CHAPTER TEN

Flow cytometry-assisted analysis of phenotypic maturation markers on an immortalized dendritic cell line

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Methods in Cell Biology, Volume 189
ISSN 0091-679X
https://doi.org/10.1016/bs.mcb.2024.05.008

Abstract

Dendritic cells (DCs), and especially so conventional type I DCs (cDC1s), are fundamental regulators of anticancer immunity, largely reflecting their superior ability to engulf tumor-derived material and process it for cross-presentation on MHC Class I molecules to $CD8^+$ cytotoxic T lymphocytes (CTLs). Thus, investigating key DC functions including (but not limited to) phagocytic capacity, expression of CTL-activating ligands on the cell surface, and cross-presentation efficacy is an important component of multiple immuno-oncology studies. Unfortunately, DCs are terminally differentiated cells, implying that they cannot be propagated indefinitely *in vitro* and hence must be generated *ad hoc* from circulating or bone marrow-derived precursors, which presents several limitations. Here, we propose a simple, cytofluorometric method to quantify phenotypic activation markers including CD80, CD86 and MHC class II molecules on the surface of a conditionally immortalized immature DC line that can be indefinitely propagated *in vitro* but also driven into maturation at will with a simple change in culture conditions. Upon appropriate scaling and automatization, this approach is compatible with high-throughput screening programs for the discovery of novel DC activators that do not suffer from batch variability and other limitations associated with the generation of fresh DCs.

1. Introduction

Dendritic cells (DCs) are major regulators of the interaction between the innate and adaptive branch of the immune response (Cabeza-Cabrerizo et al., 2021). Indeed, immature DCs constantly probe their surroundings by constantly taking up soluble material and (relatively small) particulate, processing it in the endosomal compartment and presenting it on the cell surface in association with MHC molecules (Blander, 2018; Jhunjhunwala et al., 2021; Yang et al., 2023). DCs—especially so-called type I conventional DCs (cDC1s) (Hildner et al., 2008; Johnson et al., 2022; Theisen et al., 2018)—are particularly efficient antigen-presenting cells (APCs) as they display peptides derived from extracellular material not only on MHC Class II molecules for presentation to $CD4^+$ helper (T_H) cells (as most APCs do) but also on MHC Class I molecules for presentation to $CD8^+$ cytotoxic T lymphocytes (CTLs), a process that is commonly known as antigen cross-presentation (Blander, 2018; Cruz et al., 2017; Yin et al., 2021). Cross-presentation (which generally occurs in secondary or tertiary lymphoid organs) is particularly important for the activation of both natural and therapy-driven tumor-targeting immune responses against malignant cells (Heras-Murillo et al., 2024; Jhunjhunwala et al., 2021; Woo et al., 2015; Yatim et al., 2017). In line with this notion, considerable preclinical and

clinical efforts are being dedicated at the development of immunotherapeutic strategies that, directly or indirectly, harness the immunostimulatory potential of DCs (Kyrysyuk & Wucherpfennig, 2023; Palucka & Banchereau, 2012), including (but not limited to) approaches involving the administration of exogenous or release of endogenous DC stimulators such as Toll-like receptor (TLR) agonists (Hennessy et al., 2010; Le Naour & Kroemer, 2023; Vacchelli et al., 2013a; Vanpouille-Box et al., 2019) and immunostimulatory cytokines (Deckers et al., 2023; García-Martínez et al., 2018; Mempel et al., 2024), as well as the use of patient-derived DCs as therapeutic vaccines upon *ex vivo* expansion and exposure to autologous or heterologous tumor-derived material plus immunostimulatory cues (Cultrara et al., 2023; Fucikova et al., 2022; Harari et al., 2020; Vacchelli et al., 2013b; Wculek et al., 2020).

