrifuge the suspension 6 min at 300 × *g*, 4°C, and discard the supernatant.
8. Resuspend cell pellet in 10 ml/spleen of RPMI containing 3% FBS, filter through a cell strainer (or a single sheet of nylon mesh) placed on the top of a 50-ml tube, and collect the suspension.

   Filtration can be helped by pre-hydrating the nylon mesh with a small volume of medium.
9. Properly dilute an aliquot of the single-cell suspension with trypan blue solution (Strober, 2001) and estimate the number of viable cells, avoiding red blood cell counts.

   Trypan blue colors dead cells a faint blue, and these cells should not be taken into account. Red blood cells can be recognized as small cells with a round shape and a neat, thick perimeter.

Separation of PMN-MDSCs and M-MDSCs from splenocytes

An example of gating strategy for the sorting and results analyzed with FlowJo 7.6.5 Software is shown in Figure 1.

10. Transfer 1×10^8 viable cells obtained from the spleens of tumor-bearing mice to a new 15-ml conical tube, and then add 8 to 10 ml of DPBS to wash the cells.

    In the case of spleens coming from mice with completely unknown tumors, the proportion of myeloid cells should be evaluated before staining a large sample.
11. Centrifuge the suspension 6 min at 300 × *g*, 4°C, and discard the supernatant.
12. Add 10 ml of sorting buffer and wash cells by gently pipetting up and down.
13. Centrifuge the suspension 6 min at 300 × *g*, 4°C, and discard the supernatant.

    These fractions can be used to assess their immunosuppressive activity either in vitro and in vivo.
14. Incubate samples with 10 µl Fc-receptor blocking reagent in 50 µl of sorting buffer at room temperature for 10 min.
15. Add the mixture of antibody, composed of Fixable Viability Dye (5 µl), anti-CD11b (10 µl), anti Ly6G (10 µl), and anti Ly6C (10 µl), to the tubes and incubate at 4°C for 20 min. Adjust the volume of staining mix to 100 µl with the sorting buffer. Specifically, for FACS analysis, cells are stained in 15-ml polypropylene tubes previously coated for at least 1 hr with heat-inactivated fetal bovine serum.
16. Wash twice with sorting buffer. Centrifuge the suspension 6 min at 300 × *g*, 4°C, and discard the supernatant.
17. After labeling, resuspend samples at the concentration of 30×10^6 cells/ml of sorting buffer and proceed with the FACS separation as outlined in Figure 1.
18. Filter MDSCs through a 100-µm cell strainer and isolate through MoFlo Astrios.

    Sorting should be performed with a 100-µm nozzle, setting the pressure, voltage, and cell rate as appropriate for the sorter.
19. Collect sorted M-MDSCs and PMN-MDSCs in two different 15-ml polypropylene tubes previously coated for at least 1 hr with heat-inactivated fetal bovine serum.
20. After the separation, wash and resuspend M-MDSCs and PMN-MDSCs in 10 ml of RPMI/10% FBS. Take an aliquot of the single-cell suspension, dilute it properly with trypan blue solution, and estimate the number of viable cells (Strober, 2001). Another aliquot is checked for purity with the LSRII flow cytometer.

NOTE: *All the procedures, including the sorting, are performed at 4°C, to avoid cell loss and adherence to the plastic.*

These fractions can be stained to assess the presence and distribution of markers that were used to characterize mouse MDSCs (Table 1).

BASIC PROTOCOL 2

MOUSE BM-MDSC GENERATION

This protocol is optimized to obtain bone marrow (BM)–derived MDSCs from mice as previously described (Marigo et al., 2010). BM cells are cultured for 4 days in the presence of the recombinant mouse cytokines GM-CSF and IL-6. The final cultures will contain proportions of PMN-MDSCs, M-MDSCs, and macrophages.

Materials

C57BL/6 mice (The Jackson Laboratory)
RPMI containing 3% FBS (see recipe)
Red cell ACK lysis buffer (Lonza)
RPMI containing 10% FBS (see recipe)
Premium Grade IL-6 (Miltenyi Biotec)
Premium Grade GM-CSF (Miltenyi Biotec)
DPBS without Ca or Mg (Lonza BioWhittaker, cat. no. BE17-515Q) containing 2 mM EDTA

Scissors and pliers
2-ml syringe with 26-G needle
15- and 50-ml conical tubes
Centrifuge
100-μm nylon-mesh cell strainer (BD Biosciences)
6-well culture dishes

Additional reagents and equipment to harvest bone marrow from mice (Reeves & Reeves, 1992) and to determine cell number using trypan blue dye exclusion (Strober, 2001)

Process bone marrow (BM)

1. Remove tibias and femurs from mice, and remove the muscle from the bones with scissors.

   Take care to avoid hair contamination

2. Cut the extremity of the bones with pliers and scissors under sterile conditions.
3. Flush the medium inside the bones with a small volume of RPMI containing 3% FBS, gently injected with a 2-ml syringe with a 26-G needle. Collect flushed-out material in 50-ml conical tubes containing 5 ml of RPMI containing 3% FBS

BM-MDSC generation

4. Centrifuge the suspension 6 min at 300 ×*g*, 4°C, and discard the supernatant.

   The supernatant may be discarded by decantation or aspiration.

5. For two legs, lyse red blood cells by adding a volume of 5 ml of red blood cell lysis buffer to the pelleted cells, and incubate at room temperature for 4 min.
6. Add a volume of RPMI/3% FBS equal to at least five times the volume of lysis buffer added, and mix by gently pipetting up and down.
7. Centrifuge the suspension 6 min at 300 ×*g*, 4°C, and discard the supernatant.

Table 1 Common mAbs Used for Characterization of Surface Molecules in Mouse MDSCs

Epitope	Clone	Antibody type	Company	Expression
CD11b	M1/70	Rat IgG2b,κ	ThermoFisher	+
Gr-1	RB6-85C	Rat IgG2b,κ	Biolegend	+
Ly6G	1A8	Rat IgG2a,κ	ThermoFisher	±
Ly6C	Mk1.4	Rat IgM, κ	ThermoFisher	±
CD115	AFS98	Rat IgG2a,κ	ThermoFisher	Low/−
CD16/CD32	2.4G2	Rat IgG2b,κ	BD Pharmingen	+
CD124 (IL-4Rα)	mIL4R-M1	Rat IgG2a,κ	BD Pharmingen	±
CD40	3/23	Rat IgG2a,κ	BD Pharmingen	−
CD80	16-10A1	Armenian Hamster IgG2,κ	BD Pharmingen	+
CD86	GL1	Rat IgG2a,κ	BD Pharmingen	−
MHC-I (H-2Kd)	SF1-1.1	Mouse IgG2a,κ	BD Pharmingen	+
MHC-I (H-2Kb)	AF6-88.5	Mouse IgG2a,κ	BD Pharmingen	+
MHC-II	2G9	Rat IgG2a,κ	BD Pharmingen	−
CD31	390	Rat IgG2a,κ	Biolegend	+/Low/−
F4/80	A3-1	Rat IgG2b	Serotec	Low/−
CD2	RM2-5	Rat IgG2a,λ	BD Pharmingen	Low/−
CD71	RI7217	Rat IgG2a,κ	Biolegend	Low/−
CD11c	N418/HL3	Armenian Hamster IgG/Armenian Hamster IgG1, λ2	Biolegend/BD Pharmingen	Low/−
ER-MP58	ER-MP58	Rat IgM	Serotec	+

8. Resuspend the cells obtained from two legs in 5 ml of RPMI containing 10% FBS and filter the suspension through a cell strainer.

   The volume can be scaled to meet researchers' needs; extra medium volume can also be added to rinse the cell strainer, minimizing cell loss.

9. Take an aliquot of the single-cell suspension, dilute it properly with trypan blue solution, and estimate the number of viable cells (Strober, 2001).

10. Adjust the concentration of the BM suspension to 1.5×10^6 cells/ml using RPMI/10% FBS.

11. Plate 1 ml (1.5×10^6 cells) of BM cells in each well of a 6-well plate, add 2 ml of RPMI/10% FBS in the presence of cytokines GM-CSF and IL-6, both at a final concentration of 40 ng/ml (per well), and place the culture in a 37°C, 5% CO_2 incubator for 4 days.

12. At day 4 of culture, to test the immunosuppressive properties of the cells, gently harvest BM-MDSCs with a Pasteur pipette. Wash dishes with DPBS containing 2 mM EDTA to detach and collect the remaining cells.

BASIC PROTOCOL 3

MEASURING MYELOID-INDUCED SUPPRESSION OF T CELL ANTIGEN-INDUCED PROLIFERATION *IN VITRO* BY CELL TRACE DILUTION

Immune suppression exerted by myeloid cells to the detriment of activated T cells can be measured in terms of inhibition of proliferation by evaluating dye dilution to trace multiple generations of proliferating lymphocytes.

Materials

Immunosuppressive cells, e.g., MDSCs (Basic Protocol 1 or 2)
RPMI containing 10% FBS (see recipe)
CD45.1 congenic mice; purchased from Jackson Laboratories, under the name B6.SJL-PtrcaPepcb/BoyJ.
Transgenic OT-1 mice on a C57BL/6 background bearing a αβ T cell receptor (TCR) that recognizes the K^b-restricted $OVA_{257-264}$ peptide; purchased from Jackson Laboratories, under the name C57BL/6-Tg (TcraTcrb)1100Mjb/J)
Red cell ACK lysis buffer (Lonza)
RPMI containing 3% FBS (see recipe)
Dulbecco's phosphate-buffered saline (DPBS without Ca and Mg; Lonza Biowhittaker, cat. no. BE17-515Q)
CellTrace™ Violet Cell Proliferation Kit (Molecular Probes)
Fetal bovine serum (FBS)
10 mg/ml $OVA_{257-264}$ peptide stock solution (available lyophilized from JPT Peptide Technologies)
Staining buffer (see recipe)
Fc-receptor (FcR) blocking reagent (clone 2.4G2, ThermoFisher)
Anti-CD8 (i.e., PeCy5-anti CD8a, clone 53.6.7, cat. # 15-0081-81, ThermoFisher)
Anti-CD45.2 conjugated to a brilliant fluorochrome (i.e., PE-anti CD45.2, clone 104, cat. # 12-0454-81, ThermoFisher)

96-well or 384-well flat-bottom plates
10-mm culture dish
2-ml syringe
50-ml conical tube
Refrigerated centrifuge
100-μm nylon-mesh cell strainer (BD Biosciences)

LSRII flow cytometer (BD Biosciences)
FlowJo 7.6.5 Software (TreeStar)
4-ml round-bottom tubes
TruCount Tubes (Becton Dickinson)

Additional reagents and equipment to prepare immunosuppressive cells (Basic Protocol 1 and 2), harvest spleens from mice (Reeves & Reeves, 1992), and count viable cells by trypan blue exclusion (Strober, 2001)

Day 0: Suppressive cell plating

1. Prepare MDSCs, according to Basic Protocol 1 or 2.
2. Wash cells twice in RPMI medium containing 10% FBS and resuspend in a suitable volume of RPMI containing 10% FBS.
3. Dilute an aliquot of suspension in trypan blue solution and determine viable cell concentration (Strober, 2001).
4. Adjust cell concentration appropriately with RPMI/10% FBS.

   The concentration will depend upon the design of the experiment. A good range of MDSCs is 1.5% to 24% of the effector culture cellularity. For the 96-well plate, if 0.6×10^6 effector cells are used, MDSC concentration can be adjusted to 1.44×10^6/ml, so that 0.144×10^6 cells will be plated in 100 μl (24% of the effector cells). For the 384-well plate, MDSC concentration can be adjusted to $0{,}6 \times 10^6$/ml, so that 24,000 cells will be plated in 40 μl (24% of the effector cells).

   For example, in the 96-well plate, if the experimental setup requires 3% of suppressive cells in the coculture and effector splenocytes are 0.6×10^6 cells/well, 0.018×10^6 suppressive cells will be plated per well. Adjust suppressive cell concentration to 0.18×10^6 cells/ml and plate 100 μl/well.

5. Plate myeloid cells in triplicate in a 96-well flat-bottom microplate in a volume of 100 μl (or 40 μl for 384-well plate) of RPMI/10% FBS, and place the microplate in a 37°C, 5% CO_2 incubator for at least 30 min.

   This assay requires a careful titration of the suppressive cells. The number of suppressor cells to add to each well will depend on the experimental plan. A good starting point could be to plate suppressor cells at 24% of the total cells in control cultures and serially dilute them by a factor of 2 until they represent ~1.5% of total cells. When organizing the distribution of samples, avoid using the outer wells, because these wells are more susceptible to evaporation. Outer wells can be filled with RPMI containing 3% FBS. Remember to fill at least three wells with RPMI/10% FBS without myeloid suppressor cells, to use as control cultures.

Plating splenocytes

6. Separately collect spleens from both CD45.1 and OT-1 mice (Reeves & Reeves, 1992) in a small volume of RPMI/3% FBS under sterile conditions.

   Splenocytes from OT-1 mice need to be diluted with CD45.1 splenocytes to obtain a concentration of OVA-specific $CD8^+$ T lymphocytes, which is 1% of total cultured cells (usually about 1:10 dilution is sufficient). Consequently, one spleen from OT-1 mice is sufficient for several microtiter plates. The percentage of specific OVA $CD8^+$ T lymphocytes in total splenocytes can be determined before their use by cytometry staining with anti-CD8 and anti-$V_\alpha 2$ $V_\beta 5.1/5.2$ mAbs to identify the specific T cell receptor (TCR).

7. Place spleens in a 10-mm culture dish with a small volume of RPMI/3% FBS, and gently disaggregate using the plunger of a 2-ml syringe.
8. Add 5 ml of RPMI/3% FBS to cells and collect the cells in a 50-ml conical tube by gently pipetting up and down. Repeat steps 7 and 8 until the spleen is completely disaggregated.

9. Centrifuge the suspension 6 min at 300 $\times g$, 4°C, and discard the supernatant.
10. Lyse red blood cells by adding about 5 ml of red cell lysis buffer for spleen, and incubate at room temperature for 4 min.
11. Add a quantity of RPMI/3% FBS equal to at least five volumes the quantity of lysis buffer applied, and mix by gently pipetting up and down.

    It is very important to obtain a red blood cell–free suspension (see explanation in Commentary).

