CHAPTER TEN

Protocol to assess the suppression of T-cell proliferation by human MDSC

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Methods in Enzymology, Volume 632
ISSN 0076-6879
https://doi.org/10.1016/bs.mie.2019.05.046

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Abstract

Inhibition of T-cell proliferation is the most common approach to assess human myeloid-derived suppressor cell (MDSC) functions. However, diverse methodologies hinder the comparison of results obtained in different laboratories. In this chapter, we present a T-cell proliferation assay procedure based on allogeneic MDSC and T-cells that is potentially suitable to multi-center studies. The T-cells are isolated from non-cancerous donors and frozen for later use in different research groups. We observed that pure thawed T-cells showed poor proliferative capacities. To retain proliferation, T-cell-autologous mature dendritic cells are supplemented after thawing. MDSC are isolated from clinical samples and represent the sole variant between assays. Flow cytometry is used to assess T-cell proliferation by the dilution of a tracking dye.

Abbreviations

CFSE carboxyfluorescein succinimidyl ester
COST European Cooperation in Science and Technology
DC dendritic cell
EU European Union
FBS fetal bovine serum
HS human serum
MDSC myeloid-derived suppressor cell(s)
NK natural killer
PBMC peripheral blood mononuclear cells
PBS phosphate buffered saline
RT room temperature

1. Introduction

Myeloid-derived suppressor cells (MDSC) are heterogeneous populations of cells of the myeloid lineage with strong immunosuppressive capacities. Increased numbers of MDSC have been measured in the blood and tissue of patients with diverse conditions including cancer, arthritis and infectious diseases. In cancer, the increased presence of MDSC has been

correlated with poor prognoses for disease progression and survival (Safarzadeh, Orangi, Mohammadi, Babaie, & Baradaran, 2018). Targeting the MDSC and their immunosuppressive capacities could be an attractive target for future combination immunotherapies for cancers. The characteristics of the different human MDSC and their role in diseases have been reviewed elsewhere (Amodio et al., 2019; Cassetta et al., 2019; Dorhoi et al., 2019; Umansky et al., 2019). Briefly, MDSC are broadly categorized based on their lineage of origin into monocytic, granulocytic and early stage MDSC. A defining character of all is their immunosuppressive function (Haile, Greten, & Korangy, 2012). However, both isolation strategies and functional assay procedures differ widely in the literature and among groups (Bruger et al., 2019; Cassetta et al., 2019). This complicates the comparability of MDSC sourced from different pathologies, tissues and in different groups. We here propose a protocol using one common allogeneic T-cell responder to test the suppressive function of MDSC sourced at different locations comparably.

Compared to murine MDSC, the study of human MDSC is complicated by various challenges: (i) The heterogeneity of MDSC populations and the lack of a specific cellular marker results in complex isolation strategies with high levels of variation among laboratories. While Lox-1 has been proposed as a specific MDSC marker, it does not seem to be expressed by all MDSC and is not yet widely adopted in isolation protocols (Condamine et al., 2016). (ii) Granulocytic MDSC (also known as PMN-MDSC) in particular possess a short ex vivo life-span and do not tolerate freezing, restricting the breadth of experiments that can be performed on any one sample (Trellakis et al., 2013). (iii) Patients' samples are restricted in the type and amount of tissue available for MDSC extraction. Usually, human MDSC are isolated from the peripheral blood of cancer patients at low yields. This limits the number and types of experiments and manipulations possible. Genetic, depletion or adoptive transfer models, such as in mice (Clavijo et al., 2017; Corzo et al., 2010; Pan et al., 2010), cannot be performed in humans.

Considering these restrictions, T-cell proliferation assays are the most common test used to assess MDSC-mediated suppression of T-cell functions. The principle of T-cell proliferation assays is convenient as they are fast to set up and easy to adapt according to MDSC yields: T-cells are stained with radioactive tritium or with a fluorescent tracker dye that halves in its intensity with every round of multiplication. The T-cells are co-cultured with MDSC and stimulated through CD3 and the co-stimulatory CD28

molecule. The results are read after 3–4 days of incubation. However, similarly to the isolation strategies for MDSC, many variables exist among laboratories that complicate the comparison of results obtained at different locations. These variations include the type and origin of T-cells used, e.g., $CD3^+$ or $CD8^+$ T-cells, autologous or allogeneic to the MDSC (Bruger et al., 2019).

Particularly the fragile nature of the MDSC, their low yields, and the large variation in T-cell proliferation protocols mean that MDSC suppression data from different laboratories are difficult to compare to each other. Since the MDSC themselves cannot be exchanged, a common suppression assay protocol is important to assess if MDSC share functional properties regardless of the origin of tissue or lineage. Are there distinct MDSC populations or is there a functional spectrum?

To address these questions, we established a T-cell proliferation assay protocol and reagents to share among different laboratories with in the pan-European COST Action Mye-EUNITER. Ideally, the MDSC would remain the only variant between experiments and laboratories. This approach is based on isolating and freezing T-cells from non-cancerous donors that can be shared. The following protocol has been developed and tested in the van der Bruggen laboratory in Brussels (Fig. 1). We are still validating the protocol with exchanged cells in COST Action Mye-EUNITER partner laboratories.

2. T-cell isolation and mature dendritic cell generation

T-cell proliferation is the most commonly used assays to assess the suppressive capacity of human MDSC. However, due to the large numbers of parameters involved, we observed procedural variations among laboratories (Bruger et al., 2019). Since none of the procedures is objectively superior, we agreed to adopt a common protocol in a multicenter study.

To achieve this, we advise to isolate allogeneic T-cells from non-cancerous donors (Sections 2.1–4). Specifically, in the van der Bruggen group (Brussels) we obtain up to 500 mL peripheral blood from hemochromatosis patients who have to be bled regularly as part of their routine treatment. This allows us to isolate T-cells in numbers large enough to be shared easily with other laboratories. The negative selection process, ensuring that CD3-engagement is not induced during the isolation process, is performed by separating $CD2^+$ and $CD2^-$ cells through rosetting with sheep

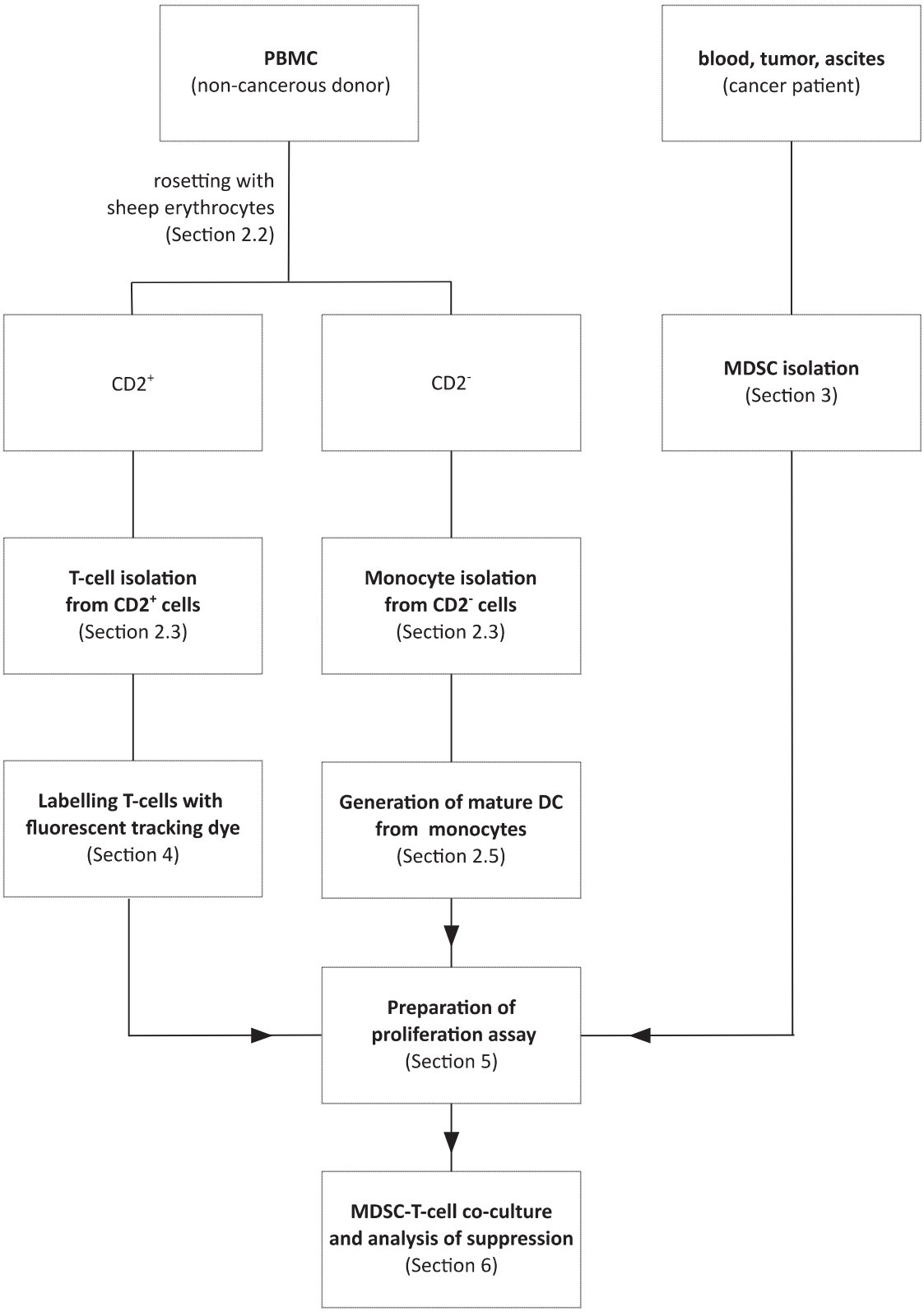

Fig. 1 Overview of the process. T-cells and monocytes are isolated from non-cancerous (here: hemochromatosis) donors. DCs are generated from the monocytes. T-cells and monocytes are frozen for future use in T-cell proliferation assays at different research groups. MDSC are isolated from cancer patients and used directly in T-cell proliferation assays.

erythrocytes, followed by the depletion of $CD56^+$ NK cells from the $CD2^+$ fraction (Sections 2.2–3). The T-cells are frozen at −80 °C and can be shared among laboratories. However, remaining myeloid cell impurities did not survive the thawing process, and, as a consequence, thawed T-cells barely proliferated. T-cell proliferation can be rescued by the addition of low numbers of autologous mature dendritic cells (DC), differentiated from the $CD2^-$ fraction (Sections 2.4–7).