To elicit potent anticancer immune responses, DCs including cDC1s must expose not only MHC Class I and II molecules, but also a series of T cell costimulating ligands on their surface, including (but not limited to) CD80, CD83 and CD86 (Jhunjhunwala et al., 2021). Such a mature configuration—which is generally associated with the expression of chemokine receptors guiding DCs to secondary or tertiary lymphoid organs (Worbs et al., 2017)—is indeed fundamental for (cross)presentation to result in robust T cell priming rather than anergy, which would instead promote immunological tolerance (Jhunjhunwala et al., 2021; Kroemer et al., 2022). Importantly, DC maturation can be elicited by a number of signals that operate as pattern recognition receptor (PRR) agonists, including pathogen-derived molecules commonly known as microbe-associated molecular patterns (MAMPs), as well as endogenous molecules that are released or exposed by stressed and dying (cancer) cells, which are commonly referred to as damage-associated molecular patterns (DAMPs) (Brubaker et al., 2015; Chen & Nuñez, 2010; Gong et al., 2020; Kroemer et al., 2022; Marchi et al., 2023). The spatiotemporally ordered emission of DAMPs by dying cancer cells is fundamental for their death to be perceived by DCs as immunogenic, and hence culminate with the initiation of a tumor-specific immune response (Kroemer et al., 2022).

Based on these observations, it is not surprising that numerous immuno-oncology studies involve the assessment of one or more DC functions. Unfortunately, DCs are terminally differentiated cells, implying that they cannot be propagated indefinitely *in vitro* but must be purified or generated *ad hoc* from circulating or bone marrow-derived precursors (Cerrato et al., 2020; Hong & Dobrovolskaia, 2024; Isnard et al., 2020). Besides being laborious and associated with a relatively poor yield, this approach suffers

from considerable batch variability, limiting its applicability to high-throughput screening platforms. A conditionally immortalized mouse DC line that can be propagated indefinitely *in vitro* but also differentiated at will *via* minimal changes in culture conditions that enable the expression of a potent oncogene (SV40 large T antigen) coupled to the repression of tumor protein p53 (TRP53, best known as p53) can at least in part circumvents this problem, *de facto* offering a tool that can be scalable for the high-throughput screening of novel molecules that influence DC functions (Zhao et al., 2021).

Here, we propose a simple, flow cytometry-assisted approach to quantify phenotypic activation markers including CD80, CD86 and MHC class II molecules on these conditionally immortalized DCs optionally exposed to maturation conditions.

2. Materials

2.1 Common disposables (*see Note 1*)

- Corning™ 96-Well, Cell Culture-Treated, V-Shaped-Bottom Microplate (#3897, Corning)
- Falcon® 12-well Clear Flat Bottom TC-treated Multiwell Cell Culture Plate, with Lid, Individually Wrapped, Sterile (#353043, Corning)
- Falcon® 15 mL High Clarity PP Centrifuge Tube, Conical Bottom, with Dome Seal Screw Cap, Sterile (#352196, Corning)
- Falcon® 5 mL Round-Bottom Polypropylene Test Tubes Without Cap (#352002, Corning)
- Falcon® 50 mL High Clarity PP Centrifuge Tube, Conical Bottom, Sterile (#352070, Corning)
- Nunc™ EasYFlask™ 75 cm^2 Cell Culture Flasks (#156499, Thermo Scientific)

2.2 Cells and reagents (*see Note 1*)

- APC/Cyanine7 anti-mouse CD11c Antibody (clone N418) (#117324, BioLegend)
- Bovine Serum Albumin (BSA) Powder—Low-Electrolyte Grade (#700-100P, GeminiBio)
- Corning™ 500 mL RPMI 1640 1× (#15-040-CV, Corning)
- Corning™ Phosphate-Buffered Saline (PBS), 1× without calcium and magnesium, pH 7.4 ± 0.1 (#21-040-CMR, Corning)