12. Centrifuge the suspension 6 min at 300 $\times g$, 4°C, and discard the supernatant.
13. Resuspend cells in 1 ml of RPMI/10% FBS, and put on ice.
14. Wash both CD45.1 and OT-1 cells by adding 10 ml of RPMI/10% FBS.
15. Centrifuge the suspensions 6 min at 300 $\times g$, 4°C, and discard the supernatant.
16. Resuspend the cells in 2 ml/spleen of RPMI/10% FBS, and filter the suspension through a cell strainer.
17. Dilute an aliquot of suspension in trypan blue solution, and determine viable cell concentration (Strober, 2001).
18. Keep CD45.1 splenocytes on ice and wash OT-1 cells twice by adding 10 ml of ice-cold DPBS (4°C) and discarding the supernatant.
19. Adjust OT-1 splenocyte concentration to 20×10^6 cells/ml concentration with room temperature DPBS and add an equal volume of DPBS containing CellTrace™ at the concentration of 5 μM (the final concentration of the suspension will be equal to half the concentration). Keep the suspension 5 min at 37°C in incubator and mix every minute.
20. Block the staining by adding an equal volume of FBS and keep at room temperature for 1 min. Centrifuge the suspension 6 min at 300 $\times g$, 4°C, and discard the supernatant.
21. Wash cells twice in DPBS with 10% FBS and resuspend in 5 ml of RPMI.
22. Check the staining with CellTrace™ by flow cytometry.

    When evaluating CellTrace incorporation, a control for autofluorescence value of unstained splenocytes should be included, to evaluate whether the difference in emission signals among unstained splenocytes and CellTrace$^+$ splenocytes is enough to be able to quantify extensive proliferation, which will bring a strong dilution of CellTrace signal. A successful staining is achieved when the histogram has a single, narrow peak.

23. Adjust the concentration of both the CD45.1 and the OT-1 splenocyte suspensions to 12×10^6 cells/ml for 96-well plate (or at 5×10^6 cells/ml for 384 wells/plate) in RPMI/10%.
24. Mix CD45.1$^+$ and CellTrace$^+$ OT-1 cells in appropriate proportions to obtain a concentration of OVA-specific CD8$^+$ T lymphocytes that is 1% of the total culture, and add $OVA_{257\text{-}264}$ peptide to a final concentration of 1 μg/ml.

    CD8$^+$OVA-TCR$^+$ percentage in OT-1 mice can be determined by FACS analysis using a simple staining with anti-CD8α and anti-anti-$V_\alpha 2$ $V_\beta 5.1/5$.antibodies. CD8$^+$OVA-TCR$^+$ double-positive cells are usually about 10% of total splenocytes in these transgenic mice, while the same cell population is almost negligible in wild-type C57BL/6 or congenic CD45.1 mice. In order to obtain a cell suspension with 1% CD8$^+$OVA-TCR$^+$ cells, 1 part of OVA splenocytes (with 10% CD8$^+$OVA-TCR$^+$cells) can be added to 9 parts of CD45.1$^+$ splenocytes (with ∼0% CD8$^+$OVA-TCR$^+$cells).

25. Immediately plate 50 μl per well of cell suspension onto the previous 96-well plate containing the immunosuppressive myeloid cells (step 5), or 20 μl per well if a 384-well plate was used.

26. Add 50 μl to 96-well plate (or 20 μl for 384-well plate) of RPMI/10% FBS containing $OVA_{257-264}$ peptide at a final concentration in each well of 1 μg/ml. Incubate for 3 days in the 37°C, 5% CO_2 incubator.

Day 3: Culture harvesting

27. Pool triplicate cultures to new 4-ml round-bottom tubes, wash samples once with staining buffer, centrifuge the suspension 6 min at 300 $\times g$, 4°C, and discard the supernatant.

28. Block nonspecific binding with 50 μl FcR blocking reagent for 10 min at 4°C.

29. Stain cells with 50 μl of a mix composed of 0.5 μl of anti-CD8 and 0.1 μl of anti-CD45.2 antibodies in 49 μl of staining buffer, for 20 min at 4°C.

    Remember to include appropriate single-staining controls.

30. Wash samples once with staining buffer, centrifuge the suspension 6 min at 300 $\times g$, 4°C, and discard the supernatant.

31. Resuspend samples in 200 μl DPBS, transfer the mix in TruCount tubes, briefly vortex and then proceed with flow cytometric acquisition and analysis.

Flow cytometric analysis

32. Perform flow cytometric acquisition and analysis.

    To detect proliferating $CD8^+$ T cells, gate cells first by their morphology, and then collect a sufficient number of events in the $CD8^+/CD45.2^+/CellTrace^+$ gate.

    After the acquisition, proceed with data analysis. Gate cells by morphology and identify $CD8^+/CD45.2^+/CellTrace^+$ population. Inside this gate, set a baseline gate on the histograms of CellTrace signal of unstimulated splenocyte controls, and copy this gate to the stimulated CD8 cells cultured in the presence or absence of MDSCs, in order to determine the percentage of proliferating T lymphocytes. With FlowJo, it is the possible to model proliferation data; FlowJo presents a graphical display as well as information about each cell generation in the subset. The proliferation platform also provides information about the fraction of cells from the original population that have divided and the number of times these cells have divided. In addition, the FlowJo Proliferation Platform draws gates that separate each cell generation.

    Results obtained can be quantitative or qualitative. See Critical Parameters and Troubleshooting.

MEASURING MYELOID-INDUCED SUPPRESSION OF T CELL PROLIFERATION *IN VITRO* BY ANTI-CD3/ANTI-CD28 STIMULATION AND EVALUATION OF [^{3}H]THYMIDINE ([^{3}H]TdR) INCORPORATION

BASIC PROTOCOL 4

Proliferation of T cells can be induced by antigen-independent stimulation, and MDSCs can exert immune suppressive functions on these cells. The proliferative arrest of actively dividing cells can also be measured as a function of [^{3}H]TdR incorporation by T cells into their DNA. The method is extremely simple and provides a quantitative and relatively rapid analysis, which can be easily applied to complex experiments as large-scale screenings of drugs and treatments.

Materials

Dulbecco's phosphate-buffered saline (DPBS; Lonza BioWhittaker, cat. no. BE17-515Q)

Anti-CD3 (2C11, ATCC)
Anti-CD28 (clone 37.5, ATCC)
Immunosuppressive (myeloid) cells, e.g., MDSCs (Basic Protocol 1 or 2)
RPMI containing 10% FBS (see recipe)
C57BL/6 mice purchased from Jackson Laboratories
RPMI containing 3% FBS (see recipe)
Red cell ACK lysis buffer (Lonza)
[^{3}H]TdR (PerkinElmer)
Serum-free RPMI (e.g., Invitrogen)
Serum-free RPMI medium (e.g., Invitrogen)
96% ethanol
MicroScint-20 scintillation fluid (PerkinElmer)

96-well flat-bottom microtiter plate
10-mm culture dish2-ml syringe
50-ml conical tubes (BD Falcon)
Refrigerated centrifuge
100-μm nylon-mesh cell strainer (BD Biosciences)
Unifilter-96 GF/C plate (PerkinElmer) with plate sticker
96-well U-bottom microtiter plate (PerkinElmer)
Plate harvester: FilterMate 196 (Packard)
TopSeal-A 96-well microtiter plate adhesive sealers (PerkinElmer)
Scintillation counting device (TopCount, PerkinElmer)
Computer running spreadsheet program, e.g., Microsoft Excel

Additional reagents and equipment to prepare immunosuppressive cells (Basic Protocol 1 or 2), harvest spleens from mice (Reeves & Reeves, 1992), and count viable cells by trypan blue exclusion (Strober, 2001)

Day 0: Culture plate coating

1. Prepare sufficient coating buffer: DPBS containing anti-CD3 (3 μg/ml final concentration) and anti-CD28 (2 μg/ml final concentration).

   Antibody concentration may be optimized on the basis of stock and supplier; a suitable volume would be 11 ml of antibody solution per plate.

2. Fill 96-well flat-bottom microtiter plates with 100 μl/well anti-CD3 and anti-CD28-containing DPBS with a multichannel pipettor; also fill an equal number of wells with 100 μl/well of DPBS without antibodies, for nonspecific proliferation measurement.

   Also remember to fill extra wells for appropriate controls based on the experimental set up, e.g., suppressive cells only.

3. Incubate the plate overnight at 4°C.

Day 1: Cell plating

Plate immunosuppressive cells

4. Prepare myeloid (immunosuppressive) cells, e.g., MDSCs, according to Basic Protocol 1 or 2 or other strategies of enrichment.

5. Wash cells twice in RPMI containing 10% FBS and resuspend them in a suitable volume of RPMI/10% FBS.

   The supernatant should be aspirated from the top; be careful to remove as much supernatant as possible without disturbing the cell pellet.

6. Resuspend cells in a small volume of RPMI/10% FBS. Dilute an aliquot of suspension in trypan blue solution and determine viable cell concentration (Strober, 2001).

7. Adjust cell concentration appropriately with RPMI/10% FBS.

   The concentration depends upon the design of the experiment. A good range of MDSCs is 24% to 1.5% of the effector culture cellularity. If 0.6 × 10^6 effector cells are used, MDSC concentration can be adjusted to 1.44 × 10^6/ml, so that 0.144 × 10^6 cells will be plated in 100 μl (24% of the effector cells).

8. Working under sterile conditions, empty 96-well microtiter plate (from step 3) by inverting it with a rapid movement.

   Work under a laminar flow hood; a plastic basin wrapped with paper towels can be used to discard the coating buffer.

9. Fill every well with 200 μl of DPBS using a multichannel pipettor and empty the plate as in step 8. Repeat at least three times to wash extensively.

   Fill the plate from the top of the well to avoid scratching the surface of the well, which might disturb the antibody coating.

10. Immediately plate suppressive cells in triplicate for specific and background proliferation in 100 μl of RPMI/10% FBS, and place the microtiter plate in a 37°C, 5% CO_2 incubator.

    When organizing the distribution of samples, avoid using the outer wells, because these wells are more susceptible to evaporation. Outer wells can be filled with sterile medium. Remember to fill at least six wells with RPMI/10% FBS without suppressive cells; these wells will be used as control cultures for the determination of specific and nonspecific proliferation. Multiply these control wells for different treatments according to the experimental setup, e.g., different inhibitors and drugs to be tested.

Plate splenocytes

11. Collect spleens from C57BL/6 mice (Reeves & Reeves, 1992) in a small volume of RPMI containing 3% FBS under sterile conditions.

    Usually one spleen from an 8-week old-mouse is sufficient for one plate.

12. Place spleens in a 10-mm culture dish with a small volume of RPMI/3% FBS and gently disaggregate them using the plunger of a 2-ml syringe.

13. Add 5 ml of RPMI/3% FBS to cells and collect the cells in a 50-ml conical tube by gently pipetting. Repeat steps 12 and 13 until complete disaggregation of the spleen is achieved.

14. Centrifuge the suspension 6 min at 300 ×*g*, 4°C, and discard the supernatant.

15. Lyse red blood cells by adding a volume of red cell ACK lysis buffer equal to the volume of the pelleted cells, and incubate at room temperature for 4 min.

16. Add a volume of RPMI/3% FBS equal to at least five times the volume of lysis buffer added, and mix by gently pipetting up and down.

17. Centrifuge the suspension 6 min at 300 ×*g*, 4°C, and discard the supernatant.

18. Wash cells with 10 ml of RPMI/10% FBS.

19. Centrifuge the suspension 6 min at 300 ×*g*, 4°C, and discard the supernatant.

20. Resuspend the cells in 2 ml of RPMI/10% FBS for each spleen and filter the suspension through a cell strainer.

The volume can be scaled to meet researchers' needs; extra medium volume can also be added to rinse the cell strainer, preventing cell loss.

21. Dilute an aliquot of the suspension in trypan blue solution and determine viable cell concentration (Strober, 2001).

22. Adjust the concentration of the splenocyte suspension to 6×10^6 cells/ml with RPMI/10% FBS.

23. Immediately plate 100 µl of cell suspension onto the previously coated microtiter plate and incubate 3 days in the 37°C, 5% CO_2 incubator.

    Pay attention to the cell distribution schema. Splenocytes will proliferate upon anti-CD3/anti-CD28 stimulation, and their proliferation will be inhibited by immunosuppressive cells; therefore, splenocytes should be distributed in previously anti-CD3/anti-CD28-coated wells, either with or without immunosuppressive cells, as test and reference wells, respectively. The proliferative behavior of MDSCs alone under these conditions should also be tested; therefore, MDSCs will be cultured with 100 µl of medium instead of effector cells.

    [^{3}H]TdR should be added the third day after culture setup. It would be useful to check the cell culture daily in order to avoid medium exhaustion by excessive proliferation of cells (signaled by medium turning yellow) or initial cell death (granular, small cells instead of cell clumps).

Day 4

24. Add 1 µCi of [^{3}H]TdR in 25 µl of serum-free RPMI to each well and incubate 18 hr in the 37°C, 5% CO_2 incubator.

    The concentration of the [^{3}H]TdR solution added to each well will thus be 0.04 µCi/µl.

Day 5

Culture harvesting

25. Load a Unifilter-96 GF/C plate and an empty clean 96-well U-bottom plate onto the plate harvester and prepare Unifilter-96 GF/C plate by washing it with distilled water.

    A wet Unifilter-96 GF/C is essential to obtain consistent results.

26. Load the culture plate onto harvester and aspirate culture medium, collecting it in the "hot" radioactive waste tank.

27. Wash the plate five times with water and aspirate, collecting the supernatant in the "hot" radioactive waste tank. Repeat another five times using aspiration, collecting the supernatant in the "cold" radioactive waste tank.

28. Remove the culture plate from the harvester and replace it with a proper vessel filled with 96% ethanol.

    In this case, a proper vessel is a container, similar in dimensions to a 96-well plate, that can be accommodated properly in the harvester; it could be, for example, a plate lid or a pipet-tip box lid with the same length and width as a 96-well plate, deep enough to contain 2 to 5 ml of alcohol.

29. Completely aspirate ethanol, collecting it in the "cold" radioactive waste tank.

30. Open the harvester and let the Unifilter-96 GF/C plate dry by continuous aspiration.

    Plate dryness is essential to obtain consistent results, since the scintillation cocktail can only be mixed with a very small amount of water and still perform correctly.