2.1 PBMC isolation from whole blood

Sharing PBMC from the same donor within multi-center studies requires the isolation of allogeneic T-cells from non-cancerous donors. We use blood obtained from hemochromatosis patients for this purpose. We first isolate the PBMC, and then the T-cells and monocytes in subsequent steps.

a. Dilute whole blood 1:3 with cold PBS + 1 mM EDTA.

b. Separate the PBMC from the erythrocytes, neutrophils and platelets by density gradient centrifugation. Add 15 mL Lymphoprep™ (Alere Technologies) or Ficoll (Sigma) into a 50 mL Falcon tube at room temperature (RT) and carefully layer the blood onto the Lymphoprep without mixing the two solutions by holding the tube at a low angle. To separate the cells by density, centrifuge the cells for 20 min at 900*g* without breaks or acceleration to avoid disrupting the density gradient established during centrifugation.

c. Following the density gradient separation the PBMC settle in a thin layer below the plasma, and above the Lymphoprep solution and erythrocyte pellet (Fig. 2). Carefully remove the PBMC layer with a pipette and place it into a fetal bovine serum (FBS)-coated 50 mL Falcon tube. The Falcon is pre-coated with FBS for 24 h at 37 °C to prevent cells from adhering to the plastic.

d. Wash the cells with cold PBS + 1 mM EDTA and centrifuge at 400*g* for 10 min. EDTA is a Ca^{2+} chelating agent that prevents platelet aggregation.

e. The PBMC settle in the pellet. Carefully remove the supernatant and as much of residual platelets that settle in a white ring above the PBMC as possible. Remaining platelets could interfere in later steps with the DC generation through CD40L and with flow cytometry analyses by interacting with antibodies. Inspect the cell solution for platelets underneath a microscope if doubtful that all platelets have been successfully

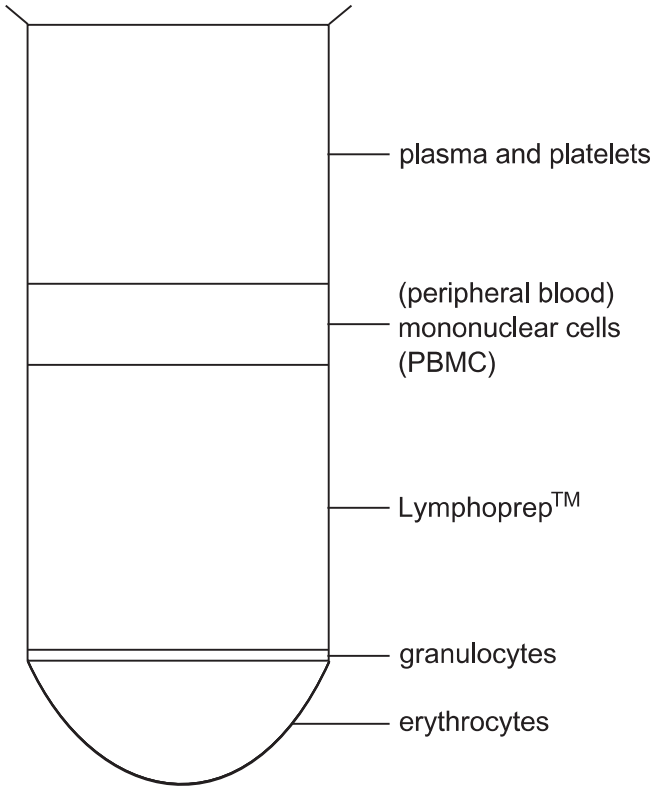

Fig. 2 Density gradient separation of peripheral blood. Whole blood diluted with cold PBS-EDTA is carefully layered onto Lymphoprep™ solution. Density separation is achieved after centrifugation at 900*g* for 20 min without acceleration or brakes. Shown is a sketch of how the blood components separate.

removed by aspiration. Should platelets remain, resuspend the PBMC in PBS + 1 mM EDTA and centrifuge them at 200*g* for 3 min and aspirate the supernatant. Platelets are too light to pellet at this speed.

f. Repeat the washing steps with 50 mL cold PBS + 1 mM EDTA twice.

g. Resuspend the PBMC in warm RPMI 1640 (Gibco) and count the cells. Retain 5×10^5 cells for flow cytometry assessment (Section 2.5).

2.2 Separation of $CD2^+$ and $CD2^-$ cells by rosetting with sheep erythrocytes

T-cells are isolated by negative selection to avoid any adverse effects caused by CD3-binding beads. Erythrocyte rosetting allows separating $CD2^+$ and $CD2^-$ cells from PBMC. CD2 is an adhesion molecule present on T-cells and natural killer (NK) cells, which spontaneously binds to the LFA3-homolog on sheep erythrocytes (Ocklind, 1988). CD2/LFA-3 interaction causes an aggregation of NK and T-cells around the

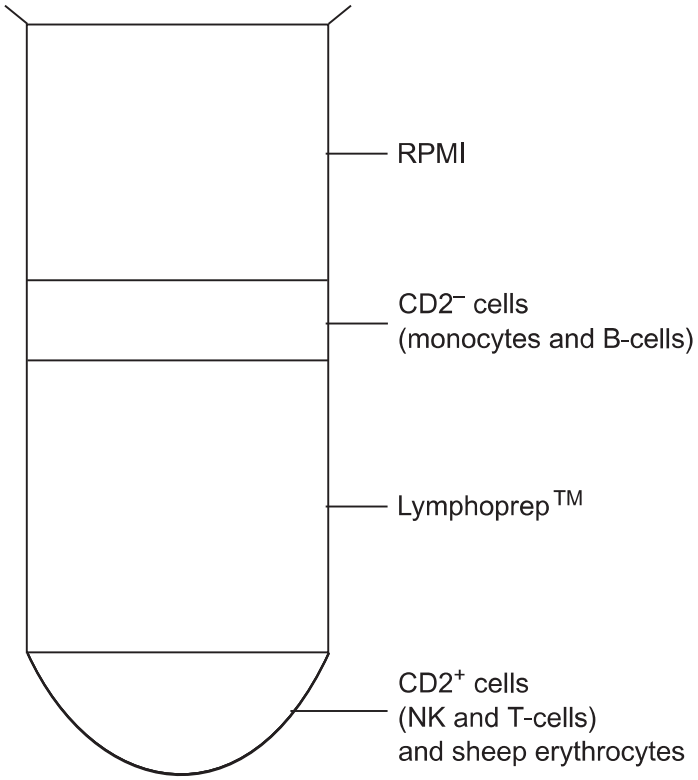

Fig. 3 Density gradient separation of $CD2^+$ and $CD2^-$ cells. Sheep erythrocytes express an LFA3-homolog that binds to human CD2 and thus causes the spontaneous aggregation of human NK and T-cells (Ocklind, 1988). The sketch indicates how $CD2^+$ and $CD2^-$ cells separate by density gradient.

erythrocytes, allowing $CD2^+$ NK and T-cells to be elegantly separated from $CD2^-$ B-cells and monocytes by a density gradient. The $CD2^+$ cells pellet together with the erythrocytes, while the $CD2^-$ cells settle in the mononuclear layer (Fig. 3). In the following sections, we first describe the preparation of sheep erythrocytes. Second, we describe the erythrocyte rosetting protocol.

2.2.1 Preparation of sheep erythrocytes

a. Use reagents equilibrated to room temperature. We use sheep erythrocytes in 50% Alsever's suspension provided by Labor Dr. Merck (E-400). 2-aminoethylisothiouronium bromide (AET) is provided by Sigma (A54601).

b. Check the hematocrit (% erythrocytes) of the sheep erythrocytes first, using the provider's website: http://www.labormerk.com/manufacturing/blood_products/

Table 1 Reference table for the resuspension of sheep erythrocytes in AET and RPMI 1640 according to the hematocrit content.

% Hematocrit	X: A.E.T. (mg)	Y: H_20 volume for AET (mL)	Z: RPMI 1640 (mL)
10	625	13.5	15.6
11	688	14.7	17.2
12	750	16.2	18.7
13	813	17.3	20.3
14	875	18.9	21.9
15	938	20.0	23.4
16	1000	21.6	25.0
17	1063	22.7	26.6
18	1125	24.3	28.0
19	1188	25.7	29.7
20	1250	27.0	31.2

c. Prepare the AET solution by dissolving **X** mg of AET in **Y** mL of milli-Q water (see Table 1). Add 1 M NaOH to bring the solution to pH 9. Note that the correct pH of the solution is of particular importance.

d. Filter the AET suspension with a 0.22 μm Steriflip (Millipore, catalog ♯ SCGP00525) into a 50 mL filter Falcon tube.

e. Resuspend sheep erythrocytes by shaking and/or pipetting, and collect 12.5 mL of the erythrocyte suspension. Wash the suspension by adding 37.5 mL of PBS and centrifuge at 910*g* for 8 min.

f. Aspirate supernatant. Leave some PBS to avoid aspirating any erythrocytes. Add the AET solution and resuspend the erythrocytes. Incubate for 15 min at 37 °C.

g. Pellet the erythrocyte suspension by centrifugation at 910*g* for 8 min. Remove AET supernatant. Resuspend in 50 mL PBS. The erythrocyte pellet should be of dense consistency and require repeated gentle agitation for complete resuspension.

h. Wash with 50 mL PBS by centrifugation at 910*g* for 8 min three times. The pellet's consistency should become less dense with each washing step. Perform an additional washing step if the supernatant is quite red which indicates erythrocyte lysis.

i. Resuspend the cells in **Z** mL of RPMI 1640 (Table 1). Store the cells at 4 °C for up to 15 days. Test the sheep erythrocytes on donor blood before use, and control the efficiency of the rosetting by flow cytometry as described in Section 2.5.

2.2.2 Separation of $CD2^+$ and $CD2^-$ cells from sheep erythrocytes

a. Prepare three solutions in advance to lyse the erythrocytes in later steps:
   i. 5 M NaCl stock solution, in distilled water, sterilized
   ii. 0.2% NaCl lysis solution (in sterile water, from stock)
   iii. 1.2% NaCl reconstitution solution (in sterile water, from stock)
b. Resuspend the PBMC in warm RPMI 1640 at 10^7 cells/mL or in at least 10 mL.
c. Add 1 mL sheep erythrocytes (preparation described in Section 2.2.1) for each 10^7 PBMC. Mix the cells well and incubate them at room temperature for 10 min.
d. The Lymphoprep density separation step is repeated with the PBMC and sheep erythrocyte suspension (Section 2.1, steps b and c).
e. The $CD2^-$ cells will settle in the PBMC layer (Fig. 3). Remove the $CD2^-$ cells, wash and count them as before (Section 2.1, steps e–g). $CD14^+$ cells are obtained from the $CD2^-$ cells (described in Section 2.4) and used to generate autologous mature dendritic cells (Section 2.6).
f. Remove the Lymphoprep solution carefully and retain the pellet. The pellet contains the sheep erythrocytes and the $CD2^+$ cells that attached to them.
g. Lyse the erythrocytes with 0.2% NaCl (prepared in Section 2.2.1) by resuspending the cells carefully but thoroughly. After 50–60 s reconstitute osmolarity with 1.2% NaCl (Section 2.2.1). Refer to Table 2 for the required volumes.

Table 2 Reference table for the use of lysis buffers according to the original volumes of blood used.

Blood volume before density gradient (mL)	0.2% NaCl (mL)	1.2% NaCl (mL)
50	7.5	17.5
40	6.0	14.0
30	4.5	10.5
20	3.0	7.0
10	1.5	3.5

h. Centrifuge for 5 min at 300*g* RT. Resuspend the pellet in warm RPMI 1640 and count the cells. Retain 5×10^5 $CD2^+$ and $CD2^-$ cells for flow cytometry assessment (Section 2.5).