- Dexamethasone (Dex) (#02190040-*CF*, MP Biomedicals) (*see Note 2*)
- Doxycycline hyclate (Dox) (#D9891, Sigma-Aldrich) (*see Note 2*)
- eBioscience™ Foxp3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent (#00-5521-00, Thermo Scientific)
- eBioscience™ Permeabilization Buffer (10×) (#00-8333-56, Thermo Scientific)
- FITC anti-mouse CD86 Antibody (clone GL-1) (#105006, BioLegend)
- Foundation™ Fetal Bovine Serum (FBS) (#900-108-500, GeminiBio) (*see Note 3*)
- Gibco™ 2-Mercaptoethanol (#21985023, Thermo Scientific) (*see Note 4*)
- Gibco™ Gentamicin (50 mg/mL) (#15750078, Thermo Scientific)
- Gibco™ HEPES (1 M) (#15630080, Thermo Scientific)
- Gibco™ Penicillin-Streptomycin-Glutamine (100×) (#10378016, Thermo Scientific)
- Gibco™ Sodium Pyruvate (100 mM) (#11360070, Thermo Scientific)
- Gibco™ Trypsin-EDTA (0.5%), no phenol red (#15400054, Thermo Scientific)
- Inducible immortalized DCs (iniDCs) (Richter et al., 2013)
- PE/Cyanine7 anti-mouse CD80 Antibody (clone 16-10A1) (#104734, BioLegend)
- PerCP/Cyanine5.5 anti-mouse I-A/I-E Antibody (clone M5/114.15.2) (#107625, BioLegend)
- Recombinant Murine GM-CSF (#315-03, PeproTech) (*see Note 5*)
- TLR4 Agonist—Ultrapure lipopolysaccharide (LPS) from *E. coli* 055:B5 (#tlrl-pb5lps, InvivoGen)
- TruStain FcX™ (anti-mouse CD16/32) Antibody (#101319, BioLegend)
- Zombie aqua™ Fixable Viability Kit (#423102, BioLegend)

2.3 Equipment (*see Note 1*)

- Benchtop centrifuge, such as Allegra X-14R Centrifuge, 120 V, 60 Hz (#A99464, Beckman Coulter)
- Flow cytometer equipped with lasers for acquisition of SSC-H, FSC-H, BL1-H (488 nm laser, 530/30 filter), BL3-H (488 nm laser, 695/40 filter), BL4-H (488 nm laser, 780/60 filter), RL3-H (637 nm laser, 780/60 filter), VL2-H (405 nm laser, 512/25 filter), such as Attune NxT flow cytometer (Thermo Scientific).
- Humidified cell culture incubator, such as Heracell™ VIOS 160i CO2 Incubator (#51030285, Thermo Scientific)

- Laboratory biosafety cabinet (Class II), such as Purifier Logic+ Class II Type A2 Biological Safety Cabinet (#320419101, Labconco)

2.4 Software

- FlowJo v. 10.9.0 (FlowJo LLC)
- Excel (Microsoft)
- Prism v 10.0.1 (GraphPad)

3. Methods

3.1 *In vitro* de-immortalization and maturation of iniDCs

1. iniDCs are routinely expanded at 37 °C under 5% CO_2 in Nunc™ EasYFlask™ 75 cm^2 Cell Culture Flasks containing 22 mL RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.3 mg/mL glutamine, 50 μg/mL gentamicin, 5 mM HEPES, 1 mM sodium pyruvate and 50 μM 2-mercaptoethanol (complete culture medium), further supplemented with 50 μM 2-mercaptoethanol, 10 ng/mL recombinant GM-CSF, 100 nM Dex and 1 μg/mL Dox (to preserve immortalization).
2. Once iniDCs reach 70–80% confluence, exhausted culture medium is transferred to a 50 mL Falcon® tube and cells are gently washed with 5 mL pre-warmed PBS at 37 °C.
3. PBS is discarded and iniDCs are detached by adding 10 mL Gibco™ Trypsin-EDTA (0.5%) solution for 5 min at 37 °C under 5% CO_2 (*see Note 6*).
4. Once iniDCs are detached (*see Note 7*), Gibco™ Trypsin-EDTA (0.5%) solution is neutralized with 5 mL exhausted culture media and the cell suspension is transferred to a 50 mL Falcon® tube.
5. IniDCs are pelleted by centrifugation for 5 min at 300 *g*, followed by aspiration of supernatants and resuspension in 1 mL complete culture medium.
6. To propagated immortalized iniDCs, 5×10^6 cells are seeded in a new Nunc™ EasYFlask™ 75 cm^2 Cell Culture Flask with 22 mL pre-warmed complete culture medium supplemented with 50 μM 2-mercaptoethanol, 10 ng/mL recombinant GM-CSF, 100 nM Dex and 1 μg/mL Dox.
7. To generate de-immortalized iniDCs (de-iniDCs), 7×10^6 cells are seeded in a new Nunc™ EasYFlask™ 75 cm^2 Cell Culture Flask with 22 mL pre-warmed complete culture medium supplemented with