Preparation of Unifilter-96 GF/C plate for reading

31. Check plate dryness.

    The Unifilter-96 GF/C plate can be observed against a light source; wells that are not properly dried will appear translucent. Wait until the plates are completely dry.

32. Apply a plate sticker on the back of the Unifilter-96 GF/C plate.

    Take care to properly position the sticker. Inappropriate positioning will result in leakage of scintillation liquid.

33. Fill each well with 25 µl Microscint-20 with a multichannel pipettor.

    Take care not to spill the liquid outside of the well rim, since this could hinder plate sealing.

34. Seal the plate with TopSeal-A plate sealer.

35. Keep the plate in the dark for at least 30 min.

    This step of incubation in the dark is necessary in order to quench the scintillation cocktail.

36. Read the plate in a TopCount scintillation counting device for 1 min/well.

37. Interpret results.

    Proliferation is represented in counts per minute (cpm), which is proportional to the [^{3}H]TdR that cells have incorporated in their DNA during the S phase of proliferation. In a spreadsheet program like Excel, calculate average cpm from triplicate cultures and subtract nonspecific proliferation. Also check for MDSC proliferation, which should be minimal.

MEASURING MYELOID CELL–INDUCED SUPPRESSION OF T CELL CYTOTOXIC ACTIVITY *IN VITRO*: INHIBITION OF ANTIGEN-INDUCED CYTOTOXIC ACTIVITY OF T CELLS IN MICROCULTURES

BASIC PROTOCOL 5

Immune suppression exerted by myeloid cells to the detriment of activated T cells could also be measured in ways that can complement simply looking at the process of T cell expansion. Assessing the cytotoxic activity of $CD8^+$ T cells, elicited by antigen-specific stimulation *in vitro*, will allow one to simultaneously assess both T cell abundance and functional effector state.

Materials

Immunosuppressive cells, e.g., MDSCs (Basic Protocol 1 and 2)
RPMI containing 10% FBS (see recipe)
10 mg/ml $OVA_{257-264}$ peptide stock solution (available lyophilized from JPT Peptide Technologies)
EL4 cell line (ATCC TIB-39™) maintained in culture in 75-cm^2 culture flasks: prepare a sufficient number of flasks containing EL4 cells, which need to be subconfluent on day 5
Dulbecco's phosphate buffered saline (DPBS without Ca or Mg; Lonza BioWhittaker, cat. no. BE17-515Q)
Fetal bovine serum (FBS)
1 mCi/ml $Na^{51}CrO_4$ (PerkinElmer)
RPMI containing 5% FBS (see recipe)
Sodium dodecyl sulfate (SDS)

96-well flat bottom microplates
50-ml conical tubes

Refrigerated centrifuge
12-ml round-bottom tubes
Microtiter plate carrier for centrifuge
LumaPlate (PerkinElmer)
Scintillation counting device (TopCount, PerkinElmer)

Additional reagents and equipment to prepare immunosuppressive cells (Basic Protocol 1 or 2), to prepare splenocytes (Basic Protocol 3), and count viable cells by trypan blue exclusion (Strober, 2001)

Day 0: Suppressive cell plating

Prepare myeloid (immunosuppressive) cells, e.g., MDSCs, according to Basic Protocol 1 or 2 and plate them by following the procedures (1 to 5) reported in Basic Protocol 3, considering a 96-well flat-bottom microplate only.

Plating effector splenocytes

Prepare splenocytes according to Basic Protocol 3 from step from 6 to 18. Continue with the steps below.

1. Adjust the concentration of both the CD45.1 and the OT-1 splenocyte suspensions (prepared as described in Basic Protocol 3) to 6×10^6 cells/ml for a 96-well plate in RPMI/10% FBS.
2. Mix CD45.1 and OT-1 splenocytes in appropriate proportions to obtain a concentration of OVA-specific $CD8^+$ T lymphocytes, which is 1% of the total culture, and add $OVA_{257\text{-}264}$ peptide for a final concentration of 1 μg/ml.

   See Basic Protocol 3, step 24, for details.
3. Immediately plate 100 μl of splenocyte suspension onto the previous microplate containing the immunosuppressive myeloid cells, and incubate for 5 days at 37°C, in the 5% CO_2 incubator.

Day 5: Target cell preparation

4. Collect EL4 in suspension cells from 75-cm^2 flasks in a 50-ml conical tube.
5. Centrifuge the suspension 6 min at 300 × *g*, 4°C, and discard the supernatant.
6. Resuspend the cells in 5 ml of 10% FBS in DPBS.
7. Dilute an aliquot of suspension in trypan blue solution and determine viable cell concentration (Strober, 2001).
8. Aliquot 6×10^6 cells to two new 12-ml round-bottom tubes. Bring volume to 10 ml with 10% FBS in DPBS.

   3×10^6 target cells are sufficient for at least 15 test plates. The quantity can be scaled when necessary; nevertheless, if more than 3×10^6 cells are needed, prepare different aliquots for the next steps.
9. Centrifuge the suspension 6 min at 300 × *g*, 4°C, and carefully discard the supernatant.

   In this step, it is essential to eliminate as much supernatant as possible, in order to improve ^{51}Cr uptake by target cells.
10. Add 10 μl of undiluted FBS to both pellets.

    If you decide to load a smaller number of cells, simply scale down FBS to obtain a 10% final concentration.

11. Add 3 μl of $OVA_{257-264}$ peptide solution (10 mg/ml) to one of the two pellets (which will be the pulsed sample).

    One pellet will not receive the peptide and will be used as control for nonspecific lysis. An EG7 cell line stably transfected to express the antigen $OVA_{257-264}$ is also available. These cells can also be used in the test to evaluate specific lysis.

12. Add 100 μl of 1 mCi/ml $Na^{51}CrO_4$ to each tube and resuspend by gentle flicking.

    Avoid the formation of bubbles.

13. Incubate 1 hr at 37°C, flicking the tube every 15 min. After 1 hr, wash the cells twice with RPMI containing 5% FBS, each time centrifuging the cells 10 min at 300 $\times g$, room temperature, removing the supernatant, and then resuspending the cells in 6 ml of RPMI/5% FBS. Determine viable cell concentration (Strober, 2001) and adjust to 0.02×10^6 cells/ml with RPMI/5% FBS.

    During the 1-hr target incubation, proceed with the rest of the protocol (step 14). If test plates (step 14) are not ready when the target incubation ends, return washed target cells to the incubator; it is extremely important to count and adjust target-cell concentration just before plating them, to improve reproducibility.

Test plate setup

14. Fill new 96-well plate (test plate) with 100 μl/well of RPMI containing 5% FBS. Resuspend cells in culture plate by gently pipetting up and down several times with a multichannel pipettor, then transfer 50 μl of culture to the first row of the test plate; pipet up and down five times to mix, and then transfer 50 μl of cells to the subsequent rows, thus obtaining an 8-point, 1:3 serial dilution (Fig. 2).

    Every well of the culture plate has to be diluted eight times; consequently, four different culture conditions in triplicate could be arranged per each test plate. See Figure 2 as an example. Remember that specific and nonspecific lysis need to be determined in separate plates, because the same culture will be tested against peptide-pulsed and unpulsed targets: therefore, every test plate has to be prepared in duplicate. Also, remember to fill at least six wells in a separate plate (three for peptide-pulsed target cells and three for unpulsed target cells) with RPMI containing 5% FBS and 1% SDS and six wells with RPMI/5% FBS without any addition of culture cells, to evaluate maximum and spontaneous ^{51}Cr release of target cells, respectively.

15. Fill plates with 100 μl of either $OVA_{257-264}$ peptide-pulsed or unpulsed EL4 cells (see steps 12 to 14).

    Remember to add peptide-pulsed and unpulsed target cells to wells set for maximum and spontaneous release evaluation.

16. Using a centrifuge with a microtiter plate carrier, spin down cells for 10 sec at 300 $\times g$, at room temperature, to bring the cells to the bottoms of the wells.

17. Incubate the plate 5 hr at 37°C in the, 5% CO_2 incubator.

Reading test plates

18. With a multichannel pipettor, transfer 30 μl of supernatant from each well of the incubated test plates to a PerkinElmer LumaPlate and let medium evaporate overnight.

    The same row of tips can be used to transfer a full plate, starting from the bottom (more diluted row) of the test plate to the top.

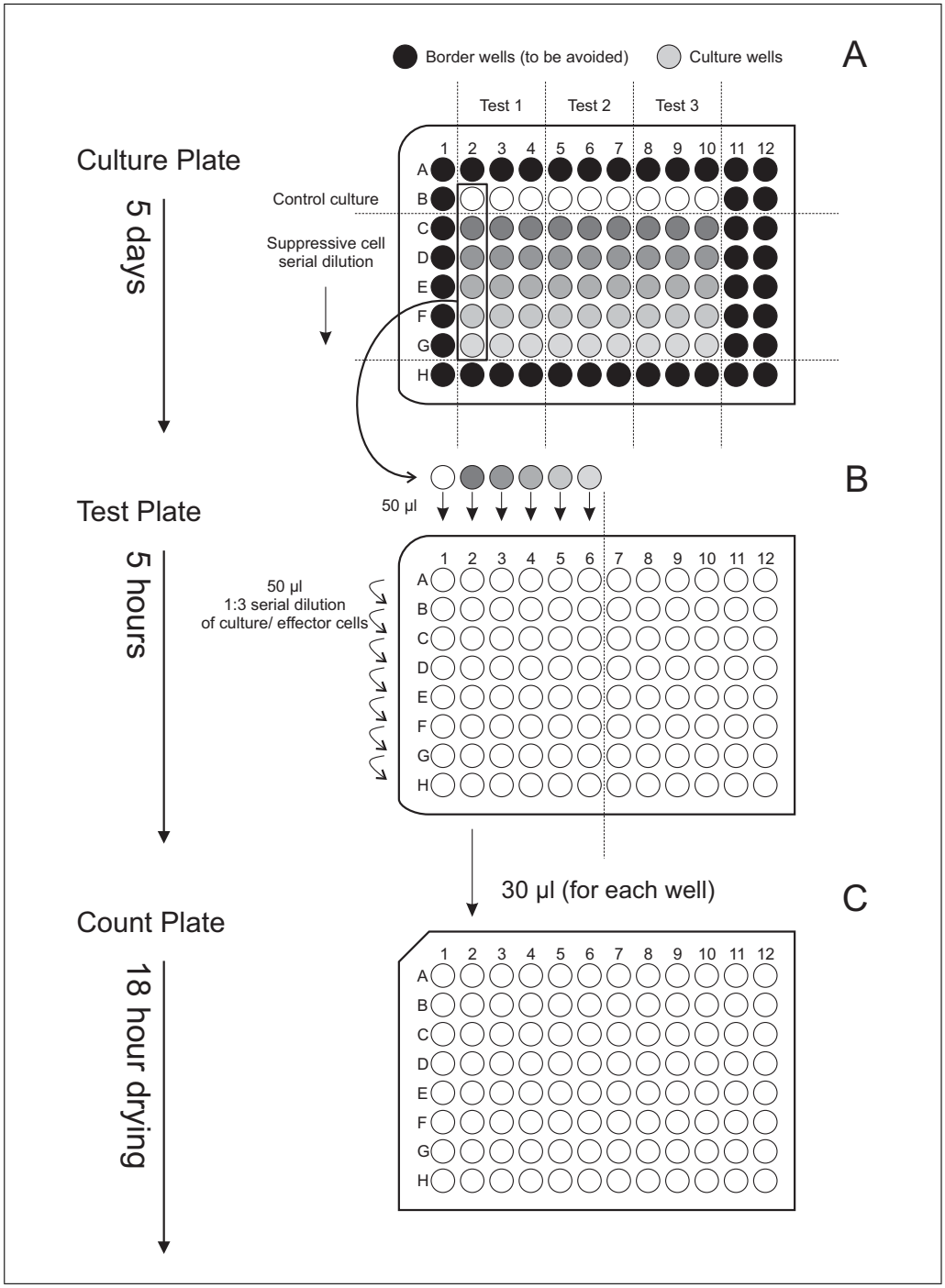

Figure 2 Sample protocol sheets for immunosuppressive assays. Design (**A**) allows measurement of the suppressive activity of 5-fold serial dilution of myeloid cells/plate. Designs (**B**) and (**C**) allow one to test the effector function of two lanes of cultures in plate A, with 8-fold serial dilutions.

19. Read LumaPlates in a TopCount scintillation counting device for 1 min/well.

Figure 2 shows a schematic representation of sample plating in the different steps of this protocol.

Interpretation of results and calculation of lytic units

^{51}Cr release due to lysis of target cells, measured by the TopCount device (see Basic Protocols 4 and 5), is expressed in counts per minute (cpm). TopCount makes it possible to set up reading protocols that can directly transform cpm values to percentage lysis,

Table 2 Common mAbs Used for Characterization of Human CD11b$^{low/-}$ BM-MDSCs and PB M-MDSCs

Epitope	Clone	Antibody type	Company	Expression[a]
CD3	UUCHT1	Mouse IgG_1, κ	Beckman Coulter	−
CD4	SK3	Mouse IgG_1, κ	BD Biosciences	−
CD8	SK1	Mouse IgG_1, κ	BD Biosciences	−
CD19	HIB19	Mouse IgG_1, κ	BD Biosciences	−
CD56	NCAM16.2	Mouse BALB/c IgG_{2b}, κ	BD Biosciences	−
CD11b	Bear1	Mouse IgG_1	Beckman Coulter	Low/−
CD16	REA423	Recombinant human IgG_1	Miltenyi Biotec	−
CD124	25463	Mouse IgG_{2a}	R&D Systems	+
CD14	M5E2	Mouse IgG_{2a}, κ	BD Biosciences	+
HLA-DR	L243	Mouse BALB/c IgG_{2a}, κ	BD Bioscience	Low/−[a]

[a]CD11b and CD16 expressions refers to BM-MDSC, while CD124, CD14, and HLA-DR expressions refer to PB M-MDSC.

and also calculate the average of triplicate measurements; calculations can otherwise be performed with a software like Excel applying the following formula:

$$\%\,\text{lysis} = \frac{\text{cpm}_{\text{experimental}} - \text{cpm}_{\text{spontaneous}}}{\text{cpm}_{\text{maximum}} - \text{cpm}_{\text{spontaneous}}} \times 100$$

Results can be displayed in a line plot with the *x* axis representing culture dilution and the *y* axis representing percentage of lysis. Nonspecific lysis, if any was detected, could be subtracted from specific lysis point by point, or otherwise could be separately displayed.