2.3 Isolation of T-cells from $CD2^+$ cells by negative selection

Only T and NK cells express CD2. T-cells are further isolated from $CD2^+$ cells by depleting NK cells using anti-CD56 magnetic beads. CD56 is a cell adhesion glycoprotein that is prototypic marker for NK cells. The purity of the isolated cells and the success of the separation are assessed by flow cytometry in Section 2.5.

a. Use $CD2^+$ cells isolated from whole blood (Sections 2.2).

b. Count the cells and resuspend 10^7 cells in 60 µL PBS + 2% human serum (HS).

c. Per 10^7 cells add 20 µL Fc receptor (FcR) blocking reagent (Miltenyi, catalog ♯ 130-059-901) and 20 µL magnetic anti-CD56 beads (Miltenyi, catalog ♯ 130-050-401).

d. Mix well and incubate for 20 min at 4 °C.

e. Separate T-cells and NK cells using either manual or automatic magnetic columns. Keep the samples cool at all times using racks pre-cooled to 4 °C (e.g., Chill 15 Rack, Miltenyi, catalog ♯ 130-092-952). Follow the manufacturer's instruction if using manual separation. If the automatic AutoMACS separator is used, use the program possel_s.

f. The $CD56^+$ NK cells will be in the positive fraction, the T-cells in the negative eluate fraction. Retain both fractions, wash them by centrifugation at 300*g* for 8 min with RPMI 1640 and count the cells.

g. Retain 5×10^5 cells to assess the purity of the cells by flow cytometry (Section 2.5).

h. Freeze the T-cells at −80 °C as described in Section 2.8.1. These T-cells will be used in the proliferation assay in Section 5.

2.4 Isolation of $CD14^+$ cells from $CD2^-$ cells by positive selection

In our experience, frozen T-cells lack myeloid cells to sustain reliable proliferation in vitro. To supplement isolated T-cells (Section 2.4) with antigen presenting cells, autologous mature DC are differentiated from monocytes. These are isolated from the $CD2^-$ fraction (Section 2.2) by anti-CD14 magnetic bead selection. The purity of the isolated cells and the success of the separation are assessed by flow cytometry in Section 2.5.

a. Use $CD2^-$ cells isolated from whole blood (Section 2.2).
b. Count the cells and resuspend 10^7 cells in 60 µL PBS + 2% HS.
c. Per 10^7 cells add 20 µL FcR blocking reagent (Miltenyi, catalog ♯ 130-059-901) and 20 µL magnetic anti-CD14 beads (Miltenyi, catalog ♯ 130-050-201).
d. Mix well and incubate for 20 min at 4 °C.
e. Isolate monocytes by either manual or automatic magnetic columns. Keep the samples cool at all times using racks pre-cooled to 4 °C (e.g., Chill 15 Rack, Miltenyi, catalog ♯ 130-092-952). Follow the manufacturer's instruction if using manual separation. If the automatic AutoMACS separator is used, use the program possel_s.
f. The $CD14^+$ monocytes will be in the positive fraction. Wash the isolated cells by centrifugation at 300*g* for 8 min with RPMI 1640 and count them.
g. Retain 5×10^5 cells to assess the purity of the cells by flow cytometry (Section 2.5).
h. Use the $CD14^+$ monocytes to generate mature dendritic cells (Section 2.6).

2.5 Control of cell purity by flow cytometry

The purity of T-cells and monocytes is controlled by flow cytometry and compared to the individual steps of the isolation process (Fig. 4). Use own flow cytometry systems, colors and compensation controls. This laboratory uses a BD Fortessa and Diva software.

T-cells should be $CD2^+$, $CD3^+$ and (in part) $CD8^+$, and $CD14^-$ and $CD56^-$. NK cells should be $CD2^+$, $CD8\alpha^+$ and $CD56^+$, and $CD3^-$ and $CD14^-$. Monocytes should be $CD14^+$, and $CD2^-$, $CD3^-$, $CD8^-$ and $CD56^-$.

Samples

i. Unstained control
ii. PBMC
iii. $CD2^+$ cells
iv. $CD56^+$ NK cells
v. $CD56^-$ T-cells
vi. $CD2^-$ cells
vii. $CD14^+$ monocytes
viii. $CD14^-$ cells

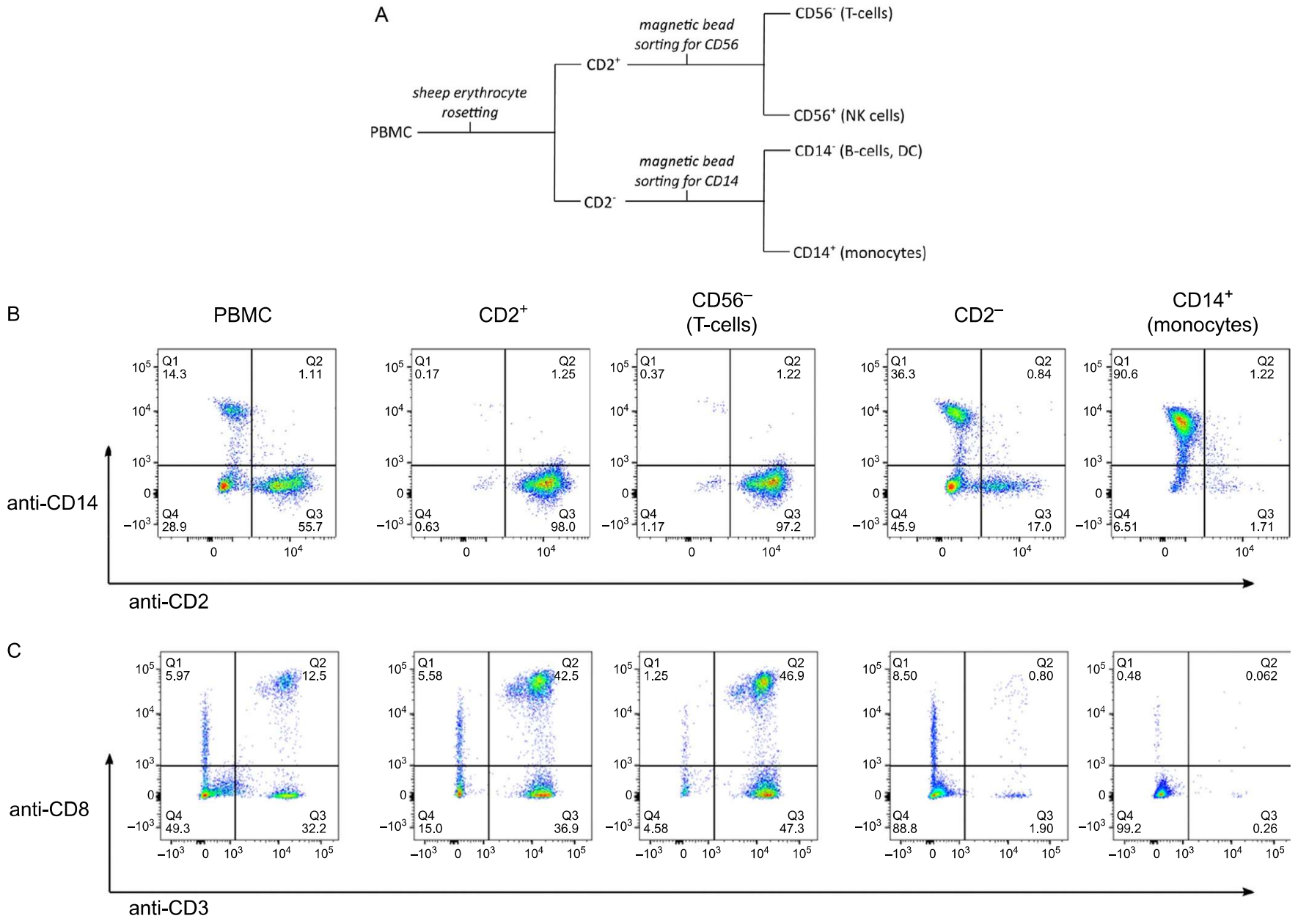

Fig. 4 See legend on next page.

Antibody-panel (BC = Beckman Coulter, BD = Becton Dickinson, BL = BioLegend):

1.	CD2	Alexa Fluor 647	clone LT2	Serotec, MCA1194A647
2.	CD3	Alexa Fluor 488	clone HIT3a	BL, 300319
3.	CD8α	PE	clone RPA-T8	BD, 555367
4.	CD14	PerCP	clone MoP9	BD, 345786

Procedure

a. The staining buffer contains PBS + 2% HS. Alternatively, the Brilliant Stain Buffer (BD, catalog # 563794) can be used.

b. Pellet the cells by centrifugation at 300*g* for 8 min. Aspirate the supernatant and resuspend the cells at 5×10^5 cells/mL (200 μL) in staining buffer. Seed 100 μL per sample into a 96-well plate or flow cytometry tubes. Add 100 μL of either staining buffer or antibody mix according to manufacturer's instructions to the cells. Incubate at RT for 20 min. Wash twice with 200 μL staining buffer by centrifugation at 300*g* for 5 min. Resuspend in 200 μL of staining buffer and proceed with flow cytometry analysis.

2.6 Generation of mature DC

Pure T-cells by themselves showed poor proliferative capacity after thawing as remaining myeloid cells do not survive the process. To overcome this problem, we generate and freeze autologous mature DC. These are thawed on the day of the proliferation assay (Section 2.7). This section describes the generation of mature DCs from monocytes over 7 days.

a. The complete medium for this section is RPMI 1640 + 10% FBS + 2 mM L-glutamine + 30 μg/mL penicillin + 100 μg/mL streptomycin. The staining buffer contains PBS + 2% HS.

Fig. 4 Assessment of T-cell and monocyte purity by flow cytometry after isolation. (A) Diagram of the separation workflow to obtain autologous T-cells and monocytes from whole blood. (B) Analysis of the expression of CD2 (NK and T-cells) and CD14 (monocytes) by PBMC and subsequently isolated populations. (C) Expression of CD3 (T-cells) and CD8 (NK and T-cells) by PBMC and subsequently isolated populations.

b. Use fresh $CD14^+$ monocytes (Section 2.4). Count the cells and resuspend them at 1×10^6 cells/mL in complete medium pre-warmed to 37 °C.

c. Gently seed 1 mL ($=1 \times 10^6$ cells) per well into a 12-well tissue culture plate.

d. Gently add 1 mL of complete medium supplemented with 200 ng/mL GM-CSF (final concentration: 100 ng/mL) and 20 ng/mL IL-4 (final concentration: 10 ng/mL). Incubate the cells at 37 °C, 5% CO_2 for 2 days.

e. On the second day of incubation, gently add 2 mL complete medium supplemented with 100 ng/mL GM-CSF (final concentration: 50 ng/mL) and 20 ng/mL IL-4 (final concentration: 10 ng/mL). Incubate the cells at 37 °C, 5% CO_2 for 3 days.

f. On the fifth day of incubation, add 50 ng/mL GM-CSF, 10 ng/mL IL-4 and 1 μg/mL biomunyl (Zambon, G00132) or 1 μg/mL LPS (Sigma) directly. Incubate the cells at 37 °C, 5% CO_2 for 2 days.

g. On the seventh day of incubation, visually inspect the cells for morphology using light microscopy. The cells should be non-adherent to the plastic and display dendrites.

h. Gently harvest the cells in 50 mL Falcons using 5 mL pipettes. Wash each well with 1 mL RPMI 1640. Count the cells and wash by centrifugation at 300*g* for 8 min.