50 μM 2-mercaptoethanol and 20 ng/mL recombinant GM-CSF. Complete de-immortalization takes 3–5 days (*see Notes 8 and 9*).

8. For flow cytometry experiments, iniDCs or de-iniDCs are detached as detailed in steps 2–5, resuspended in complete medium (for iniDCs supplemented with 50 μM 2-mercaptoethanol, 10 ng/mL recombinant GM-CSF, 100 nM Dex and 1 μg/mL Dox; for de-iniDCs supplemented with 50 μM 2-mercaptoethanol and 20 ng/mL recombinant GM-CSF) at a density of 3×10^5 cells/mL and seeded in Falcon® 12-well Clear Flat Bottom TC-treated Multiwell Cell Culture Plates (1 mL/well)
9. Maturation of iniDCs and de-iniDCs is induced by adding 100 ng/mL ultrapure LPS to the culture medium for 24–72 h or any potential compound of interest (*see Note 10*).

3.2 Staining

1. Exhausted culture medium is transferred to a Falcon® 5 mL Round-Bottom Polypropylene Test Tube.
2. PBS is discarded and cells are detached by adding 1 mL Gibco™ Trypsin-EDTA (0.5%) solution for 5 min at 37 °C under 5% CO_2 (*see Note 11*).
3. Once cells are detached (*see Note 7*) Gibco™ Trypsin-EDTA (0.5%) solution is neutralized with 1 mL exhausted culture media and the cell suspension is transferred to a Falcon® 5 mL Round-Bottom Polypropylene Test Tube.
4. Cells are pelleted by centrifugation for 5 min at 300 g, followed by aspiration of supernatants and resuspension in 200 μL pre-warmed PBS and transferred to Corning™ 96-Well, Cell Culture-Treated, V-Shaped-Bottom Microplate (*see Note 12*).
5. Plates are centrifuged for 5 min at 300 *g* at room temperature and supernatants are discarded by inversion.
6. Each well is dispensed with 25 μL Zombie aqua™ Fixable Viability Kit (1:200 in PBS) and plates are incubated at 4 °C in the dark for 15 min, followed by rinsing with 175 μL pre-warmed PBS.
7. Plates are centrifuged for 5 min at 300 *g* at room temperature and supernatants are discarded by inversion.
8. Each well is dispensed with 25 μL Murine TruStain FcX™ (1:200 in PBS supplemented with 0.5% (*w/v*) BSA) and plates are incubated at 4 °C in the dark for 15 min.
9. Each well is dispensed with 25 μL PBS supplemented with 0.5% (w/v) BSA plus 1:25 (v:v) PE/Cyanine7 anti-mouse CD80 Antibody, 1:25

(v:v) FITC anti-mouse CD86 Antibody, 1:25 (v:v) APC/Cyanine7 anti-mouse CD11c Antibody and 1:312 (v:v) PerCP/Cyanine5.5 anti-mouse I-A/I-E Antibody and plates are incubated at 4 °C in the dark for 25 min (*see Notes 13 and 14*).

10. Each well is washed with 150 μL PBS supplemented with 0.5% (w/v) BSA and plates are centrifuged for 5 min at 300 *g* at room temperature.
11. Supernatants are discarded by inversion and cells are resuspended in 200 μL eBioscience™ Foxp3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent, followed by plate incubation at 4 °C in the dark for 30 min (*see Note 15*).
12. Plates are centrifuged for 5 min at 300 *g* at 4 °C, supernatants are discarded by inversion, and cells are resuspended in 200 μL 1× eBioscience™ Permeabilization Buffer.
13. Finally, plates are centrifuged for 5 min at 300 *g* at 4 °C, supernatants are discarded by inversion, and cells are resuspended in 200 μL PBS supplemented with 0.5% (w/v) BSA.