Another way to represent the results, which is more straightforward and also makes it possible to average more experiments, is the calculation of Lytic Units (L.U.). This involves determination of the culture dilution necessary to obtain a given lysis percentage, usually 30% (L.U.30) or 50% (L.U.50). Such determinations could be achieved using any software allowing for nonlinear regression. Data are initially arranged in a table with two columns, the first indicating the amount of culture applied to the test (on the basis of the dilution), the other the percentage of specific lysis (see the two far-right columns of Table3).

The amount of culture that gives a lysis of 30% can thus be determined by applying a four-parameter logistic regression, or another of the proposed models, that better fit the experimental results, obtaining a measurement of L.U.30. Normalize this number on a per culture basis. To get the number of L.U.30 contained in the culture:

$$\text{no. of L.U.}_{30} = \frac{1}{\text{L.U.}_{30}}$$

Normalize the test culture against the control culture without myeloid suppressor cells:

$$\%\ \text{L.U.}_{30} = \frac{\text{no. of L.U.}_{30}^{\text{exper}}}{\text{no. of L.U.}_{30}^{\text{ctrl}}}$$

This will give a measure of the percentage of L.U.30 in test culture compared to control culture and represent a direct measure of how much inhibition is provided by the presence of different numbers of myeloid suppressors. Amount of culture in the first column can

Table 3 Example of the Determination of the Culture Dilution Necessary to Obtain a Given Lysis Percentage[a]

Dilution number	Amount of culture	Percent lysis
1st	1.00E+00	97
2nd	3.33E-01	83
3rd	1.11E-01	80
4th	3.70E-02	60
5th	1.23E-02	31
6th	4.12E-03	15
7th	1.37E-03	7
8th	4.57E-04	4

[a]Regarding the amount of culture (see equations in Support Protocol), we assume that the initial undiluted culture is 1 and that the second well, i.e., 1:3 of the initial culture, will be 0.33 and so on. If the reference culture, without suppressive cells, produces 30% lysis of target cells at the fifth dilution, 1 liter.U.30 of the culture will be 0.0123 (1/81) of the initial culture and the number of lytic units per culture will be 1/0.0123 = 81. At the same time, if a culture containing suppressive cells produces 30% lysis of target cells at the third dilution, 1 liter.$U._{30}$ of this experimental culture will be 0.11 (1/9) of the initial culture and the number of lytic units per culture will be 1/0.11 = 9. The % $L.U._{30}$ of the experimental culture in relation to the reference culture will therefore be 9/81 × 100 = 11%.

also be reported as absolute number of cells by counting the cells in the wells of culture plates before dilution.

BASIC PROTOCOL 6

MEASURING MYELOID CELL–DEPENDENT *IN VIVO* TOLERANCE BY ADOPTIVE TRANSFER

Immune suppression can also be studied *in vivo*, and the method is certainly more challenging than the *in vitro* setup. We previously presented a method (Marigo et al., 2010; also see previous version of this unit; doi: 10.1002/0471142735.im1417s91) to evaluate *in vivo* tolerance, by which normal healthy recipients were antigen stimulated and where MDSCs were adoptively transferred. This protocol permits the evaluation of tolerance in a situation of stimulation relatively free from the multiple variables present during either tumor growth or infectious diseases. Instead, the protocol reported below is extended to tumor-bearing mice where MDSCs are expanded *in vivo*, during tumor development, and the antigen is expressed by the tumor and is cross-presented to lymphocytes. The experiment must take into account at least three groups of animals: a group of tumor-free mice, one of tumor bearing mice and a group of vaccinated tumor-bearing mice (Ugel et al., 2012).

Materials

Dendritic cell (DC) preparation started 5 days before beginning this protocol (Inaba, Swiggard, Steinman, Romani, Schuler, & Brinster, 2009)
EG7-OVA cells (ATCC no. CRL-2113^{TM}) stably expressing $OVA_{257-264}$
Serum-free RPMI 1640 medium (e.g., Invitrogen)
CD45.2$^+$C57BL/6 mice purchased from Jackson Laboratories
RPMI containing 3% FBS (see recipe)
CD45.1$^+$ transgenic OT-1 mice obtained in our animal facility by crossing the two strains purchased from Jackson Laboratories
CD8α$^+$ T cell isolation kit (Miltenyi Biotech)
RPMI containing 10% FBS (see recipe)
1 mg/ml LPS stock solution (Sigma, cat. no. L-4516)
10 mg/ml $OVA_{257-264}$ peptide stock solution (available lyophilized from JPT Peptide Technologies)

Dulbecco's phosphate-buffered saline (DPBS; Lonza BioWhittaker, cat. no. BE17-515Q)
BD Golgi Stop (BD Biosciences)
FcR blocking reagent (clone 2.4G2, ThermoFisher)
Anti–mouse CD8 (i.e., PeCy5-anti CD8a, clone 53.6.7; ThermoFisher, Catalog # 15-0081-81)
Anti–mouse CD451.1 conjugated to a brilliant fluorochrome (i.e., PE-anti CD45.1, clone A20; ThermoFisher, Catalog # 12-0453-81)
BD Cytofix/Cytoperm kit (BD Biosciences)
Anti–mouse IFN-γ (i.e., FITC-anti IFN-γ, clone XMG1.2; ThermoFisher, Catalog # 11-7311-41) or rat IgG1 as isotype control (ThermoFisher)

50-ml conical tubes
1-ml syringe
18-G and 26-G needles
10-mm culture dish
2-ml syringe
Refrigerated centrifuge
Infrared heat lamp
Mouse restraining device
100-μm nylon-mesh cell strainer (BD Biosciences)
12-ml and 4-ml round-bottom tubes
48-well culture plate
96 well U-bottom microtiter plate
Microtiter plate carrier for centrifuge
LSRII flow cytometer (BD Biosciences)
FlowJo 7.6.5 Software (TreeStar)
Red cell ACK lysis buffer (Lonza)

Additional reagents and equipment for culturing dendritic cells Inaba et al., 2009), harvesting spleens and other lymphoid organs from mice (Reeves & Reeves, 1992), counting viable cells by trypan blue exclusion (Strober, 2001), flow cytometry, injection of mice, to prepare immunosuppressive cells (Basic Protocol 1 or 2), euthanasia of mice (Reeves & Reeves, 1992), and flow cytometric IFN-γ intracellular staining

NOTE: 5 days before starting, set up a dendritic cell (DC) culture from mouse bone marrow as described in Inaba et al. (2009), which will be used to vaccinate some groups of control mice.

Day 0

1. Collect EG7-OVA suspension cell line expressing $OVA_{257-264}$ from 75-cm^2 flasks in a 50-ml conical tube.
2. Gently pipet cells up and down with a 1-ml syringe and an 18-G needle.
3. Load the syringe and tap bubbles, change the needle to a 26-G size.
4. Dilute an aliquot of suspension in trypan blue solution, and determine viable cell concentration (Strober, 2001).
5. Adjust EG7-OVA cell concentration to 10×10^6 cells/ml with serum-free RPMI and inject 100 μl (10^6 EG7 cells) subcutaneously into the flank of at least 5 mice per group of $CD45.2^+$ C57BL/6 mice.

   Also prepare uninjected controls.

Day 7

6. Collect spleens (Reeves & Reeves, 1992) from OT-1CD45.1$^+$ mice in a small volume of RPMI containing 3% FBS under sterile conditions.
7. Place spleens in a 10-mm culture dish with a small volume of RPMI containing 3% FBS and gently disaggregate using the plunger of a 2-ml syringe.
8. Add 5 ml of RPMI containing 3% FBS to cells and collect the cells in a 50-ml conical tube by gently pipetting. Repeat until the spleen pulp is completely disaggregated.
9. Centrifuge the suspension 6 min at 300 $\times g$, 4°C, and discard the supernatant.
10. Dilute an aliquot of suspension in trypan blue solution, and determine viable cell concentration (Strober, 2001).
11. Prepare CD8$^+$ T cells by means of CD8α^+ T cell isolation kit from Miltenyi Biotech.

    Alternatively, the whole spleen of the OT-1 CD41.1$^+$ mouse could be used without any sorting. Determine abundance of CD8$^+$ T cells by means of cytometric staining with anti-CD8 and anti-$V_\alpha 2$ $V_\beta 5.1/5.2$, and collect an amount of total splenocytes that corresponds to 5$\times$ 10^6 CD8$^+$/$V_\alpha 2$ $V_\beta 5.1/5.2$ cells.

12. Wash enriched CD8$^+$ T cells with at least 10 ml fresh serum-free RPMI 1640 medium twice, each time by centrifuging cells 6 min at 300 $\times g$, 4°C, and carefully discarding the supernatant.
13. Resuspend cells in a small volume of serum-free RPMI.
14. Dilute an aliquot of suspension in trypan blue solution, and determine viable cell concentration (Strober, 2001).
15. Adjust concentration to 25$\times$ 10^6 cells/ml with serum-free RPMI, place cells in a 50-ml conical tube, and put the tube on ice.
16. Warm CD45.2$^+$C57BL/6 mice under an infrared heat lamp for about 10 min.

    Do not overheat mice, and do not exceed the correct number of mice per cage during warming steps.

17. Gently pipet cells up and down with a 1-ml syringe and a 18-G needle.
18. Load the syringe, change the needle to a 26-G size, and tap bubbles out.

    Take care to eliminate bubbles thoroughly; otherwise, mice might die from air embolism.

19. Place the mouse in the restraining device and inject 200 µl of cell suspension (5 $\times$ 10^6 cells/mouse in the tail vein).

    For randomization purposes, label mice before cell injection and keep them together in the same cage.

Day 8

DC maturation

20. Gently remove supernatant medium from the DC culture. Take care to avoid scraping the plate while removing the supernatant.
21. Feed cells with 1 ml of 10RPMI/10% FBS medium containing 1 µg/ml LPS (add from 1 mg/ml stock) and 2 µg/ml $OVA_{257\text{-}264}$ peptide (add from 10 mg/ml stock).
22. Incubate the cell culture at 37°C, in a 5% CO_2 incubator.

Day 9

DC preparation

23. Gently remove cells from plate culture, washing wells several times with DPBS, and transfer cell suspension to a 50-ml conical tube.
24. Centrifuge the suspension 6 min at 300 ×*g*, 4°C, and discard the supernatant.
25. Wash cell pellet by adding 10 ml serum-free RPMI; repeat steps 19 and 20 twice.
26. Resuspend mature DC preparation in a small volume of serum-free RPMI.
27. Dilute an aliquot of suspension in trypan blue solution, and determine viable cell concentration (Strober, 2001).
28. Adjust concentration to 10×10^6 cells/ml with serum-free RPMI, place cells in a 50-ml conical tube, and put them on ice.
29. Check mature DC preparation by flow cytometry for CD11c expression and MHC II and CD80/CD86 up-regulation relative to immature DCs, as described in Inaba et al., (2009).

Perform DC vaccination

This procedure is used as a control to make sure that the *in vivo* tolerance is maintained in the presence of further stimulation of $CD8^+$ T lymphocytes.

30. Gently pipet mature DCs up and down with a 1-ml syringe and 18-G needle.
31. Load the syringe, change the needle to a 26-G size, and tap to remove bubbles.
32. Inoculate 100 μl of DC suspension subcutaneously into each mouse flank.

Day 14

Lymph node cell preparation

33. Euthanize mice and collect inguinal, axillary, and brachial lymph nodes (Reeves & Reeves, 1992) from both sides in a small volume of RPMI/3% FBS in a 48-well plate, separately for each mouse.
34. Put lymph nodes on a nylon mesh and disaggregate mechanically with the plunger from a 2-ml syringe.
35. Let disaggregated cells pass through the nylon mesh, washing with 5 ml of RPMI containing 3% FBS. Repeat steps 34 and 35 for every lymph node until complete disaggregation is achieved.
36. Centrifuge the suspension 6 min at 300 ×*g*, 4°C, and discard the supernatant.

    Aspiration of supernatant with a glass Pasteur pipet attached to a vacuum pump minimizes cell loss.

37. Add 10 ml of RPMI/10% FBS to wash pellet.
38. Centrifuge the suspension 6 min at 300 ×g, 4°C, and discard the supernatant.
39. Resuspend cell pellet in a small volume of RPMI/10% FBS and filter through a nylon mesh into a 12-ml round-bottom tube.
40. Dilute an aliquot of suspension in trypan blue solution, and determine viable cell concentration (Strober, 2001).
41. Adjust concentration to 5×10^6 cells/ml with RPMI/10% FBS and place cells on ice.

Lymph node cell staining

42. Plate lymph node cell suspension in 100 μl of RPMI/10% FBS per well of a 96-well U-bottom microtiter plate.

    Usually, 12 replicates, subdivided into 6 wells for specific-peptide stimulation and 6 wells for either unstimulated or unrelated peptide-stimulated control culture, should be sufficient.

43. Add 100 μl of RPMI/10% FBS containing 2 μg/ml $OVA_{257-264}$ peptide (add from 10 mg/ml stock) to half of the wells and 100 μl of RPMI/10% FBS without peptide (or unrelated peptide) to the other half.

    IL-2 could be also added, at a final concentration of 20 U/ml, to each well to increase the signal in subsequent flow cytometric analysis.

44. Incubate plate at 37°C, in a 5% CO_2 incubator 6 to 8 hr before Golgi stop treatment.

    Ex vivo peptide stimulation must be carried out for at least 18 hr, and BD Golgi Stop treatment should not exceed 8 to 12 hr. It may be necessary to initially test and coordinate these concomitant steps in the initial setup experiments.

BD Golgi stop incubation

45. Prepare sufficient 11× BD Golgi Stop stock solution according to the manufacturer's instructions in RPMI/10% FBS and distribute among the wells of the plate at 20 μl/well.

    The BD Golgi Stop datasheet suggests using 4 μl for 6 ml (final volume); hence, to fill an entire plate, prepare 1.986 ml of RPMI/10% FBS medium and add 14.7 μl BD Golgi Stop.