i. Resuspend the cells at 1×10^6 cells/mL in RPMI 1640. Remove 5×10^5 cells for phenotypic assessment by flow cytometry. Freeze the rest at −80 °C as described in Section 2.8.2.

j. To control the cells' phenotype, pellet the cells by centrifugation at 300*g* for 8 min. Aspirate the supernatant and resuspend the cells at 2.5×10^5 cells/mL (200 μL) in staining buffer. Seed 100 μL per sample into a 96-well plate or flow cytometry tubes. Add 100 μL of either staining buffer or antibody mix to the cells. Incubate on ice for 20 min. Wash twice with 200 μL staining buffer by centrifugation at 300*g* for 5 min. Resuspend in 200 μL staining buffer and proceed with flow cytometry analysis.

k. Use own flow cytometry systems, colors and compensation controls. This laboratory uses a BD Fortessa and Diva software.

l. The mature dendritic cells are $CD3^-$, $CD19^-$ and $CD14^-$, and $CD1a^+$, $CD11b/c^+$ and $CD83/86^+$ (Fig. 5).

m. Samples

i. Unstained control

ii. Stained sample

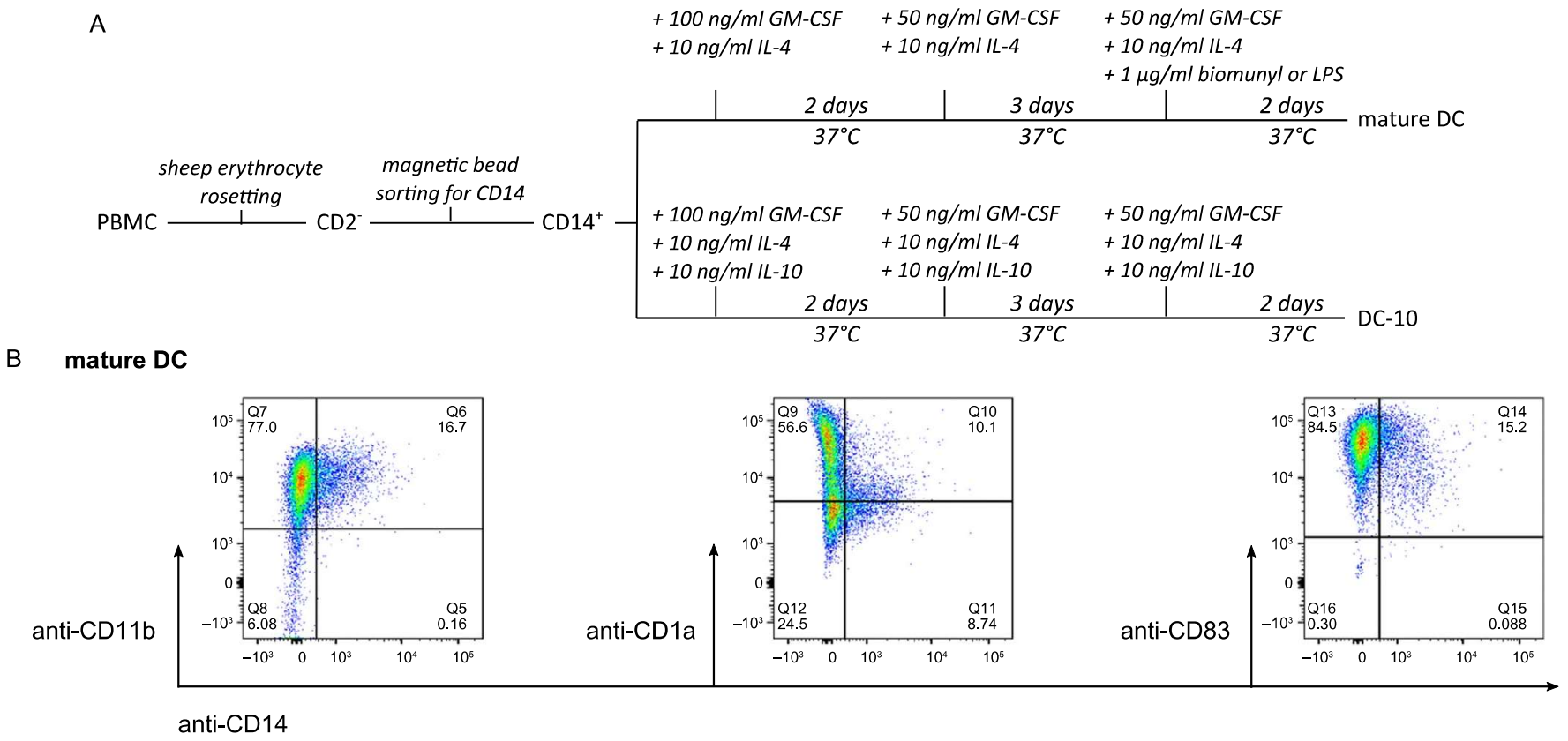

Fig. 5 Phenotypes of mature DC and DC-10s. (A) Workflow of DC differentiation protocol. Phenotypes of (B) mature DC and (C) DC-10 after 7 days of differentiation.

C **DC-10**

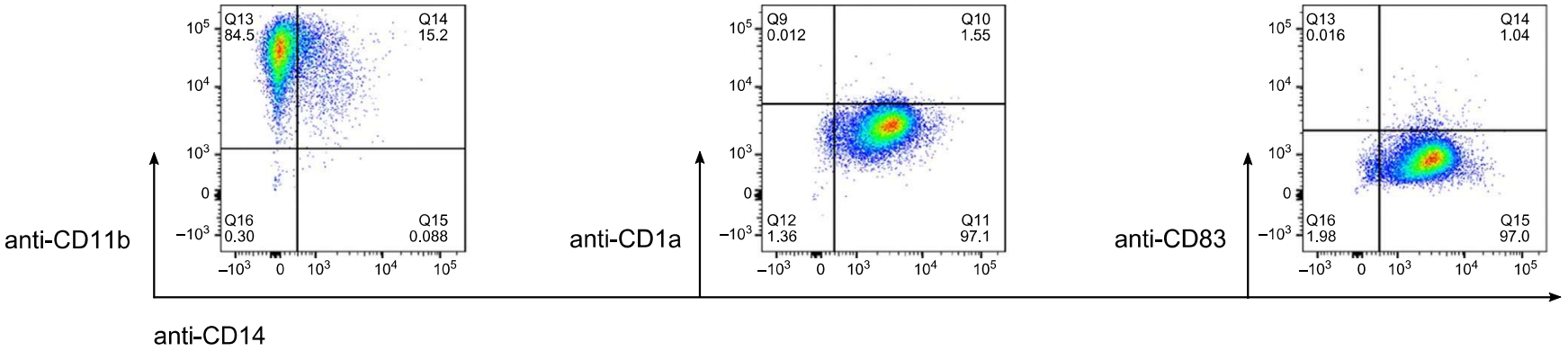

Fig. 5—Cont'd

Antibody-panel (BC = Beckman Coulter, BD = Becton Dickinson, BL = BioLegend):

1.	CD3 (lineage control—T-cells)	Alexa Fluor 488	clone HIT3a	BL, 300319
2.	CD19 (lineage control—B-cells)	BV510	clone SJ25C1	BD, 562947
3.	CD1a	PE-Cy5	clone HI149	BD, 555808
4.	CD11b	PE	clone ICRF44	BD, 555388
5.	CD14	APC-Cy7	clone HCD14	BL, 325619
6.	CD83	APC	clone HB15a	BC, IM2218

2.7 Immunosuppressive DC-10

We recommend validating the proliferation assay with immunosuppressive DC-10 as a suppression control. Thus, if cells isolated as MDSC are not suppressive in the system, the procedure itself can be excluded as a cause. This section describes the generation of immunosuppressive DC-10 from monocytes (Gregori et al., 2010).

a. The complete medium for this section is RPMI 1640 + 10% FBS + 2 mM L-glutamine + 30 µg/mL penicillin + 100 µg/mL streptomycin. The staining buffer contains PBS + 2% HS.

b. Use the freshly $CD14^+$ monocytes (Section 2.4). Count the cells and resuspend them at 1×10^6 cells/mL in complete medium pre-warmed to 37 °C.

c. Gently seed 1 mL (=1×10^6 cells) per well into a 12-well tissue culture plate.

d. Gently add 1 mL complete medium supplemented with 200 ng/mL GM-CSF (final concentration: 100 ng/mL), 20 ng/mL IL-4 (final concentration: 10 ng/mL) and 20 ng/mL IL-10 (final concentration: 10 ng/mL, Invitrogen). Incubate the cells at 37 °C, 5% CO_2 for 2 days.

e. On the third day of culture, gently add 2 mL complete medium supplemented with 100 ng/mL GM-CSF (final concentration: 50 ng/mL), 20 ng/mL IL-4 (final concentration: 10 ng/mL) and 20 ng/mL IL-10 (final concentration: 10 ng/mL) without further manipulation to avoid cell activation. Incubate the cells at 37 °C, 5% CO_2 for additional 2 days.

f. On the fifth day of culture, add 50 ng/mL GM-CSF, 10 ng/mL IL-4 and 10 ng/mL IL-10 directly to the wells without further manipulation to avoid cell activation. Incubate the cells at 37 °C, 5% CO_2 for additional 2 days.

g. On the seventh day of culture, visually inspect the cells for morphology using light microscopy. The cells should be non-adherent to the plastic or other cells, and display dendrites.

h. Gently harvest the cells into 50 mL Falcons using 5 mL pipettes. Wash each well with 1 mL RPMI 1640. Count the cells and wash by centrifugation at 300*g* for 8 min.

i. Resuspend the cells at 1×10^6 cells/mL in RPMI 1640. Remove 5×10^5 cells for phenotypic assessment by flow cytometry. Freeze the rest at −80 °C as described in Section 2.8.2.

j. To control the DC-10 phenotype, pellet the cells by centrifugation at 300*g* for 8 min. Aspirate the supernatant and resuspend the cells at 2.5×10^5 cells/mL (200 µL) of staining buffer. Seed 100 µL per sample into well of a plate or flow cytometry tubes. Add 100 µL of either staining buffer or antibody mix to the cells. Incubate on ice for 20 min. Wash twice with 200 µL of staining buffer by centrifugation at 300*g* for 5 min. Resuspend in 200 µL of staining buffer and proceed with flow cytometry analysis.

k. Use own flow cytometry systems, colors and compensation controls. This laboratory uses a BD Fortessa and Diva software.

l. The DC-10 cells are $CD3^-$, $CD19^-$, $CD1a^-$, and $CD14^+$ $CD83^+$ $CD86^+$, $CD11b^+$ and $CD11c^+$ (Fig. 5).

m. Samples

i. Unstained control

ii. Stained sample

Antibody-panel (BC = Beckman Coulter, BD = Becton Dickinson, BL = BioLegend):

1.	CD3 (T cells)	Alexa Fluor 488	clone HIT3a	BL, 300319
2.	CD19 (B cells)	BV510	clone SJ25C1	BD, 562947
3.	CD1a	PE-Cy5	clone HI149	BD, 555808
4.	CD11b	PE	clone ICRF44	BD, 555388
5.	CD14	APC-Cy7	clone HCD14	BL, 325619
6.	CD83	APC	clone HB15a	BC, IM2218

2.8 Freezing and thawing of cells

In a multi-center study, the MDSC should ideally be the only variable parameter. To achieve this, the T-cells and mature DC isolated in Sections 2.3 and 2.6 are shared with each laboratory. Here, we describe the freezing and thawing protocols.