3.3 Acquisition and gating

1. Laser voltage (gain) and compensation are set on unstained cells as well as cells stained with each fluorochrome separately (*see Note 13*).
2. Acquire >10,000 events per sample, ideally at a flow rate <500 events/s (*see Notes 16 and 17*).
3. Gating should be performed as followed (Fig. 1):
   a. Cells are identified on FSC-H vs SSC-H dot plots, excluding events with low FSC-H and SSC-H (debris) (Fig. 1A).
   b. On cells, singlets are identified on FSC-A vs FSC-H dot plots, including events exhibiting a direct proportionality between these two parameters (Fig. 1B).
   c. On singlets, live cells are identified on FSC-H vs VL2-H dots plots, including events that stain negatively for Zombie aqua™ (Fig. 1C).
   d. On live cells, DCs are identified on FSC-H vs RL3-H dot plots, including events that stain positively for APC/Cy7 ($CD11c^+$) based on fluorescence minus one (FMO) controls (*see Note 13*) (Fig. 1D).
   e. On $CD11c^+$ DCs, MHC Class II-expressing cells are identified on FSC-H vs BL3-H dot plots, including events that stain positively for PerCP/Cy7 ($MHC\ II^+$) based on FMO controls (*see Note 13*) (Fig. 1E).

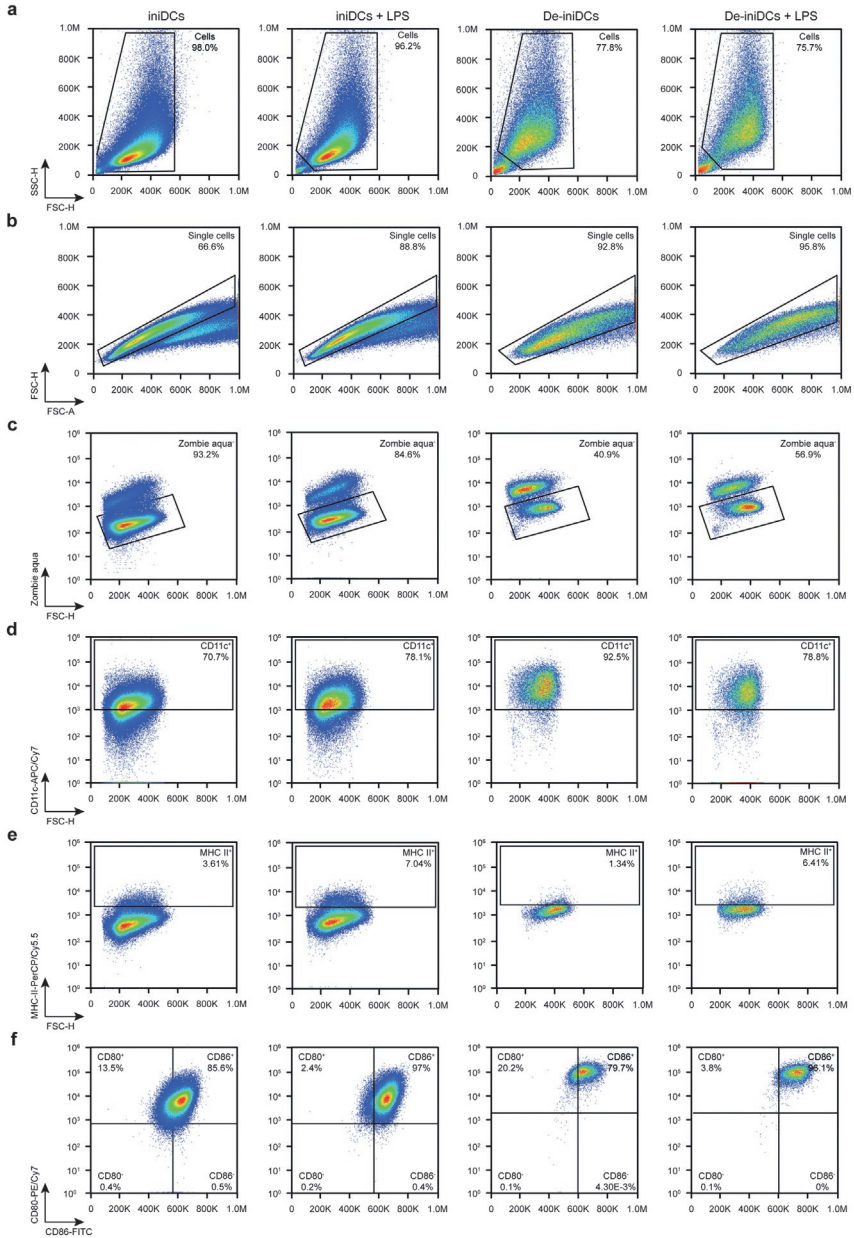

Fig. 1 Gating strategy. iniDCs were maintained in culture, optionally converted to de-iniDCs and exposed to lipopolysaccharide as detailed in Section 3.1, then stained with antibodies specific for CD11c, CD80, CD86 and MHC Class II molecules plus Zombie aqua as detailed in Section 3.2, followed by fluorescence acquisition and gating as detailed in Section 3.3. (A) Debris exclusion. (B) Singlet gating on cells. (C) Zombie aqua^{-} (live) cell gating on singlets. (D) CD11c^{+} (DC) cell gating on live cells. (E) MHC II^{+} cell identification on DCs. (F) CD80^{+} and/or CD86^{+} cell identification on DCs. Representative dot plots are shown, numbers indicate the percentage of events within each gate.

f. On $CD11c^+$ DCs, CD80- and/or CD86-expressing cells are identified on BL1-H vs BL4-H dot plots, including events that stain positively for FITC ($CD86^+$) and/or Pe/Cy7 ($CD80^+$) based on FMO controls (*see Note 13*) (Fig. 1F)

g. Data can be represented as percentage of positive cells (Figs. 1 and 2) and/or mean fluorescence intensity (MFI) for the entire cell population of interest (Fig. 2).