46. Incubate 8 to 12 hr at 37°C, in a 5% CO_2 incubator.

Day 15

47. Transfer the cells to new 4-ml round-bottom tubes.

48. Wash samples once in DPBS, centrifuge the suspension 6 min at 300 ×g, 4°C, and discard the supernatant.

49. Resuspend cell pellets in 200 μl DPBS and plate 100 μl of cell suspension in duplicate on a new 96-well U-bottom microtiter plate.

    It is necessary to split every sample into two aliquots in order to stain them with IFN-γ-specific antibody and matched isotype control. Remember to save a small aliquot of each sample and pool together. These extra samples will be used to perform single staining controls.

50. Centrifuge the plate in a microtiter plate carrier 2 min at 300 ×g, 4°C, and discard the supernatant by decanting.

51. Incubate the cells with 2 μg of FcR blocking reagent (2.4G2 mAb), in a volume of 50 μl per well of DPBS for 20 min at room temperature.

    It may be necessary to titrate the antibody to determine the appropriate amount.

52. Stain cells with 50 μl DPBS per well containing anti-CD8-PeCy5 (0.5 μl) and anti-CD45.1-PE (0.1 μl) for 20 min at 4°C.

    Remember to include appropriate single-staining controls. It may be necessary to titrate the antibodies.

53. Wash samples once with 100 μl/well DPBS, centrifuge the suspension 6 min at 300 ×g, 4°C, and discard the supernatant. Repeat washing with 200 μl DPBS, centrifuging under the same conditions.

54. Add 100 μl/sample Fixation/Permeabilization solution (from BD Cytofix/Cytoperm kit), and gently resuspend with a multichannel pipettor. Incubate 20 min at 4°C.

55. Wash samples once with 100 μl of 1× BD Perm/Wash buffer (from BD Cytofix/Cytoperm kit), centrifuge the plate 2 min at 300 ×g, 4°C, and discard the supernatant. Repeat washing with 100 μl of 1× BD Perm/Wash buffer and decant the supernatants.

56. Stain cells with 50 μl BD Perm/Wash buffer containing in our case rat anti–mouse IFN-γ FITC (0.75 μl/well) or rat IgG1 FITC isotype control (0.75 μl/well) for 30 min at 4°C.

    Remember to include appropriate single staining controls. It may be necessary to titrate antibody amount.

57. Wash samples once with 100 μl 1× BD Perm/Wash buffer, centrifuge the plate 2 min at 300 ×g, 4°C, and discard the supernatant by decanting. Repeat washing with 100 μl 1× BD Perm/Wash buffer, and centrifuge again as before.

    In order to detect intracellular IFN-γ, gate cells first by their morphology, and then collect as many events as possible in the $CD8^+$/$CD45.1^+$ gate. After the acquisition, proceed with data analysis. For each sample, either stained with specific antibody or isotype control, provide the same gating schema, taking care to adapt gates for minor changes. Gate cells by morphology and identify $CD8^+CD45.1^+$ populations. Inside this gate, set a baseline gate on negative IFN-γ staining using the isotype control, and copy this gate to the sample stained with the specific antibody, in order to determine the percentage of IFN–γ-releasing lymphocytes.

IDENTIFICATION AND SORTING OF HUMAN M-MDSC CELLS FROM PERIPHERAL BLOOD (PB) OF CANCER PATIENTS TO MEASURE THEIR IMMUNOSUPPRESSIVE ACTIVITY

BASIC PROTOCOL 7

The aim of this protocol is to obtain $CD14^+$ cells enriched for M-MDSCs from PB of cancer patients, and to preserve their functional activity in order to use them for *in vitro* functional assays. PBMCs are obtained after centrifugation and sedimentation of whole blood on a Ficoll gradient (see Mandruzzato et al., 2009).

The selection of fluorochrome-labeled antibodies to identify M-MDSCs has to be performed based on optimal signal strength and minimal spectral overlap; the optimal concentration of each antibody must be evaluated in titration experiments.

Materials

Anticoagulated whole blood obtained from cancer patients
Sorting buffer (see recipe)
Human FcR blocking reagent (Miltenyi Biotec)
CD14 FITC (clone M5E2, BD Biosciences)
HLA-DR APC (clone L243, BD Biosciences)
Complete IMDM medium (see recipe)

15- and 50-ml polypropylene conical tubes
Refrigerated centrifuge
12 × 75–mm polypropylene tubes pre-coated for at least 1 hr with heat-inactivated fetal bovine serum (FBS)

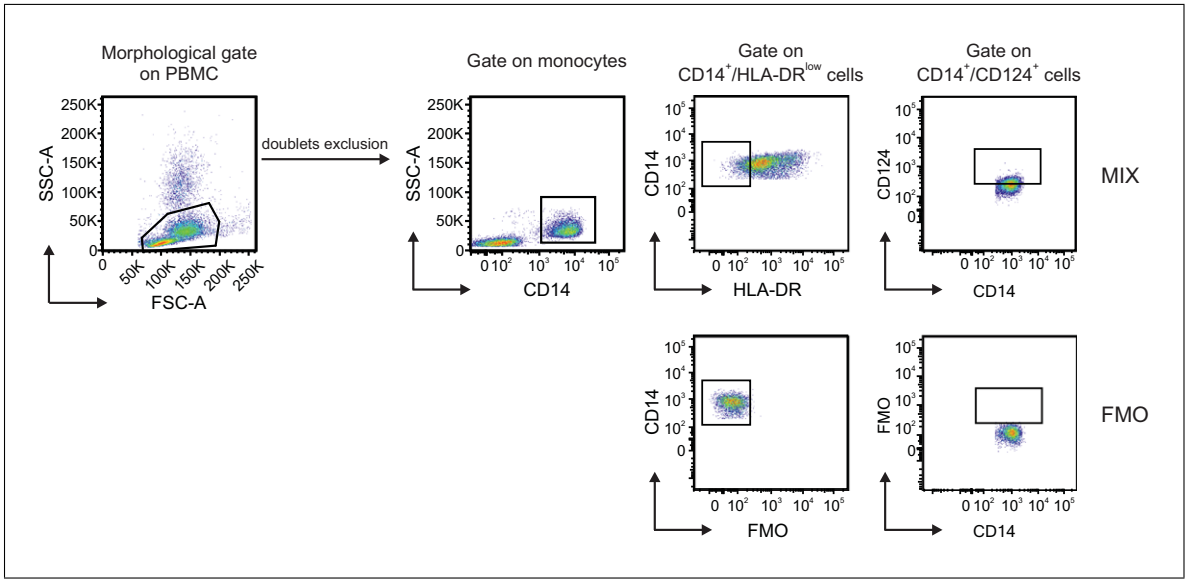

Figure 3 Gating strategy for the definition of M-MDSCs in human PBMCs. PBMCs were labeled with anti-CD14, anti-HLA-DR, and anti-CD124, and then analyzed by flow cytometry. After a morphological gate on PBMCs and doublets exclusion, monocytes were gated as $CD14^+$ cells, and then the gate for HLA-DR^{low} cells and $CD124^+$ cells was set based on the FMO control.

Additional reagents and equipment for isolating PBMC from whole blood (Mandruzzato et al., 2009) and counting viable cells (Strober, 2001)

NOTE: All the procedures, including sorting, are performed at 4°C, to avoid cell loss and adherence to the plastic.

Determination of M-MDSCs expression level on PBMCs

The following antibodies were used to characterize blood M-MDSCs in melanoma (Damuzzo et al., 2016) and meningioma (Pinton et al., 2018) patients: CD14-FITC, HLA-DR-APC and CD124 PE. As the down-regulation of HLA-DR and CD124 expression are important parameters to determine two subsets of M-MDSCs, it is important to use FMO controls to define HLA-DR and CD124 gates as shown in Figure 3. The protocol for PB-M MDSC staining is described below:

1. Isolate PBMCs from whole blood of cancer patients (see Mandruzzato et al., 2009). Collect cells in 12 × 75–mm tubes for FACS analysis and wash them with sorting buffer. Centrifuge 6 min at 300 × *g*, 4°C, and discard the supernatant.
2. After the centrifugation, tubes are subsequently incubated with Fc-receptor blocking reagent diluted 1/25 at 4°C for 10 min.
3. Add the mixture of antibodies (CD14/HLA-DR/CD124) to the tubes in a final volume of 100 µl sorting buffer and incubate at 4°C for 20 min.

   Common mAbs used for characterization of human $CD11b^{low/-}$ BM-MDSCs and PB M-MDSCs are listed in Table 2.

4. At the end of the incubation time, wash cells with sorting buffer. Centrifuge the suspension for 6 min at 300 × *g*, 4°C, and discard the supernatant.
5. Resuspend samples in 250 µl of sorting buffer, and proceed with flow cytometric acquisition and analysis.

Flow cytometry analysis of PB M-MDSCs

An example of gating strategy, analyzed with FlowJo 7.6.5 software, is shown in Figure 3. The expression of HLA-DR and CD124 markers is evaluated on $CD14^+$ whole monocytes cells gated on singlet cells and on the basis of FMO signal.

Separation of PB M-MDSCs

6. Stain whole PBMCs (isolated from blood of cancer patients; see step 1) with anti-CD14 and anti-HLA-DR antibodies using the same staining protocol described above. Specifically, for FACS analysis, cells are stained in 12 × 75–mm polypropylene tubes pre-coated for at least 1 hr with heat-inactivated fetal bovine serum (FBS).

7. After labeling, filter cells through a 100-µm cell strainer and isolate $CD14^+$/HLA-DR^{low} cells by FACS sorting.

   Sorting conditions (nozzle, pressure, voltage at the deflection plates, cell rate) should be set up in order to assure maximal monocyte viability after separation. To this purpose, the staining of monocytes with a viability dye 24 and 48 hr after cell sorting is strongly recommended in order to ascertain that a lack in the immunosuppressive activity is real and not influenced by cell death.

8. After the separation, wash and resuspend PB M-MDSCs in 10 ml of complete IMDM. Properly dilute aliquots of the single-cell suspension with trypan blue solution and estimate the number of viable cells (Strober, 2001).

   FACS sorting of $CD124^+/CD14^+$ M-MDSCs is challenging due to low intensity and unimodal expression of CD124. Therefore, in patients with an expansion of $CD124^+/CD14^+$M-MDSCs, total monocyte fraction can be isolated by immunomagnetic sorting using anti-CD14 microbeads (Miltenyi Biotec) and the manufacturer's protocol. In this case, it would be better to test more than one monocyte: T cell ratio, in order to avoid a lack of immunosuppressive activity due to a high dilution of M-MDSCs in the culture.

HUMAN BM-MDSC GENERATION

BASIC PROTOCOL 8

MDSCs can also be expanded *in vitro* starting from bone marrow (BM) cell aspirate from healthy donors, as previously described (Marigo et al., 2010), using the protocol described below. Specifically, we culture BM cells depleted of T, B and NK cells for 4 days with recombinant human (rh) G-CSF and GM-CSF. At the end of the culture, a heterogeneous cell culture is obtained, enriched for BM-MDSCs that are contained in the most immature fraction (Solito et al., 2011b). To purify BM-MDSCs, the cell culture is depleted of the most mature myeloid cells ($CD11b^+$) to obtain $CD11b^{low/-}$ BM-MDSCs.

Materials

Bone marrow aspirates (see information in step 1)
10× red blood cell lysis buffer (see recipe)
Complete IMDM (see recipe; FBS should be previously tested in order to guarantee a proper proliferation of BM-MDSCs)
Sorting buffer (see recipe)
Human CD3 Microbeads (Miltenyi Biotec)
Human CD19 Microbeads (Miltenyi Biotec)
Human CD56 Microbeads (Miltenyi Biotec)
Human CD11b Microbeads (Miltenyi Biotec)
Human FcR blocking reagent (Miltenyi Biotec Catalog #130-059-901)
CD11b-PE (clone Bear1, Beckman Coulter)
CD16-FITC (clone REA423, Miltenyi Biotec)

Anti–CD3 mAb (optional)
Anti–CD19 mAb (optional)
Anti–CD56 mAb (optional)

100-μm nylon-mesh cell strainer (BD Biosciences)
15- and 50-ml polypropylene conical tubes
Refrigerated centrifuge
LD column (Miltenyi Biotec)
MACS separator (Miltenyi Biotec)
LSRII flow cytometer (BD Biosciences)
FlowJo 7.6.5 Software (TreeStar)
24-well tissue culture plate
12 × 75–mm tubes

Additional reagents and equipment for counting cells (Strober, 2001)

Isolation of BM-MDSCs

1. Samples are obtained from BM aspiration both from pediatric patients with normal cytologic characteristics and from adult patients undergoing orthopedic implants. Blood aspirates are collected in tubes containing K_2EDTA for BM from adults and sodium citrate for pediatric BM. Samples are filtered through a 100-μm cell strainer into 50-ml polypropylene tubes, and red blood cells are removed using a hypotonic solution of ammonium chloride (red blood cell lysis buffer).

   The amount of the lysis buffer and the incubation time may be optimized on the basis of BM source. As an alternative source to obtain MDSCs, it is possible to use cultured stem cells ($CD34^+$ cells), which are available from different companies (e.g., AllCell). In our experience, the in vitro differentiation induces the acquisition of immunosuppressive activity and MDSC-associated markers in the cell population that down-regulates the CD34 marker.

2. Wash the cells with complete IMDM. Centrifuge 6 min at 300 × *g*, 4°C, and discard the supernatant.

3. Resuspend cells in 10 ml of complete IMDM.

4. Dilute an aliquot of suspension in trypan blue solution, and determine viable cell concentration (Strober, 2001).

5. Incubate 10^7 BM cells in 40 μl of sorting buffer with a mix containing 20 μl of CD3 microbeads, 20 μl of CD19 microbeads, and 20 μl of CD56 microbeads at 4°C for 15 min.

   Proportionally scale up the amount of sorting and staining buffer and microbeads on the basis of total number of cells that need to be depleted. For less than 10^7 BM cells keep the same amount of reagents.

6. Wash the cells with sorting buffer, centrifuge 6 min at 300 × *g*, 4°C, aspirate the supernatant and resuspend the pellet in 500 μl of sorting buffer, then proceed with magnetic separation as described below. For more details, see the protocol from Miltenyi Biotec for magnetic separation with LD columns.

7. Place LD column in the magnetic field of a suitable MACS separator and prepare the column by rinsing it with 2 ml of sorting buffer.