2.8.1 T-cells

a. Prepare freezing serum, which is composed of HS + 20% DMSO, and store it at 4 °C. Use the freezing serum at 4 °C.
b. Count T-cells (refer to Section 2.3) and centrifuge cells at 400*g* for 7 min.
c. Resuspend cells in cold RPMI without serum at 10^7 cells/mL.
d. Dilute the cell suspension 1:1 with freezing serum for a final concentration of 5×10^6 cells/mL.
e. Quickly transfer 1 mL to each cryovial.
f. Transfer the vials to −80 °C. We advise keeping the freezing process as temperature-controlled as possible. To achieve that, we recommend using Mr. Frosty™ Freezing Container (Thermo Scientific™ 5100-0001).
g. Thaw the T-cells the day before the planned proliferation assay. Rest the cells at 37 °C, 5% CO_2 overnight. Use FBS-coated 15 mL Falcon tubes and 48-well tissue culture plates.
h. For thawing the cells, use 12 mL pre-warmed RPMI 1640 + 5% HS + 30 µg/mL penicillin + 100 µg/mL streptomycin + 5 U/mL DNase I (Worthington) + 5 U/mL IL-2.
i. Thaw the cells half-way by holding the cryovial into a warm water-bath. Resuspend the half-thawed drop-wise cells in 10 mL warm medium.
j. Centrifuge the cells at 300*g* for 8 min. Aspirate the supernatant and resuspend the cells in 1 mL medium +5 U/mL IL-2. Transfer the cells into a 48-well plate and incubate at 37 °C, 5% CO_2 overnight.

2.8.2 Dendritic cells

a. Prepare freezing serum, which is composed of FBS + 20% DMSO and store it at 4 °C. Use freezing serum at 4 °C.
b. Count DC (refer to Sections 2.6 and 2.7) and centrifuge cells at 400*g* for 7 min.
c. Resuspend cells in cold RPMI without serum at 1×10^6 cells/mL.
d. Dilute the cell suspension 1:1 with freezing serum for a final concentration of 5×10^5 cells/mL.
e. Quickly transfer 1 mL to each cryovial.

f. Transfer the vials to −80 °C. We advise keeping the freezing process as temperature-controlled as possible. To achieve that, we recommend using Mr. Frosty™ Freezing Container (Thermo Scientific™ 5100-0001).
g. Transfer cells into liquid nitrogen after 24 h.
h. Thaw the mature dendritic cells on the day of the planned proliferation assay. Rest the cells at 37 °C, 5% CO_2 for at least 4 h before use. Use FBS-coated 15 mL Falcon tubes and 48-well tissue culture plates.
i. For thawing the cells, use 12 mL pre-warmed RPMI 1640 + 10% FBS + 30 µg/mL penicillin + 100 µg/mL streptomycin + 5 U/mL DNase I.
j. Thaw the cells half-way by holding the cryovial into a warm water-bath. Resuspend the half-thawed cells drop-wise in 10 mL warm medium.
k. Centrifuge the cells at 300*g* for 8 min. Aspirate the supernatant and resuspend the cells in 1 mL medium supplemented with 50 ng/mL GM-CSF and 10 ng/mL IL-4 for mature DC, and 50 ng/mL GM-CSF, 10 ng/mL IL4 and 10 ng/mL IL-10 for DC-10, respectively. Transfer the cells into a 48-well plate and incubate at 37 °C, 5% CO_2 for at least 4 h.

3. MDSC isolation

The aim of the protocol presented is to compare the suppressive function of MDSC isolated from different tissues and pathologies and in different research groups. Since there is no single marker that distinguishes MDSC, the isolation strategies reported vary greatly among laboratories. Here, we describe the methods used by Annika Bruger and Pierre van der Bruggen (Brussels) to isolate MDSCs from the blood, ascites and tumor tissue of cancer patients. Each of these tissues requires slightly different preparation steps, e.g., tumors or tumor biopsies specifically need to be dissociated. All isolation methods require density gradient separation steps to isolate low-density granulocytic MDSC from high-density granulocytes.

The MDSC are sorted from the tissue samples by flow cytometry (Section 3.4). The same panel of markers and gating strategy for all tissue samples should be used. The anti-CD206 marker for macrophages can be replaced with an additional granulocytic marker, anti-CD66b, in blood samples. This serves as an extra check on the identity of granulocytic MDSC.

3.1 MDSC preparation from blood

Human MDSC are most commonly isolated from the blood of cancer patients. The MDSC are isolated from the mononuclear layer following a density gradient separation. Furthermore, no CD45 stain is necessary to

isolate hematopoietic cells. Additional cell types such as dendritic cells or early MDSC could be isolated instead. However, the sample sizes obtainable with blood are usually small, ranging from 20 to 100 mL, and MDSC presence in blood is rare (0.01–0.1% of $CD45^+$ cells) (own observations). This limits the yields of MDSC and thus the range of viable subsequent experiments.

a. Dilute whole blood 1:3 with PBS + 1 mM EDTA at room temperature. Do not use cold PBS + 1 mM EDTA as this might activate granulocytes and gMDSC.

b. Perform density gradient separation on the PBMC suspension as previously described in Section 2.1, steps b and c.

c. Following the density gradient separation, the PBMC settle in a thin layer below the serum, and above the Lymphoprep solution and erythrocyte pellet (Fig. 2). Carefully remove only the PBMC layer with a pipette and place it into an FBS-coated 50 mL Falcon tube.

d. Wash the cells with PBS + 1 mM EDTA and centrifuge at 300*g* for 10 min.

e. The PBMC settle in the pellet. Carefully remove the supernatant and as much of the white platelet ring covering the PBMC pellet as possible.

f. Repeat the washing steps with 50 mL PBS + 1 mM EDTA twice. Resuspend the PBMC a third time and centrifuge them at 200*g* for 3 min if platelets still remain.

g. Refer to MDSC staining protocol in Section 3.4.

3.2 MDSC preparation from ascites

Some cancers, in particular ovarian cancers, lead to an accumulation of fluid, called ascites, in the abdomen of patients in later stages of disease. In the clinic, ascites are usually collected and discarded. Ascites can be obtained in large volumes (up to 10 L, own experience) and can contain up to one trillion cells (including tumor cells). This enhances the MDSC yields obtainable. Cells from ascites are harvested by centrifugation and the MDSC are isolated from the mononuclear layer following density separation. A stain for CD45 (hematopoietic cells) is required, as epithelial or cancer cells could be present. Furthermore, due to the advanced stage of disease in most patients, patient consistency is difficult to maintain.

a. Pour the ascitic fluid into 200 mL flasks. Pellet the cells by centrifugation at 400*g* for 25 min. Aspirate the supernatant and repeat the harvesting step until no ascitic fluid remains.

b. Pool the cells, collect them in an FBS-coated 50 mL Falcon tube and wash with PBS + 1 mM EDTA and centrifuge at 400*g* for 8 min. Do not use cold PBS + 1 mM EDTA, as this might activate granulocytes and gMDSC.
c. Should the pellet contain many erythrocytes, lyse them with 3 mL 0.2% NaCl by resuspending the cells in the solution carefully and thoroughly. After 50–60 s of lysis reconstitute osmolarity with 7 mL 1.2% NaCl.
d. Resuspend the cells in warm RPMI 1640 to a total volume of 35 mL.
e. Perform density gradient separation on the cell suspension as previously described in Section 2.1, steps b and c.
f. Following the density gradient separation, the mononuclear cells settle in a thin layer below the medium, and above the Lymphoprep solution and erythrocyte pellet (in ascites this can vary in size, depending on how many erythrocytes are suspended in the ascitic fluid). Carefully remove only the PBMC layer with a pipette and place it into an FBS-coated 50 mL Falcon tube.
g. Wash the cells with warm PBS + 1 mM EDTA and centrifuge at 300*g* for 10 min.
h. The PBMC settle in the pellet. Carefully remove the supernatant.
i. Repeat the washing steps with 50 mL PBS + 1 mM EDTA twice.
j. Refer to staining protocol in Section 3.4.

3.3 MDSC preparation from solid tissue

Samples from tumors or tumor biopsies are rare, difficult to obtain and usually small, which limits the expected MDSC yield. Moreover, solid tissue samples require the most manipulation for MDSC isolation, as the tissue has to be gently dissociated. MDSC are isolated from the mononuclear fraction following density gradient separation. A stain for CD45 is required, as epithelial or cancer cells could be present.

a. We work with a hospital in very close proximity, and we receive tumor samples within 30 min after extraction. The specimens are placed in a 50 mL flask in PBS and transported on ice.
b. Place the tumor and any accompanying PBS into a sterile Petri dish on ice underneath a flow hood. Cut the tumor into very small pieces (1–2 mm) using a sterile scalpel. The pieces should be small enough to not block a 10 mL pipette.

c. Place the tumor pieces and fluid into a MACS dissociator C tube (Miltenyi, catalog ♯ 130-093-237). Add DNase I at 5 U/mL and Liberase™ TL and DL (thermolysin-dispase-low, both Sigma and Roche, catalog ♯ 05401020001 and LIBDL-RO) at 0.5 and 0.26 U/mL, respectively.

d. Dissociate the tumor cells using the pre-set program "h_tumor_01" on the gentleMACS™ dissociator (Miltenyi, catalog ♯ 130–093-235) for 45 s.

e. Incubate the tumor cells in the dissociator tube under agitation for 45 min at 37 °C.

f. Dissociate the tumor cells using the dissociator's pre-set program "h_tumor_01" for 45 s.

g. Transfer the tumor cells into an FBS-coated 50 mL Falcon tube. Rinse the dissociation tube with equal volume of human serum and add this to the tumor cells in the Falcon tube. The human serum stops the digestion by the liberase. Pellet the cells by centrifugation at 400*g* for 10 min.

h. Resuspend the tumor cells with 50 mL PBS + 1 mM EDTA per Falcon tube and pass the cells successively through a 70 and 40 μm filters, washing the cells by centrifugation at 400*g* for 8 min in between. Count and collect the cells through centrifugation at 400*g* for 8 min.

i. Resuspend the cells in warm RPMI 1640 to a total volume of 35 mL.

j. Perform density gradient separation on the cell suspension as previously described in Section 2.1, steps b and c.

k. Following the density gradient separation the mononuclear cells settle in a thin layer below the medium, and above the Lymphoprep solution and erythrocyte pellet (which is expected to be small in tumors). Carefully remove only the PBMC layer with a pipette and place it into an FBS-coated 50 mL Falcon tube.

l. Wash the cells with warm PBS + 1 mM EDTA and centrifuge at 300*g* for 10 min.

m. The PBMC settle in the pellet. Carefully remove the supernatant.

n. Repeat the washing steps with 50 mL PBS + 1 mM EDTA twice.

o. Refer to MDSC staining protocol in Section 3.4.

3.4 MDSC isolation by flow cytometry sorting

No single marker specific for MDSC has yet been discovered. Gating and isolation strategies for human MDSC thus vary widely within the literature. Bronte et al. (2016) proposed general and minimal guidelines for the detection and isolation of human MDSC. Working along these guidelines, we present here the gating and sorting strategy used in the van der Bruggen

group as an example. Up to four populations can be sorted simultaneously by flow cytometry with a FACS Aria III (Fig. 6).

a. The staining buffer contains PBS + 2% HS. Alternatively, the Brilliant Stain Buffer (BD, catalog# 563794) can be used.

b. Count the cells and resuspend in staining buffer +20% FcR blocking reagent (Miltenyi, catalog ♯ 130-059-901) in 50 μL per 10^7 cells.

c. While the cells are incubating with the FcR blocking reagent prepare the staining mix for the MDSC isolation in 50 μL of staining buffer per 10^7 cells.

d. Adapt the panel to in-house requirements for machine settings and compensation matrices. Shown below is the panel used in the van der Bruggen group (Brussels) as an example.

e. Samples

i. Unstained control

ii. FMO controls

iii. Full stain

Antibody-panel (BC = Beckman Coulter, BD = Becton Dickinson, BL = BioLegend):

1.	Live cell stain	efluor 780		Affimetrix
2.	Lineage stain:			
	CD3 (T-cells)	BV510	clone HIT3a	BD 564713
	CD19 (B-cells)	BV510	clone SJ25C1	BD 562947
	CD56 (NK cells)	BV510	clone NCAM16.2	BD 563041
3.	HLA-DR	PE-Cy7	clone L243	BL 307616
4.	CD11b	APC	clone ICRF44	BD 550019
5.	CD14	PerCP	clone MΦP9	BD 345786
6.	CD15	PE	clone HI98	BD 555402
7.	CD16	BV421	clone 3G8	BD 562878
8.	CD33	BV711	clone WM53	BD 563171
9.	CD45	Alexa-Fluor 700	clone HI30	BD 304024
10.	CD66b (blood)	FITC	clone 80H3	BC IM0531U
	or CD206 (tissues)	FITC	clone 19.2	BD 551135

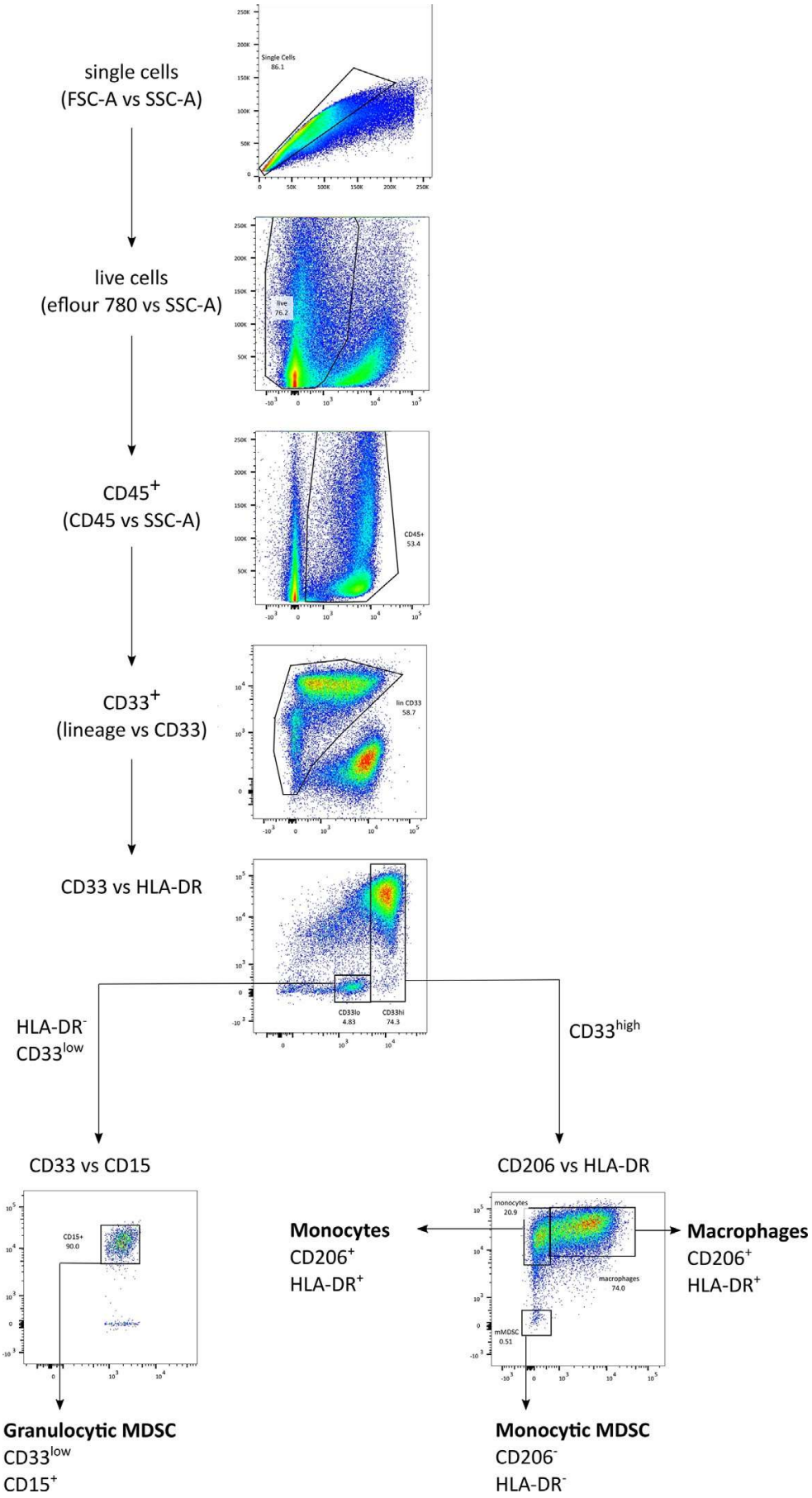

Fig. 6 MDSC gating and sorting strategy. Gating strategy employed in van der Bruggen laboratory in Brussels to isolate MDSC, monocytes and macrophages from blood, tumor and ascites. Further markers, such as CD11b, CD14, CD16 and CD66b, are used but not shown.

f. Incubate cells with 50 µL antibody mix per 10^7 cells (total: 100 µL) at RT in the dark for 20 min.
g. Wash the cells twice by centrifugation at 300*g* for 7 min with staining buffer.
h. Resuspend the cells at 500 µL per 10^7 cells or according to the sorter's cell density specifications in staining buffer.
i. This laboratory uses a BD FACS Aria III on the lowest speed setting with the largest nozzle, and Diva software.
j. For the gating strategy refer to Fig. 6.
k. Collect the MDSC, monocytes and macrophages in FACS tubes pre-coated with 1 mL FBS. Refer to Table 3 for an example of the yields obtainable from all three tissues from one ovarian cancer patient. Mind factors that could influence cell purity after the sorting procedure, including isolating multiple cell types simultaneously, debris, and cancer cells.
l. Use MDSC immediately. The cells, particularly gMDSC, cannot be frozen and have a short life span ex vivo.

4. Labeling of T-cells with fluorescent tracking dye

T-cell proliferation can be assessed in various ways. The most popular methods are labeling T-cells with radioactive thymidine or with fluorescent tracking dyes. For multi-center studies, we propose to use the latter. The T-cells are labeled with tracking dyes such as CFSE or CMFDA at the

Table 3 Example of monocytic cell yields from one ovarian cancer patient.

		Blood	Tumor	Ascites
Cells sorted	*(input)*	18 million	50 million	15 million
Monocytes	Yield	72,900	12,300	40,100
	Purity (% $CD45^+$)	*91%*	*92%*	*91%*
Macrophages	Yield	–	377,000	24,100
	Purity (% $CD45^+$)	–	*96%*	*93%*
Monocytic MDSC	Yield	487	6400	1200
	Purity (% $CD45^+$)	–	*38%*	–
Granulocytic MDSC	Yield	39,700	60,500	24,800
	Purity (% $CD45^+$)	*72%*	*88%*	*85%*

beginning of the experiment. Similar chemical properties mean these dyes are highly suitable to track cell proliferation. First, acetate groups on the dyes allow their entrance into the cells. Within the cytosol, esterases cleave off acetate groups on the dyes, rendering them cell impermeable. Second, the dyes covalently bond to several cytosolic proteins, including some with low turnover rates. Both events result in a stable high fluorescence within the cells. With each division, the dyes' intensities are halved between the two daughter cells. Thus, progressive rounds of division can be monitored by flow cytometry through the dilution of the tracking dye. With optimal labeling, up to eight peaks (or seven rounds of division) can be observed with CFSE (Baker, Sullam, & Levin, 1997; Quah & Parish, 2012; Quah, Warren, & Parish, 2007). T-cells can divide once every 24 h (own observation). In our experience, the best results are obtained after 4 days of incubation, or about three rounds of divisions. At this point, the dye dilution results in nicely separated peaks and proliferating cells are not depleted of nutrients yet.

a. Use T-cells isolated in Section 2.3 and thawed 1 day in advance as described in Section 2.8.

b. Complete medium is RPMI 1640 + 5% HS + 30 µg/mL penicillin + 100 µg/mL streptomycin + 5 U/mL IL-2.

c. Count the T-cells. Collect the cells in a 15 mL Falcon tube and pellet the cells by centrifugation at 300*g* for 8 min. Discard the supernatant and resuspend the cells in warm RPMI 1640 at a concentration of 2×10^6 cells/mL.

d. Dilute the tracking dye (we use CMFDA, Life Technologies, at 0.5 µM/mL final concentration) in warm RPMI 1640. Optimize to own specific flow cytometry settings.

e. Add diluted tracking dye to the T-cells at a ratio of 1:1. Mix gently and incubate for 20 min at 37 °C in the dark. Mix gently occasionally.

f. Fill the Falcon tube with complete medium and wash the labeled T-cells once by centrifugation at 300*g* for 8 min. Resuspend the T-cells at 2×10^6 cells/mL in complete medium. Rest the cells at 37 °C, 5% CO_2 until seeding for the proliferation assay (proceed to Section 5.2).

5. Preparation of assay

T-cell proliferation assays contain many variables that diverge widely among different laboratories. The possible variables include the type of assay

plate, the type of medium, the amount of IL-2 supplemented, the type of stimulation used, and the type of T-cell chosen (Bruger et al., 2019). We present our protocol set-up for multi-center use next.

5.1 Preparation of the plate for T-cell stimulation

a. Complete medium consists of RPMI 1640 + 5% HS + 30 µg/mL penicillin + 100 µg/mL streptomycin + 5 U/mL IL-2. Filter medium and warm to 37 °C before use. No supplementation with arginine, asparagine and tryptophan is required since RPMI 1640 already contains concentrations of the amino acids exceeding natural conditions. Low amounts of IL-2 are added to prevent the T-cells from starving of the cytokine, and to avoid an IL-2 sink effect.

b. The proliferation assay is assembled from components prepared at different times. The wells of the assay contain in 200 µL total (Fig. 7):
   - **i.** *T-cells*: labeled, 30,000 or 50,000 per well (Section 4)
   - **ii.** *Mature DC*: 1000 per well (Sections 2.6 and 2.8)
   - **iii.** *anti-CD28 antibodies*: soluble, 2 µg/mL final concentration (Section 5.1, step h)
   - **iv.** *MDSC/myeloid cells/DC-10*: variable concentrations (Section 3)

c. The T-cells are stimulated through anti-human CD3 (clone OKT3, BioLegend, catalog # number 317315) and anti-CD28 (clone CD28.2, BD Pharmingen, catalog # 555725) antibodies. These antibodies target the TCR and the co-stimulatory molecule CD28, respectively. The anti-CD3 antibody is immobilized to the tissue culture plate as T-cells require the stable cross-linking of TCRs and prolonged dwell times for activation. The anti-CD28 antibody is supplied in solution.