8. Apply cell suspension onto the column; collect unlabeled cells that pass through and wash the column twice with 1 ml of sorting buffer.

9. Wash cells with 10 ml complete IMDM.

10. Resuspend cells in 10 ml of complete IMDM and dilute an aliquot of suspension in trypan blue solution, and determine viable cell concentration (Strober, 2001).

11. Take an aliquot of cell suspension and check the enrichment of the myeloid subsets by flow cytometric analysis.

    Assess the loss of the lymphocyte region from depleted BM-myeloid cells. The assessment of the morphological features of the depleted samples through flow cytometry may be sufficient for many applications. For transcriptomic analysis, stain depleted myeloid cells with a mixture of antibodies containing anti-CD3/CD19/CD56. For the details of the staining protocol, see Basic Protocol 7.

12. Plate cells (2×10^6 cells per well) into a 24-well tissue culture plate in complete IMDM in the presence of rhG-CSF and rhGM-CSF (40 ng/ml) for 4 days, at 37°C with 8% CO_2.

    During this period, it may be useful to observe cells, in order to monitor their growth. Usually it is not necessary to split cultures unless the growth (signaled by medium turning yellow) is excessive. The presence of small areas rich in clusters of myeloid cells with granular aspect indicates that G-CSF/GM-CSF treatment is supporting myeloid survival and proliferation, since this morphology is not present in untreated cultures.

Isolation of CD11b$^{low/-}$ BM-MDSCs

13. After 4 days, harvest cell cultures in 50-ml polypropylene tubes and wash them with complete IMDM. Dilute an aliquot of the single-cell suspension with trypan blue solution, and estimate the number of viable cells (Strober, 2001).

    Usually, the number of viable BM-MDSCs recovered is around 60% of the initial number of plated cells, while recovery of CD11b$^{low/-}$ cells is 6% to 7% of total BM-MDSCs after G-CSF/GM-CSF treatment.

14. Incubate 10^7cells in 80 µl of sorting buffer with 20 µl of CD11b microbeads at 4°C for 15 min.

    Scale up the amount of sorting buffer and CD11b microbeads on the basis of total number of cells that need to be depleted. For less than 10^7 BM cells, keep the same amount of reagents.

15. Wash the cells with sorting buffer, centrifuge 6 min at $300 \times g$, 4°C, aspirate the supernatant, and resuspend the pellet in 500 µl of sorting and straining buffer. Perform magnetic separation as described below. For more details, see the protocol from Miltenyi Biotec for magnetic separation with LD columns.

16. Place LD column in the magnetic field of a suitable MACS separator and prepare the column by rinsing it with 2 ml of sorting buffer.

17. Apply cell suspension onto the column; collect unlabeled cells that pass through and wash the column twice with 1 ml of sorting buffer.

18. Wash cells with complete IMDM.

19. Resuspend cells in 10 ml of complete IMDM and dilute an aliquot of suspension in trypan blue solution, and determine viable cell concentration (Strober, 2001).

    The number of viable cells recovered depends on the cellularity and maturation profile of the BM, but the range is usually between 10^5 and 5×10^6 CD11b$^{low/-}$ cells.

20. Check the loss of the most mature myeloid cells from the cell culture by flow cytometry (Fig. 4). Collect cells in 12 × 75–mm tubes for FACS analysis and wash with sorting buffer. Centrifuge 6 min at $300 \times g$, 4°C, and discard the supernatant.

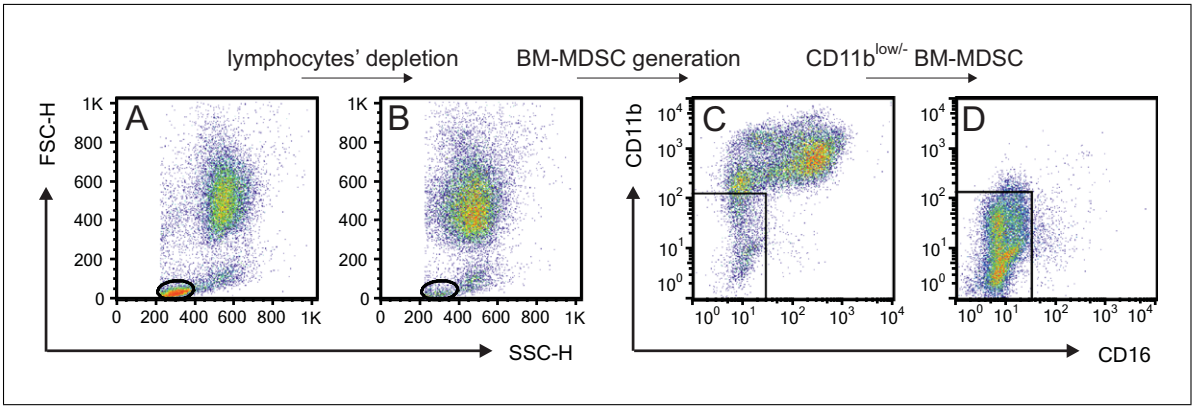

Figure 4 MDSC generation and separation from human BM cells cultured *in vitro*. The first two plots show the morphology of human BM cells before (**A**) and after (**B**) lymphocyte depletion. Lymphocyte-depleted BM cells are then cultured for 4 days with 40 ng/ml G-CSF and GM-CSF to generate BM-MDSCs. The maturation profile is characterized by the CD16 and CD11b markers as shown in (**C**). The most immature subset of BM-MDSCs, identified as $CD11b^{low/-}/CD16^{-}$, is then separated by immunomagnetic sorting using anti-CD11b microbeads (**D**).

21. Incubate the tubes with Fc-receptor blocking reagent diluted 1/25 at 4°C for 10 min. Add the mixture of antibodies (CD11b/CD16) to the tubes and incubate at 4°C for 20 min. Adjust the volume of staining mix to 100 µl with sorting buffer.

    Common mAbs used for characterization of human $CD11b^{low/-}$ BM-MDSCs and PB M-MDSCs are listed in Table 2.

22. At the end of the incubation time, wash the cells with sorting buffer. Centrifuge the suspension 6 min at 300 × *g*, 4°C, and discard the supernatant.

23. Resuspend the cells in 250 µl of sorting buffer and perform flow cytometric acquisition and analysis.

Flow cytometry analysis of $CD11b^{low/-}$ BM-MDSCs

Figure 4 shows an example of lymphocyte depletion and the gating strategy for $CD11b^{low/-}$ BM-MDSC isolation, analyzed with FlowJo 7.6.5 Software.

BASIC PROTOCOL 9

EVALUATION OF THE SUPPRESSION INDUCED BY HUMAN MDSC ON THE PROLIFERATION OF T CELLS STIMULATED BY ANTI-CD3/ANTI-CD28 AND MEASURED BY CELL TRACE DILUTION

Immune suppression exerted by MDSCs on activated T cells can be measured in terms of inhibition of T cell proliferation. The method can be applied to complex experiments for large-scale screenings, and takes into consideration a basic property of myeloid-dependent suppression: the ability to induce proliferative arrest of actively dividing cells.

Materials

Anti-CD3 (0.6 to 5 µg/ml) clone OKT3, obtained after expansion and purification of a commercially available hybridoma
Dulbecco's phosphate-buffered saline (DPBS without Ca and Mg; Lonza BioWhittaker, cat. no. BE17-515Q)
Complete IMDM medium (see recipe)
Immunosuppressive (myeloid) cells, e.g., PB M-MDSCs or $CD11b^{low/-}$ BM-MDSCs (Basic Protocol 7 and 8)
5 µg/ml anti-CD28, clone CD28.2 BioLegend, Catalog #302923)
PBMC from healthy donors (see Mandruzzato et al., 2009, for isolation technique)
CellTrace™ Violet Cell Proliferation Kit (Molecular Probes)

Fetal bovine serum (FBS; Gibco or Sigma-Aldrich); FBS used for functional assay should be tested to be sure that immunosuppression is detectable and not overcome by an excessive T cell proliferation
Human FcR blocking reagent (Miltenyi Biotec Catalog #130-059-901)
Sorting buffer (see recipe)
CD3-conjugated to a brilliant fluorochrome (i.e., PC7-anti CD3, clone UCHT1, Catalog # 737657, Beckman Coulter)

24-, 96-, or 384-well flat-bottom microtiter plate
50-ml polypropylene conical tubes (BD Falcon)
Refrigerated centrifuge
TruCount tubes (Becton Dickinson)
LSRII flow cytometer (BD Biosciences)
FlowJo 7.6.5 Software (TreeStar)

Additional reagents and equipment to prepare immunosuppressive cells (Basic Protocol 7 and 8), and to count viable cells by trypan blue exclusion (Strober, 2001)

Day 0: Culture plate coating

1. Prepare sufficient coating buffer with anti-CD3 (0.6 to 5 μg/ml final concentration in DPBS).

   Antibody concentration may be optimized on the basis of stock and supplier.

   Usually, we prefer to perform titrations by testing four or five concentrations of anti-CD3 mAb. As an example, it is possible to monitor T cell proliferation by culturing the cells for 4 days in the presence of 5, 2.5, 1.2 and 0.6 μg/ml of anti-CD3. Additionally, it is useful to perform these titration experiments in the presence of suppressive cells, in order to be sure to choose the concentration of anti-CD3 that allows a good proliferation of $CD3^+$ cells and at the same time makes it possible to appraise suppression of T cell proliferation.

2. Fill 96-well flat-bottom microtiter plates with 200 μl/well of the coating buffer using a multichannel pipettor. Alternatively, fill 384-well flat-bottom microtiter plates with 80 μl/well coating buffer with a multichannel pipettor. Fill an equal number of wells with 200 μl/well or 80 μl/well of DPBS without antibodies, for background proliferation measurement.

   Also, remember to fill extra wells for appropriate controls based on the experimental setup, e.g., suppressive cells only.

3. Incubate the plate overnight at 4°C or alternatively at 37°C for 1 hr.

4. Fill every well with 200 μl/well (96-well plate) or 80 μl/well (384-well plate) of IMDM medium using a multichannel pipettor and empty the plate by inverting it with a rapid movement. Repeat at least two times, to wash the wells extensively.

   Fill the plate from the top of the well to avoid scratching the surface of the well, which might alter the antibody coating.

5. Incubate the plate for 1 hr with 200 μl/well or 80 μl/well of complete IMDM medium containing 10% FBS. After incubation and two washes of the plate with medium, the plate is ready for the assay.

Day 1: Cell plating

Plate immunosuppressive cells

6. Prepare PB- derived M-MDSCs (Basic Protocol 7) or $CD11b^{low/-}$ BM-MDSCs (Basic Protocol 8) or other strategies of enrichment. Wash the cells with complete medium.

The supernatant should be aspirated from the top; be careful to remove as much supernatant as possible without disturbing the cell pellet.

7. After counting cells with trypan blue (Strober, 2001), resuspend them in a suitable volume of 150 μM arginine RPMI containing 10% FBS to adjust cell concentration.

   The concentration will depend upon the design of the experiment. A good range of MDSCs is 50% of the total culture cellularity. If 1× 10^5 anti-CD3/CD28 PBMCs are used in a 96-well plate, MDSC concentration can be adjusted to 2× 10^6/ml, so that 1× 10^5 cells will be plated in 50 μl (50% of total cultured cells). Alternatively, 0.25 × 10^5 anti-CD3/CD28 PBMCs can be used in 384-well plate, and MDSC concentration can be adjusted to 1.25× 10^6/ml, so that 0.25× 10^5 cells will be plated in 20 μl (50% of total cultured cells).

8. Work in sterility, empty the 96-well microtiter plate or 384-well microplate (from step 5) by inverting it with a rapid movement.

9. Immediately plate suppressive cells in triplicate or duplicate for specific and background proliferation in 50 μl/well (96-well plate) or 20 μl/well (384-well plate), and place the microtiter plate in a 37°C, 5% CO_2 incubator.

   When organizing the distribution of samples, avoid using the outer wells, because these wells are more susceptible to evaporation. Outer wells can be filled with sterile DPBS or medium. Remember to fill at least three wells with 10% medium without suppressive cells; these wells will be used as control cultures for the determination of background proliferation. Multiply these control wells for the different treatments according to the experimental setup, e.g., different inhibitors and drugs to be tested.

Prepare responder PBMCs

10. Thaw PBMCs of healthy donors in complete IMDM medium, under sterile conditions and dilute an aliquot of suspension in trypan blue solution, and determine viable cell concentration (Strober, 2001).

11. Adjust the concentration of PBMCs to 2 × 10^7 cells/ml in DPBS and the concentration of CellTrace to 2× in DPBS; quickly mix equal volumes of PBMCs and CellTrace and incubate for 5 min at 37°C. Add 1/5 of the total volume of FBS, centrifuge 6 min at 300 ×*g*, 4°C, and plate stained PBMCs at a concentration of 3× 10^6 cells/well in a 24-well plate with complete IMDM medium containing 10% FBS.

    The concentration of CellTrace depends on the stock of the reagent and the sensitivity of cells used for the staining, and the reagent should be titrated upon arrival. Usually a range of 0.5 to 2 μM is used.

12. Incubate CellTrace$^+$ PBMCs for at least 1 hr in incubator at 37°C, and check the incorporation by flow cytometry.

    To evaluate CellTrace incorporation, the control of autofluorescence value of unstained PBMCs should be included to evaluate whether the difference of emission signals among unstained PBMCs and CellTrace$^+$ PBMCs is enough to quantify a sustained proliferation, represented by a strong dilution of CellTrace signal.

13. Harvest PBMCs, count them (Strober, 2001), adjust their concentration at 2 × 10^6 cells/ml for a 96-well plate or 1.25× 10^6 cells/ml for a 384-well plate in 150 μM arginine RPMI containing 10% FBS. Plate them in triplicate or duplicate for specific and background proliferation in 100 μl/well (96-well plate) or 20 μl/well (384-well plate).

    Pay attention to the cell distribution scheme. PBMCs will proliferate upon anti-CD3/anti-CD28 stimulation, and their proliferation will be inhibited by immunosuppressive cells; therefore, PBMCs should be distributed in previously anti-CD3-coated wells, either with

or without immunosuppressive cells, as test and reference wells, respectively. Reference wells will contain 1× 10^5 (96-well plate) or 0.25×10^5 anti-CD3/CD28-activated PBMCs (384-well plate). The proliferative behavior of MDSCs alone under these conditions should also be tested; therefore, MDSCs will be cultured with additional 100 µl (96-well plate) or 40 µl (384-well plate) of medium instead of anti-CD3/CD28-activated PBMCs.