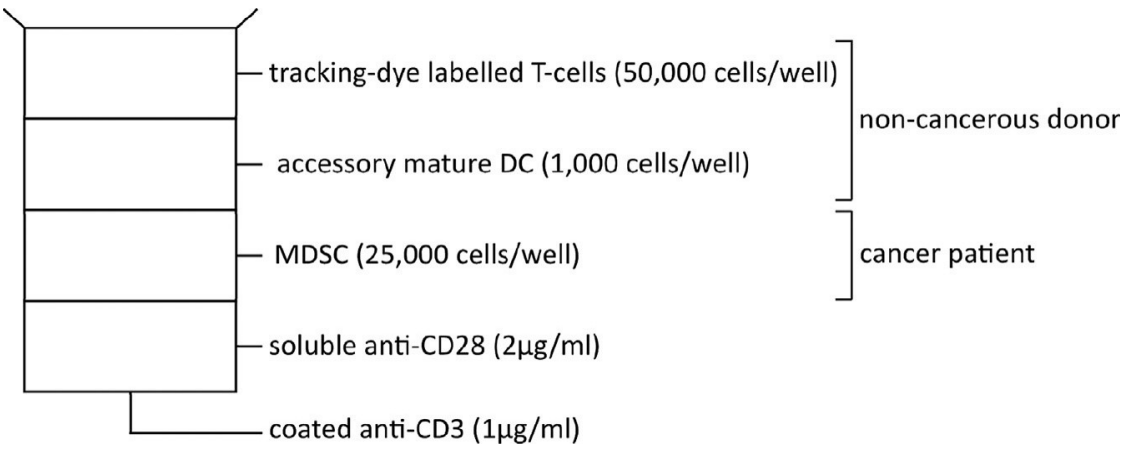

Fig. 7 Proliferation assay well design.

Controls

d. Control wells consist of the following:
   - **i.** No stimulation, no MDSC, no accessory mature DC
   - **ii.** No stimulation, no MDSC, plus accessory mature DC
   - **iii.** Anti-CD3-anti-CD28 stimulation, no MDSC, no accessory mature DC
   - **iv.** Anti-CD3-anti-CD28 stimulation, no MDSC, plus accessory mature DC

e. Add complete medium to control wells instead of stimulation or myeloid cells.

Anti-CD3 stimulation

f. Coat the wells of the flat-bottomed 96-well tissue culture plate by seeding 1 μg/mL anti-CD3 (clone: OKT3) diluted in sterile PBS at 100 μL/well. Use sterile PBS only for control wells without stimulation. Incubate the plate at 37 °C for at least 4 h. Keep sterile.

g. Gently remove all solution from the wells before seeding cells or anti-CD28 antibodies. Do not wash wells.

Anti-CD28 stimulation

h. Dilute anti-CD28 in pre-warmed complete medium to a concentration of 8 μg/mL (4 × final concentration = 2 μg/mL) and seed 50 μL/well. Use complete medium without anti-CD28 antibody in control wells.

5.2 Cells

T-cells

a. Count tracking dye-labeled T-cells (Section 4) and resuspend at $1\times$ or 0.6×10^6 cells/mL in complete medium. Seed at 30,000 or 50,000 cells/well.

Mature dendritic cells

b. Count the mature DC thawed and rested in Section 2.8. Harvest 2×10^5 mature DC into a FBS-coated 15 mL Falcon tube and collect by centrifugation at 300*g* for 8 min. Resuspend the cells in 10 mL complete medium (20,000 cells/mL). Seed the cells at 1000 cells/well. Use complete medium instead of myeloid cells in control wells.

Isolated MDSC, monocytes and macrophages

c. Use count provided by sorting machine.

d. Pellet the MDSC in the FACS tubes by centrifugation at 300*g* for 8 min. Transfer, handle and centrifuge the cells as little as possible to avoid activating or losing the cells.

e. Resuspend the cells in complete medium as previously calculated. Seed the cells at desired concentration (e.g., 25,000 cells/well). Use complete medium instead of MDSC in control wells.

6. MDSC-T-cell co-culture and analysis of suppression

6.1 Incubation

a. The cells are co-incubated for 4 days at 37 °C, 5% CO_2. After 4 days about three cycles of divisions have been concluded.

b. The tracking dye is diluted by 50% with each cycle of division but remains constant in non-proliferating cells. Since flow cytometry analysis is usually performed on log scales, 2–3 division cycles result in a dilution of the tracking dye that provides a good separation between signals from proliferating and non-proliferating cells. Too few division cycles result in higher concentrations of the tracking dye in proliferating cells, which could make difficult separating proliferating from non-proliferating peaks. Too many division cycles result in a larger separation of the peaks, but also in higher cell concentrations per well and nutrient depletion from the medium. This could result in increased cell death.

c. Proceed to reading the results by flow cytometry (Section 6.2).

6.2 Flow cytometry analysis

a. Inspect wells visually by light microscopy.

b. The following procedures are not performed under sterile conditions.

c. Dilute FcR blocking reagent 1:10 in PBS + 2% HS. Add 20 µL per well and incubate on the bench for 5 min.

d. In the meantime, prepare the antibody master mix in PBS + 2% HS. Calculate adding a total volume of 20 µL per 200 µL well. Previously test optimal antibody dilutions or refer to manufacturer's instructions.

1.	Live cell stain	e.g., efluor 780		Affimetrix
2.	CD3	PE	clone UCHT1	BD 555333
3.	CD8	APC	clone RPA-T8	BD 555369
4.	Sphero™ Rainbow Calibration Particles			BD 556288

e. The Rainbow beads are added as normalization constant at 5000 beads/well.

f. Add 20 µL of antibody master mix per well. Mix by transferring cells to a 96-well plate adequate for flow cytometry acquisition. Incubate at room temperature in the dark for 15 min.

g. Wash twice by centrifugation at 300*g* for 5 min and resuspend cells in 200 µL PBS + 2% HS.

h. Proceed to flow cytometry acquisition according to in-house machine specifications and settings. This laboratory uses a Fortessa flow cytometer and Diva software.

6.3 Data analysis

The van der Bruggen laboratory (Brussels) uses FlowJo software v10.0.7 (Treestar, Ashland, OR, USA) for flow cytometry data analysis and Excel (Microsoft) for further analysis and presentation of numeric results. Alternatively, FlowJo's incorporated proliferation modeling tool and the associated proliferation index are commonly used for analysis.

Flow cytometry analysis (on FlowJo)

a. Use compensation controls acquired to create compensation matrix.

b. Gate successively for the lymphocytes (FSC/SSC), single cells (FSC-A/FSC-H), live T-cells (viability dye/anti-CD3), and the proliferating cells (tracking dye/histogram). Finally, gate on proliferating (tracking dye^{low}) and non-proliferating (tracking dye^{high}) populations (Fig. 8). An additional step for the CD8^{+} T-cells can be inserted (anti-CD8/anti-CD3), followed by the assessment of the tracking dye dilution.

c. Create a gate for the Rainbow beads.

d. Apply the gates to all samples. Control all samples and the imposed gating. Correct if necessary.

e. Extract numerical data for the following parameters for each sample (e.g., as an Excel file):

- **i.** Number of Rainbow beads.
- **ii.** Number of live T-cells.
- **iii.** Number of non-proliferating cells (tracking dye high).
- **iv.** Median of non-proliferation cells (tracking dye high).
- **v.** Number of proliferating cells (tracking dye low).
- **vi.** Median of proliferation cells (tracking dye low).

Proliferation assay data analysis

a. Import FlowJo data as described in previous section:

- **i.** Number of Rainbow beads
- **ii.** Number of live T-cells

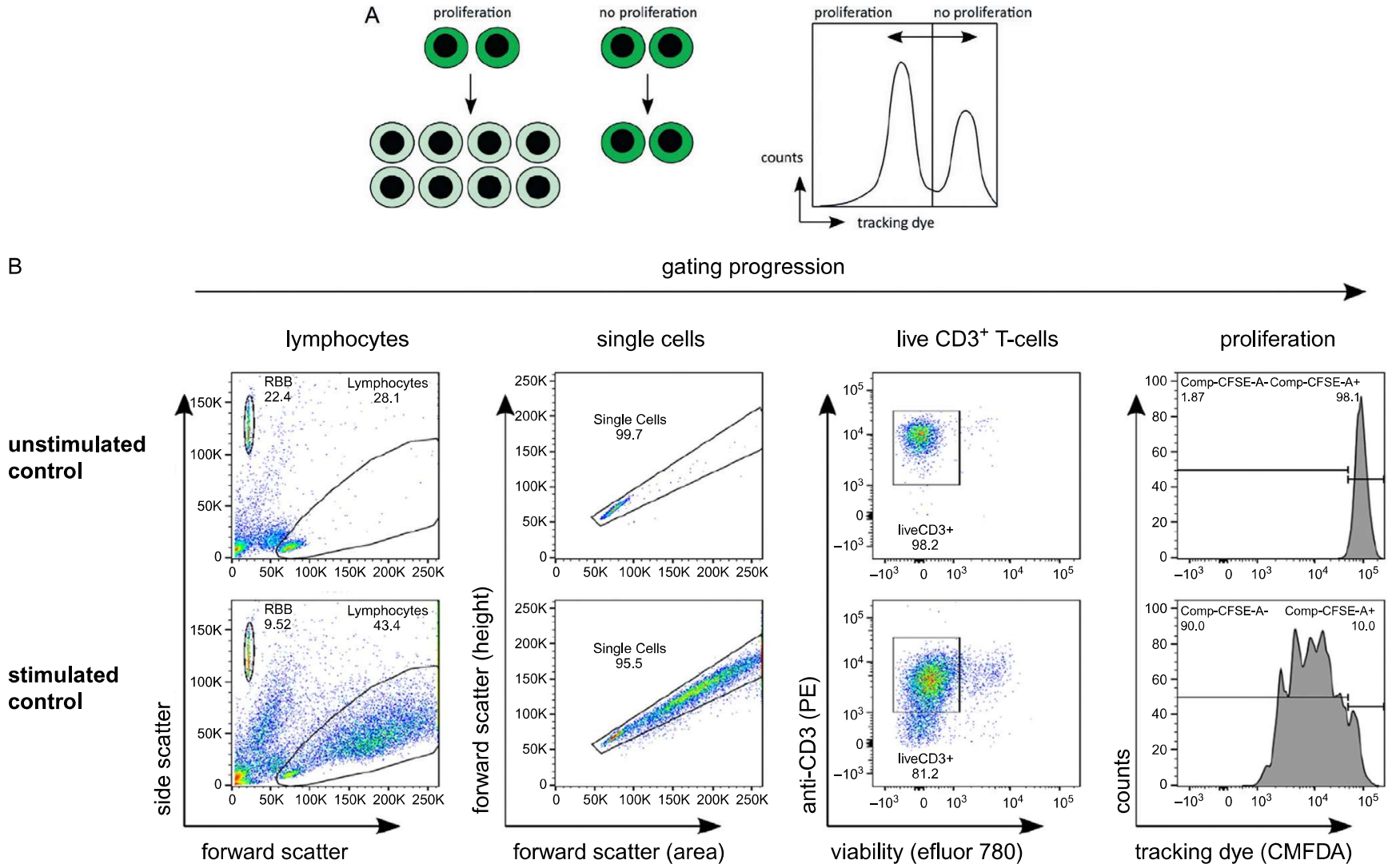

Fig. 8 Example of flow cytometry analysis of proliferation assay results. (A) Principle of proliferation assay. T-cells are labeled with a tracking dye (e.g., CFSE or CMFDA). The intensity of the dye decreases by a factor of two with each division within proliferating cells. Non-proliferating cells retain high concentrations of the dye. The results are acquired using flow cytometry. (B) Flow cytometry gating strategy. After 4 days the T-cells are stained with a viability dye, and anti-CD3 and anti-CD8 antibodies. First, lymphocytes are selected according to their cell size. Next, single cells and then live $CD3^+$ cells are selected. Finally, proliferation is assessed by tracking dye dilution. Extra steps can be inserted to focus on $CD8^+$ or $CD8^-$ cells specifically. (C) Example of the flow cytometry analysis of a MDSC-suppression assay.

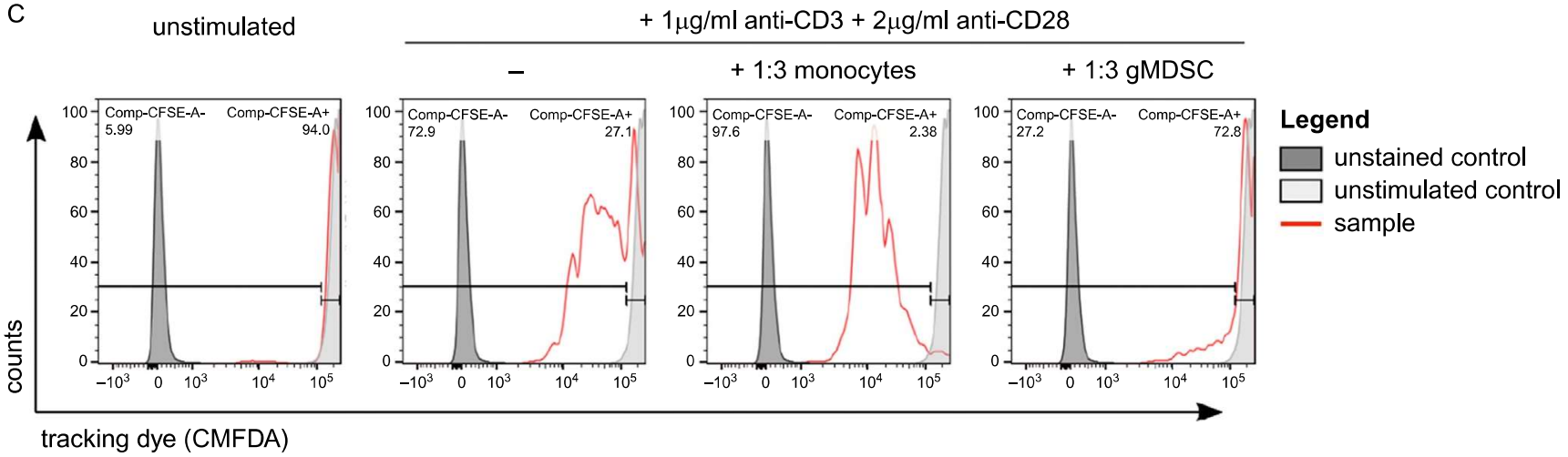
C
unstimulated
+ 1μg/ml anti-CD3 + 2μg/ml anti-CD28
–
+ 1:3 monocytes
+ 1:3 gMDSC
Comp-CFSE-A-
Comp-CFSE-A+
5.99
94.0
72.9
27.1
97.6
2.38
27.2
72.8
counts
tracking dye (CMFDA)
Legend
unstained control
unstimulated control
sample

Fig. 8—Cont'd

iii. Number of non-proliferating cells (tracking dye^{high}).
iv. Median of non-proliferation cells (tracking dye^{high}).
v. Number of proliferating cells (tracking dye^{low}).
vi. Median of proliferation cells (tracking dye^{low}).

b. Normalize the total live cell numbers, and the proliferating and non-proliferating counts to the 5000 Rainbow beads added per sample.

i.

$$cell\ numbers = \frac{total\ lymphocyte\ count}{bead\ count} \times 10{,}000\ (\text{ref : total bead number})$$

c. Calculate:

i.

$$number\ of\ divisions = log2\left(\frac{median\ non-proliferating\ cells}{median\ proliferating\ cells}\right)$$

ii.

$$\#of\ initial\ cells\ that\ have\ undergone\ division = \frac{count\ proliferating\ cells}{2^{number\ of\ divisions}}$$

iii.

$$\%\ of\ initial\ cells\ that\ have\ divided = \frac{count\ proliferating\ cells}{2^{number\ of\ divisions}} \Bigg/ \left(count\ of\ non-proliferating\ cells + \frac{count\ proliferating\ cells}{2^{number\ of\ divisions}}\right) \times 100$$

(explanation: *initial cells that have divided/total number of cells at outset* × *100)*

d. Present data as (Fig. 9):

a. change in absolute cell numbers.
b. number of divisions over time.
c. percentage of dividing cells.

Comparing different experiments

a. To compare results from separate experiments set the proliferation of the stimulation controls (T-cells, + stimulation, no myeloid cells) as 1.

b. Normalize all other results to the control.

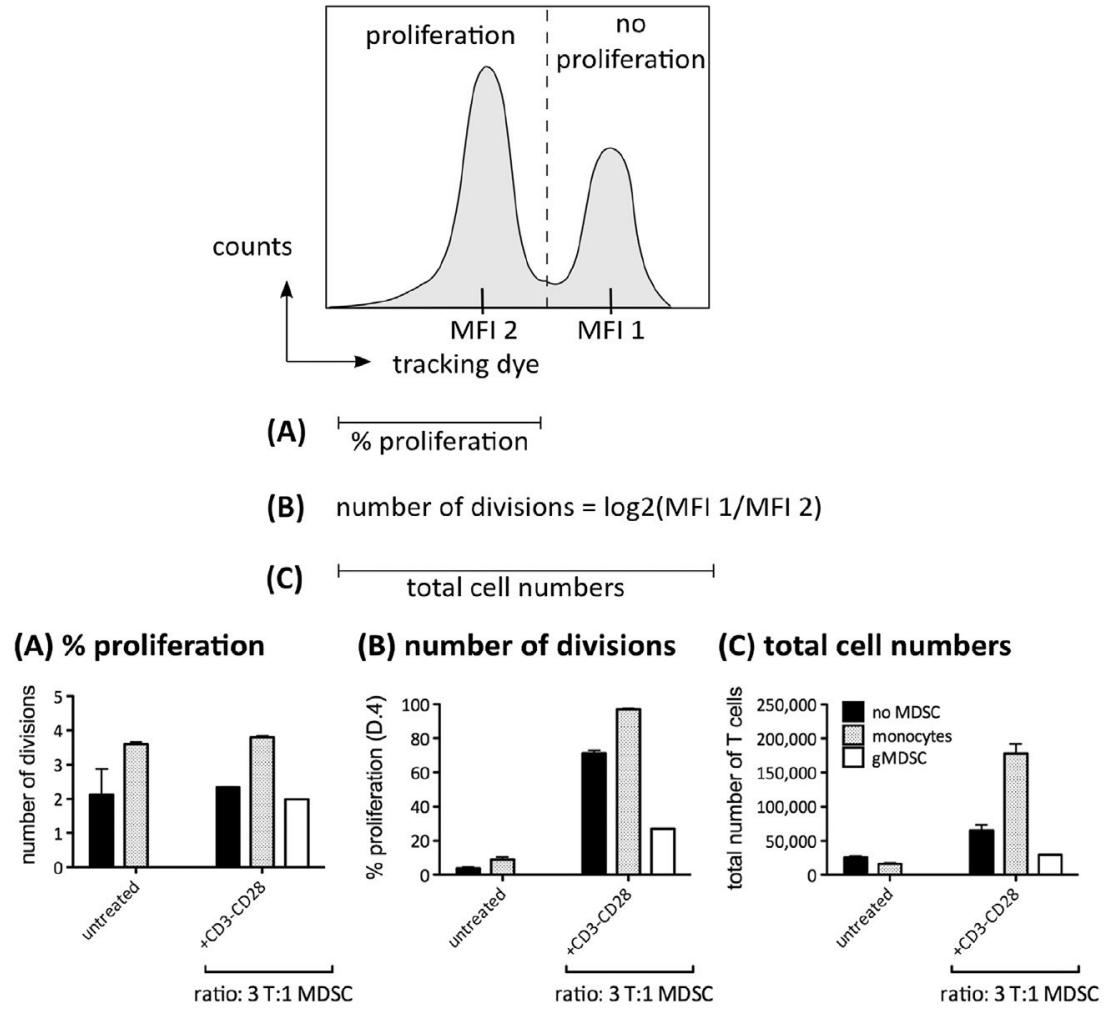

Fig. 9 Example of further analysis of proliferation assay results. Flow cytometry data is exported into excel files or similar. MDSC-mediated suppression of T-cell proliferation can then be assessed using different parameters. (A) The percentage of T-cells that proliferated. This can be calculated for the cells acquired at the end of the assay (Day 4, shown here) or calculated back to represent the fraction of the original cells in the assay (Day 0). (B) Fewer divisions can indicate that the cell cycle progression in inhibited. (C) The total number of cells present within wells after 4 days can indicate unexpected levels of apoptosis, either by strong levels of proliferation that depletes nutrients from the medium, or by interactions with myeloid cells.

7. Concluding remarks

The method presented here offers an approach to assess the suppressive functions of human MDSC through T-cell proliferation assays that can be applied to multi-center studies. At present, comparing studies of human MDSC functions is difficult due to the variation in the procedures of both MDSC isolation and function (Bruger et al., 2019). The reliable comparison

of the function of MDSC isolated from different sources and in different research groups requires the MDSC themselves to be the only variable within the assay. We achieve this by isolating T-cells from allogeneic non-cancerous donors in one laboratory, freezing the cells and shipping them to participating groups. However, the isolated T-cells are poor proliferators after thawing. We solved this issue by the addition of low numbers of allogeneic in vitro generated mature DC that restore T-cell proliferation. Moreover, mature DC can be aliquoted, frozen, shared and thawed. Further aspects including the type of consumables and antibodies are standardized within the group. However, best shipping conditions for the cells still need to be determined to successfully validate the method in partner laboratories.

Acknowledgments

This chapter follows work supported by grant #7571 from the Région Wallonne Program BIOWIN: Convention IT-TARGETS (Belgium) and grant #1610119 from the Région Wallonne Program WALINNOV: Convention IMMUCAN (Belgium). Pierre van der Bruggen was supported by research program PDR T.0232.19 from the "Fonds de la Recherche Scientifique" (Belgium). Christophe Vanhaver was supported by a PhD fellowship grant from FRIA/FNRS (Belgium).

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