14. Add 1 µl/well (96-well plate) or 0.4 µl/well (384-well plate) of soluble anti-CD28 (5 µg/ml). Adjust the volume of each well to 200 µl (96-well plate) or 80 µl (384-well plate), and place the microtiter plate in a 37°C, 5% CO_2 incubator for four days.

Day 4

Culture harvesting

15. Pool triplicates or duplicates in new 4-ml round-bottom tubes, wash samples once with sorting and staining buffer, centrifuge the suspension 6 min at 300 ×*g*, 4°C, and discard the supernatant.

16. Block nonspecific binding with 25 µl FcR blocking reagent for 10 min at 4°C.

17. Stain cells with anti-CD3 (see Table 2) for 20 min at 4°C; in addition, anti-CD4 and CD8 can be added to antibody mix together with anti-CD3 mAb in the same tube, in order to analyze immunosuppression among CD4 and CD8 subsets. Adjust the volume of staining mix to 100 µl with sorting and staining Buffer.

The optimal concentration of CD3, CD4, and CD8 must be evaluated in titration experiments, by analyzing the positive and negative signal of the antibody staining after using seven different concentrations (starting from twice the concentration recommended by the datasheet, dilute 1:2 six times). Remember to include appropriate single-staining controls. The use of anti-CD3 conjugated to a brilliant fluorochrome is recommended in order to clearly distinguish proliferating T cells (that have diluted CellTrace signal) from CellTrace-negative myeloid cells that often present an autofluorescence higher than that of T cells.

18. Wash samples once with sorting and staining buffer, centrifuge the suspension for 6 min at 300 ×*g*, 4°C, and discard the supernatant.

19. Resuspend samples in 250 µl DPBS, transfer the mix in TruCount tubes, gently vortex them, and proceed with flow cytometric acquisition and analysis.

Flow cytometry analysis

20. Perform flow cytometry acquisition and analysis.

To detect $CD3^+$ proliferating T cells, gate cells first by morphology, and then collect a sufficient number of events in the $CD3^+$/$CellTrace^+$ gate. After the acquisition, proceed with data analysis. Gate cells on morphology and identify $CD3^+CellTrace^+$ population. Inside this gate, set a baseline gate on the histograms of CellTrace signal of unstimulated PBMC control, and copy this gate to the stimulated PBMCs cultured in the presence or absence of M-MDSCs, in order to determine the percentage of proliferating T lymphocytes. With FlowJo software, it is possible to model proliferation data; FlowJo presents a graphical display as well as information about each generation in the subset. The proliferation platform also provides information about the fraction of cells from the original population that have divided, and the number of times these cells have divided. In addition, the FlowJo Proliferation Platform draws gates that separate each generation.

21. Proliferation of T cells is evaluated by assessing the signal of CellTrace on $CD3^+$ cells, and considering as proliferating the cells present from generation G2 onwards (see Fig. 5 panel A), or calculating the absolute number of $CD3^+$cells in each sample using TruCount tubes. In both cases, data are normalized assuming the proliferation of T cells cultured alone as 100%.

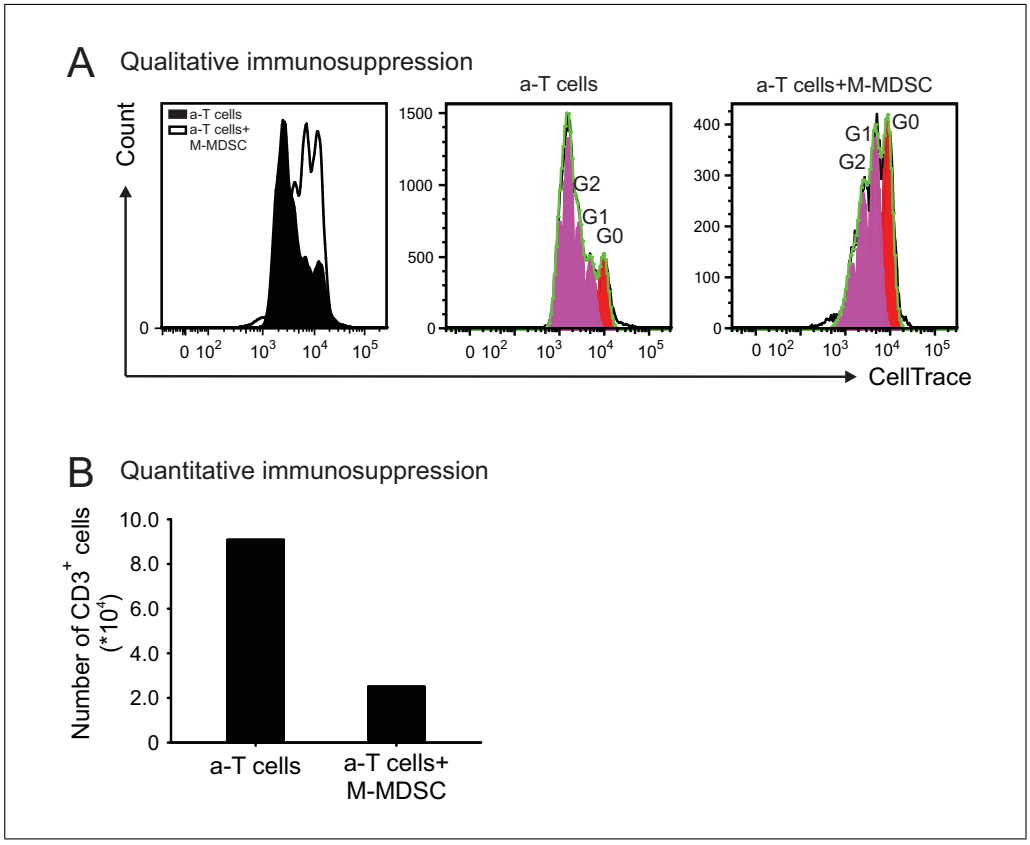

Figure 5 Representative examples of two different ways to evaluate immunosuppression. The left plot of panel (**A**) represents the overlay between CellTrace profile of activated T cells (a-T cells) cultured alone (black peak) or in the presence of MDSCs (white peak). This graph allows to appreciate the increase in the number of undivided cells (higher CellTrace fluorescence) in the presence of MDSCs as compared to T cells alone. This type of immunosuppression was defined as "qualitative" and is quantified by FlowJo proliferation tool, considering as "proliferating" the T lymphocytes belonging to generation G2 onwards and comparing these percentages in the absence (middle plot) or in the presence (right plot) of MDSCs. The histogram in panel (**B**) instead shows the reduced number of $CD3^+$ T cells quantified by TruCount tubes when activated T cells are cultured in the presence of MDSCs. This type of immunosuppression is defined as "quantitative."

REAGENTS AND SOLUTIONS

150 μM arginine RPMI containing 10% FBS

Arginine-free RPMI (Biological Industries, Kibbutz Beit Haemek, Israel), supplemented with:
150 μM arginine
10% FBS
10 U/ml penicillin and streptomycin
0.01 M HEPES
Store up to 3 days at 4°C

Complete IMDM medium

Iscove's Modified Dulbecco's Medium (IMDM; Gibco Invitrogen) supplemented with:
10% heat-inactivated FBS (Gibco)
0.01 M HEPES
0.55 mM arginine (Sigma-Aldrich)
0.24 mM asparagine (Sigma-Aldrich)
1.5 mM glutamine (Sigma-Aldrich)

100 U/ml penicillin/streptomycin
1× 2-mercaptoethanol
Store up to 3 days at 4°C

Red blood cell lysis buffer, 10×

0.15 M NH_4Cl
0.01 mM $KHCO_3$
0.1 mM disodium EDTA
Store up to 6 months at 4°C

RPMI medium containing 3%, 5%, and 10% FBS

RPMI 1640 medium (e.g., Invitrogen) supplemented with:
3%, 5%, or 10% fetal bovine serum (FBS)
1× sodium pyruvate (e.g., Invitrogen)
1× penicillin-streptomycin (e.g., Invitrogen)
1× L-glutamine (e.g., Invitrogen)
1× 2-mercaptoethanol (e.g., Invitrogen)
Store up to 3 days at 4°C

Sorting buffer

Dulbecco's phosphate-buffered saline (DPBS; Lonza BioWhittaker, cat. no. BE17-515Q) containing:
0.5% (w/v) bovine serum albumin (BSA; Sigma-Aldrich, cat. no. A4503)
2 mM disodium EDTA
Store up to 7 days at 4°C

Staining buffer

Supplement Dulbecco's phosphate-buffered saline (DPBS; Lonza BioWhittaker, cat. no. BE17-515Q) to 0.5% (w/v) with bovine serum albumin (BSA; Sigma-Aldrich, cat. no. A4503). Store up to 7 days at 4°C.

COMMENTARY

Background Information

The definition of MDSCs has been and is the subject of many studies and many reviews. It is becoming increasingly clear that a simple phenotypic analysis is not sufficient to define one or more MDSC subsets, especially in human patients (Bronte et al., 2016; Damuzzo et al., 2015; De Sanctis et al., 2016b; Solito et al., 2014). Initial efforts to harmonize their phenotype have demonstrated a large complexity and variance in the definition of all MDSC subsets, with the largest variance within the PMN subsets (Mandruzzato et al., 2016), and it is essential to combine their phenotype with a functional assay or with a biochemical and molecular characterization, in order to prove their characteristic key element, which is their suppressive ability. In fact, the ability to suppress T cell proliferation and activation is considered the "gold standard" for defining MDSCs (Bronte et al., 2016).

MDSCs restrain the immune response through many different mechanisms. MDSCs can block lymphocyte proliferation by depleting amino acids critically needed for their fitness. Indeed, they can reduce the level of cysteine through altered transport (Srivastava, Sinha, Clements, Rodriguez, & Ostrand-Rosenberg, 2010) and activate enzymes like indoleamine 2,3-dioxygenase1 (IDO1), which catabolizes l-tryptophan to kynurenines (Orabona et al., 2011), arginase 1 (ARG1), to produce ornithine, and urea or/and nitric oxide synthase 2 (NOS2), to generate nitric oxide (Bronte et al., 2005). The nutrient deprivation results in T cell proliferation arrest. Moreover, it has been reported that through the action of ARG1 and NOS2, which could act separately or in a combined fashion, l-arginine metabolism affects different T cell molecular pathways, such as translational control of the ζ-chain of CD3 and blockade of the JAK/STAT5 signaling cascade (Bronte et al., 2005).

Furthermore, activation of NOS2 by MDSCs induces the generation of nitric oxide (NO), as well as reactive oxygen species (ROS) and peroxinitrites (RNS). These short-lived compounds induce the nitration of different targets such as chemokines and TCR, which contribute to blocking T cell migration and cytotoxic effect against tumor, cause T cell apoptosis, and inhibit production of cytokines such as IL-2, which is fundamental for T cell antitumor functions (De Sanctis et al., 2014; Molon et al., 2011; Nagaraj et al., 2007).

MDSCs at the tumor site can increase the expression of programmed death ligand 1/2 (PD-L1/2) on their membrane surface, which can drastically down-regulate an anti-tumor T cell–mediated reactivity by interacting with the receptor expressed on tumor-infiltrating T cells such as programmed death 1 (PD-1). Indeed, in a mouse model of ovarian cancer, exhaustion of tumor-infiltrating lymphocytes correlated with the expression of PD-L1/2 by tumor cells and tumor-derived myeloid cells. However, treatments combining vaccines (or costimulatory antibodies) with antibodies blocking PD-1 or PD-L1, but not PD-L2, were able to expand the antigen-specific $CD8^+$ T cells and trigger tumor rejection (Duraiswamy, Freeman, & Coukos, 2013). Furthermore, it was demonstrated that PD-L1 up-regulation on MDSCs is transcriptionally induced by the hypoxia-inducible factor-1α (HIF-1α) at the tumor site. Blocking PD-L1 decreases MDSC-mediated T cell suppression under hypoxia by down-regulating IL-6 and IL-10 produced by MDSCs (Noman et al., 2014). In this respect, recent clinical trials with PD-1/PD-L1 blockade therapy have shown unprecedented durable response in patients with a variety of cancers despite only a minority of patient benefits from such therapy; for these reasons, several studies are investigating the contributions of PD-L1 signaling during checkpoint blockade therapy, especially to examine the cell populations that respond to PD-L1. Very recently, PD-L1 in tumor cells has been shown to be dispensable for the responses to PD-L1 blockade therapy after tumor establishment in three different models (MC38, A20, and E.G7). Moreover, PD-L1 appeared not only to be highly expressed in myeloid cells, but also to contribute to the inhibition of T cell activation, since blocking PD-L1 signaling by Ab releases such inhibition, leading to better T cell activation (Tang et al., 2018). Recently, we analyzed the mechanisms involved in the interplay between MDSCs and activated T cells. We found that activated T cells release IL-10 following interaction with MDSCs which, in turn, induce STAT3 phosphorylation on MDSCs, then leading to PD-L1 expression. Moreover, we observed that the expression of ligands PD-L1 and MHC class II on *in vitro*–induced MDSCs or on MDSCs from tumor microenvironment of melanoma patients is related to an increased expression of their respective receptors, PD-1 and LAG-3, on T cells, the inhibitory molecules associated with T cell dysfunctions (Pinton et al., 2016).

The immune response can also be tuned by MDSCs supporting the generation of other suppressive cells. MDSCs promote expansion of Treg through mechanisms not completely understood, which likely include CD40-CD40L interactions, TGFβ and IL-10 release and direct antigen presentation, and the expression of ARG1 (Gabrilovich, Ostrand-Rosenberg, & Bronte, 2012; Serafini, Mgebroff, Noonan, & Borrello, 2008). Moreover, MDSCs promote macrophage reprogramming toward an M2 phenotype typical of tumor associated macrophages (TAMs) through cell-cell contact cross-talk. IL-10 produced by MDSCs induces a reduced ability by macrophages to produce IL-2, which in turn stimulates IL-10 production by MDSCs (Sinha, Clements, Bunt, Albelda, & Ostrand-Rosenberg, 2007). The altered balance between IL-12/IL-10 in the tumor microenvironment skews T cell immunity toward a tumor-promoting Type 2 response and decreases DC maturation, which is critical for priming T lymphocyte responses (Ostrand-Rosenberg, Sinha, Beury, & Clements, 2012).

Many interactions, similarities, and relationships between MDSCs and TAMs are emerging, which require clarification. Indeed, it has been advanced that MDSCs and TAMs employ different mechanisms to control immune responses, but the possibility that some MDSC subsets might give rise to TAMs complicates separation of these two cell types, especially within the tumor environment (Sica et al., 2007; Ugel, De Sanctis, Mandruzzato, & Bronte, 2015).

In this unit, we report some protocols to accurately test the suppressive abilities of isolated mouse and human M-MDSCs on T lymphocytes. The accuracy and reproducibility with which immunosuppressive measures are carried out become fundamental when drug tests are required for the development of therapeutic approaches to counteract MDSC functions.

We present three methods to evaluate, either *in vitro* or *in vivo*, immune suppression by myeloid cells isolated from mice to the detriment of $CD8^+$ T lymphocytes. The proliferation assay using flow cytometry can be easily performed in many laboratories, in particular to evaluate the proliferation in response to antigen-specific stimuli. Proliferation assays by [^{3}H]TdR incorporation represent a very simple and robust method to quantitatively evaluate the suppressive activity on T cells effected by a nonspecific stimulus (anti-CD3 and anti-CD28 mAbs) to compare many different treatments. On the other hand, these assays can be easily modified to assess alloantigen stimulation (Marigo et al., 2010; also see previous version of this unit; doi: 10.1002/0471142735.im1417s91).

Basic Protocol 1, with its simplicity, could be useful to assess large numbers of experimental conditions, such as large screenings of new drugs, or when limited amounts of cells, as in the case of rare populations, are available. It must be pointed out, however, that it only provides information about T cell proliferation but is completely blind to the lymphocyte effector function.

Microcultures with myeloid suppressors, followed by evaluation of cytolytic activity by ^{51}Cr release, offer the possibility to investigate T cell–specific effector function and its modulation/inhibition by immunomodulatory cells, both preserving the simplicity of proliferation assay and allowing the investigator to work with numerous variables and rare cell populations, which could be difficult to establish under complex *in vivo* conditions. The chromium release assay is even more laborious than the [^{3}H]TdR uptake assay, but offers a more specific and extended readout. Lytic unit transformation, which measures the extent of suppression normalized to the internal control without suppressive cells, provides a more straightforward representation of the results and allows the comparison and averaging of results from different experiments. Evaluation of L.U. represents a more effective measure than either the proliferation assay or single effector-to-target ratio cytotoxicity value, since it includes an estimation of both functional activity (cytolytic activity of cultures) and cell proliferation (number of cells recovered in each culture).

Basic Protocol 6 makes it possible to study tolerogenic activity of immunosuppressive populations *in vivo*. It could be demanding in terms of time and cost, since it requires a consistent number of immunosuppressive cells per mouse and only allows the investigator to process a few experimental conditions within a single experiment. Flow cytometric evaluation of the expression of both CD8 and CD45.1 markers in the context of a $CD45.2^+$ recipient mouse makes it possible to carefully estimate the relative abundance of transferred T cells within the total $CD8^+$ T cell number in the sample; this assessment could provide information about elimination and/or proliferative blockade of the antigen-specific $CD8^+$ T cells promoted by the immunosuppressive mouse environment. The percentage of $CD8^+$/$CD45.1^+$ cells could also be used to calculate the total amount of $CD8^+$/$CD45.1^+$ cells per recipient mouse, taking into account the total number of cells obtained from explanted lymph nodes. Moreover, intracellular staining of IFN-γ will provide a measurement of the activation state of transferred effector cells.

It is not clear whether MDSCs isolated from tumor-free naïve mice are immunosuppressive (Bronte, 2009; Greifenberg et al., 2009; Ribechini, Leenen, & Lutz, 2009). It appears that the assay used might influence the extent of suppression. The use of artificially high numbers of MDSC subsets *in vitro* might unveil inhibitory properties that are intrinsic to these subpopulations, but which will not be manifest *in vivo*. In particular, a strong activation of effector T cells (such as that induced by antibodies or alloantigens) might also trigger inhibitory pathways in naïve myeloid cells. In this sense, the proliferation assay reported here tends to overestimate the immunosuppressive strength. In our experience, the *in vitro* parameter that better correlates with the immunosuppressive activity of *in vivo*–transferred myeloid cells is the capability of these cells to inhibit the effector function of antigen-specific T cells in microcultures when added at low percentages (1% to 6% of total cells in culture), as previously described (Dolcetti et al., 2010).

We have discussed protocols for both *in vitro* and *in vivo* assays based on the use of specific TCR transgenic $CD8^+$ T cells, but it is clear that these protocols can be adapted to other combinations of TCRs and mouse backgrounds, provided that preliminary experiments are performed to establish the amount of DCs necessary to induce priming of transferred $CD8^+$ T cells. Moreover, in the assays described here, we primarily used naïve $CD8^+$ T cells, but memory/effector T cells could be

isolated from previously primed mice or easily obtained by a previous, short-term stimulation of naïve $CD8^+$ T cells with the antigenic peptide in the absence of myeloid suppressors.

Basic Protocols 7, 8 and 9 describe procedures to identify the expansion of human monocytic MDSCs and isolate them from the peripheral blood of cancer patients, as well as to expand $CD11b^{low/-}$ BM-MDSCs *in vitro* and purify MDSCs in order to evaluate their suppressive function, a mandatory property to define myeloid subsets as MDSCs (Bronte et al., 2016). In some cases, these myeloid populations can increase in response to tumor-derived factors released in the tumor microenvironment, and the opportunity to monitor their level in whole blood through flow cytometry might contribute to give them a prognostic value. Flow cytometry analysis of PB-isolated M-MDSCs is rapid and requires a small amount of cells, thus allowing preservation of all the material obtained from whole blood for cell separation and suppression assay.

Critical Parameters and Troubleshooting

For the reasons discussed in Background Information, the most important parameter to consider in evaluating the suppressive activity of myeloid cells is the amount of cells required to give a complete inhibition of the *in vitro* assay. Although this principle applies to the *in vivo* assay, technical limitations might preclude *in vivo* titration as well.

It is of capital importance to make use of excellent-quality FBS in all cultures; in our experience, sera need to be relatively low in growth factors, since their abundance could interfere with suppressive activity by myeloid cells by simply activating/differentiating MDSCs. It is our practice to screen different lots of FBS to ensure that they do not interfere with the suppressive assay. Briefly, we compare the new FBS batches with the one currently in use in the lab by setting up the suppressive assays described in Basic Protocols 3 and 5 for mouse cells and Basic Protocol 9 for human cells. We select the FBS batch used for cultures whose suppressive activity was similar to those containing the previous FBS. Also, attention should be given to the red cell lysis step, since it has been reported that hemoglobin can scavenge nitric oxide (Azarov et al., 2005), altering suppressive phenomena that rely on NOS2 and ARG1 activity. Some articles report immunosuppressive assays in which T cell proliferation is obtained using beads coated with anti-CD3 and anti-CD28 antibodies. This stimulation needs to be carefully considered, because it has been recently demonstrated that it could lead to artifactual T lymphocyte suppression due to sequestration of beads and their phagocytosis by MDSCs (Davis, Silvin, & Allen, 2017).

Cell density is a critical parameter for microcultures; thus, particular attention should be paid to cell counts, dilutions, and plating. It is useful to check the percentage of $CD8^+$ T cells that are positive for $V_\alpha 2$ $V_\beta 5.1/5.2$ in transgenic OT-1 total spleen, and adjust the dilution rate of OT-1 cells to feeder C57Bl/6 cells, in order to obtain the proper state of activation after 5 days of culture.

Latent immunosuppressive programs might be artificially activated either by sorting procedures or *in vitro* manipulation, a possibility that must be considered for all separating procedures based on antibodies binding to surface molecules. Relevant changes in the amount of either mAbs or beads used for cell sorting of MDSCs should thus be tested accurately, especially in view of the demonstration that some antibodies such as anti-Gr-1 mAb can trigger a signaling cascade in target cells (Ribechini et al., 2009).

Critical parameters in *in vivo* experiments are usually the number of transferred cells and the proper preparation/maturation of DCs. Moreover, the quality of transferred cells requires attention; sometimes, these cells are very sticky and tend to form clumps, and i.v. inoculation of clumped cells might compromise recipient mouse survival. Maintaining cells on ice during i.v. inoculation prevents clump formation; clumps could also be disaggregated with gentle pipetting, carefully avoiding damage to cells with small-gauge needles.

Antigen specificity is another issue related to the very same biology of suppressor cells that, as with other cellular inhibitors of immune responses, seems to act through both antigen-specific and nonspecific mechanisms (Solito, Bronte, & Mandruzzato, 2011a). We and others favor the idea that the immune-suppressive mechanisms of MDSCs do not require direct presentation of the antigen, even though this might be a rate-limiting step under some experimental conditions where the encounter with rare $CD8^+$ T cells in lymph nodes might be favored by direct antigen presentation. Although the immune-suppressive mechanisms are mostly antigen-independent, antigen activation is mandatory since MDSCs do not affect resting T cells. The protocols described here can be adapted to evaluate

direct antigen presentation by carefully pulsing MDSCs, antigen-presenting cells, or both with either relevant or irrelevant antigenic peptides.

It is interesting to note that proliferation/suppression can be evaluated by quantifying the absolute T cell number, and/or by CellTrace profile. In fact, over the years, we have observed, in mouse and human experiments, that in some cases suppression appears as a reduction in the absolute number of T cells (quantitative suppression) and sometimes as a delay in the CellTrace profile (qualitative suppression). Sometimes MDSC-mediated immune suppression is both qualitative and quantitative, but in many cases, it is one or another (see Fig. 5). Therefore, it would be better to routinely use CellTrace-based proliferation together with the count of absolute numbers of T cells with a standard procedure based on TruCount tube, thus ensuring the possibility of evaluating both. All these considerations suggest that would be important to standardize the conditions for MDSC phenotyping and suppression within and across laboratories in order to define minimal essential criteria enabling reproducible results.

Anticipated Results

The advent of multi-parameter flow cytometry has greatly enhanced the possibility to better characterize the expanded and infiltrating myeloid subsets in cancer models and patients. Moreover, the chance to isolate highly purified immune suppressive subsets by FACS sorting, in addition to immunomagnetic methods, gives us the opportunity to facilitate downstream studies, for example through the characterization of their transcriptome to define molecular targets. Immunosuppressive assays based on CellTrace dilution evaluated by FACS methods are technically very accurate and can be easily reproduced in different laboratories.

[^{3}H]Thymidine incorporation in a 96-well plate usually produces a readout of about 150–200 $\times$ 10^3 cpm, while the negative controls fall on the order of 1/100 of the stimulated cells. A properly developed control microculture could induce the lysis of about 70% to 90% of target cells. Usually, the first and second dilution of the culture will be at plateau, and the lysis curve should reach half of the plateau at about fourth-fifths dilution, achieving the baseline at the seventh dilution. Nonspecific lysis should be $<$30% in the first dilutions and rapidly reach background at the third dilution. Nonspecific lysis may be proportional to specific lysis, and instead of subtracting background from specific lysis, it could be a better approach to minimize nonspecific lysis utilizing a different target cell line that is less sensitive to NK activity. If lytic unit representation is chosen, it could also be informative to take a look at the line plot for each sample, in order to assure proper regression of the results and the consistency of estimated L.U.

A proper immunization with DCs will result in the accumulation of CD8$^+$/CD45.1$^+$ cells to represent $\sim$2% to 6% of total lymph node cellularity. More than $\sim$60% of CD8$^+$/CD45.1$^+$-gated cells should be positive for IFN-γ staining. The samples that have not received *ex vivo* peptide stimulation usually do not give high background staining compared to matched isotype control.

Time Considerations

The protocols presented here are quite time consuming, and they also require incubations lasting several days. The presented strategy for mouse MDSC enrichment takes $\sim$3 hr to be completed, while the strategy for human M-MDSCs isolation from peripheral blood takes $\sim$5 hr. CellTrace proliferation assays, [^{3}H]TdR proliferation assay, and the ^{51}Cr release test setup can be completed in at a couple of hours for simple experiments, but could take longer depending on the number of different conditions that need to be tested. [^{3}H]TdR readout could be completed in less than 1 hr, taking into account the quenching period, while the CellTrace proliferation assay takes $\sim$2 hr. Chromium release can be more time consuming, since target cell pulsing usually needs $\sim$1.5 hr to complete pulsing, washing, and cell counting. Dilution of CTL cultures could be performed during target pulsing, and, usually, the setup of a commonly sized experiment with 15 to 20 different experimental conditions in triplicate could be completed within the time of pulsing. After 5 hr of effector/target co-culture, the supernatant transfer could take at least 30 min. LumaPlates need to be read the following day.

In vivo experiments are quite demanding; challenging steps usually include the achievement of a suitable amount of suppressive cells, which could take several hours of sorting, and the single-recipient processing of lymph nodes, which could take about 6 hr for a simple experiment with about 20 recipients. Flow cytometric acquisition could also take a long time, depending on the

frequency of $CD8^+CD45.1^+$ events to be collected.

Recently, it has emerged that the immunosuppressive mechanisms mediated by MDSCs could be different for different tumors. In the future, it may be necessary to reevaluate the technical measures to optimize the isolation of MDSCs and standardize the appropriate functional tests by comparing the districts where these cells are present and different tumor types (Damuzzo et al., 2015; Kumar, Patel, Tcyganov, & Gabrilovich, 2016).

Conflicts of Interest

Authors declare that they do not have any conflicts of interest.

Acknowledgements

This work was supported by grants from the Cancer Research Institute Clinic and Laboratory Integration Program (CLIP Call 2017) Grant (funded by Cancer Research Institute U.S.A.); Italian Association for Cancer Research (AIRC, grants 18603 to V.B., 17400 to S.M. and Multi Unit Airc 5×1000 grant 12182); EuroNanoMed III-2016 [funded by Ministry of Education, University and Research (MIUR, Italy) CUP: B35D18000000006]; and University of Padova grant CPDA-144873.

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