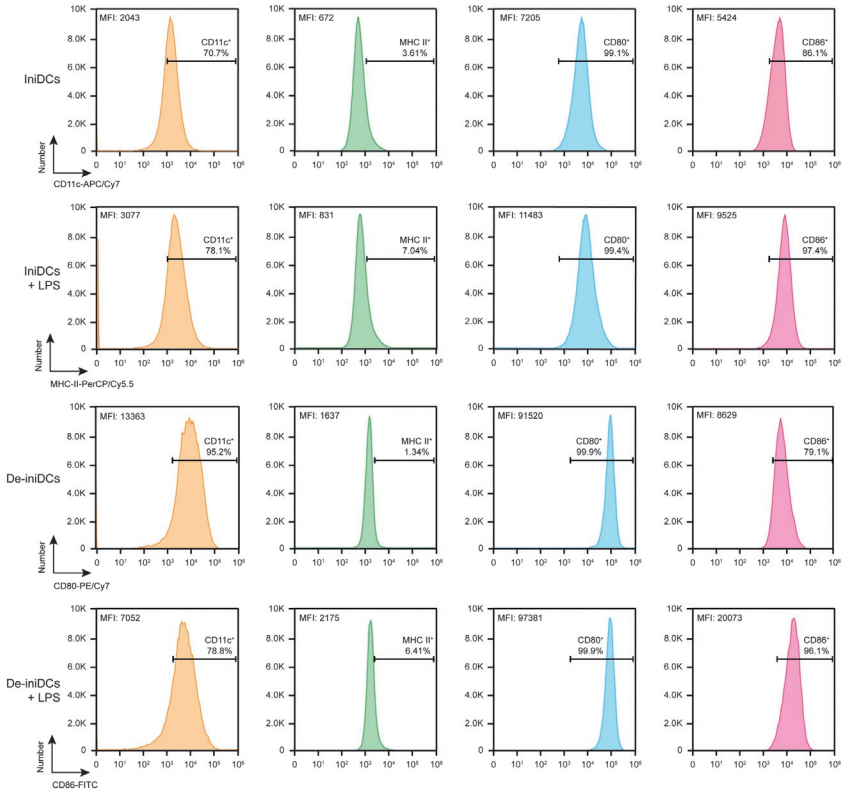

Fig. 2 Phenotypic maturation markers on iniDCs and de-iniDCs. iniDCs were maintained in culture, optionally converted to de-iniDCs and exposed to lipopolysaccharide as detailed in Section 3.1, then stained with antibodies specific for CD11c, CD80, CD86 and MHC Class II molecules plus Zombie aqua as detailed in Section 3.2, followed by fluorescence acquisition and gating as detailed in Section 3.3. Representative histograms are shown, numbers indicate the percentage of events within each gate and MFI for the entire cell population.

4. Notes

1. Virtually equivalent disposables, reagents and equipment can be purchased from alternative sources, generally at comparable cost.
2. The Dex and Dox stock solution (100 μM Dex plus 1 mg/mL Dox in PBS) is stable at 4 °C for a maximum of 3 months.
3. Prior to first use, FBS must be decomplemented at 56 °C for 30 min in a water bath. This prevents the potential activation of the complement system (Mastellos et al., 2024), which may have toxic effects of iniDCs and de-iniDCs.
4. 2-Mercaptoethanol can cause respiratory tract and skin irritations, and hence it should be manipulated with appropriate certified personal protective equipment (PPE).
5. Recombinant GM-CSF is stable at −20 °C but repeated freeze-thawing cycles should be avoided to minimize degradation.
6. Gibco™ CTS™ TrypLE™ Select Enzyme (#A1285901, Thermo Scientific) or other commercially available reagents can be employed as alternatives to Gibco™ Trypsin-EDTA (0.5%) solution.
7. As FBS leftover may inhibit the enzymatic activity of the Gibco™ Trypsin-EDTA (0.5%) solution, it is recommendable to visually ensure complete detachment by light microscopy before proceeding.
8. De-iniDCs stop proliferating 48 h upon Dex/Dox withdrawal.
9. De-iniDCs are larger than iniDCs and tend to exhibit increased adherence to the culture substrate, a phenotype that is partially lost upon LPS stimulation (*see also Note 11*)
10. IniDC maturation can be achieved as early as 12 h after incubation with the appropriate dose of LPS.
11. Falcon® Cell scrapers (#353085, Corning) may also be used to detach de-iniDCs after incubation in Gibco™ Trypsin-EDTA (0.5%) solution (*see also Note 9*).
12. Falcon® 5 mL Round-Bottom Polypropylene Test Tubes can also be employed for staining, adjusting total reagent volume to 200–250 μL per sample.
13. It is fundamental to include samples stained with single fluorochromes as well as FMO samples to adjust gain, compensation, and gating.
14. Staining samples in the dark is important to minimize fluorochrome photobleaching, which may negatively affect acquisition.

15. This step can also be performed at 4 °C in the dark overnight.
16. The acquisition of a high number of events per sample is important to enable proper gating and analysis.
17. An excessive flow rate may result in suboptimal acquisition, in turn negatively affecting gating and analysis.

5. Concluding remarks

The protocol described herein provides a straightforward approach to harness iniDCs and their de-immortalized counterparts to cytofluorometrically quantify phenotypic DC maturation markers including CD80, CD86 and MHC Class II molecules. This strategy has recently been harnessed with success to implement a CRISPR/Cas9 screening that unveiled the ability of BCL2 apoptosis regulator (BCL2) to endogenously suppress DC activation by preserving mitochondrial integrity and hence limiting the spontaneous release of interferogenic mitochondrial DNA species (Miller et al., 2021) in the cytosol (Kepp et al., 2023; Zhao et al., 2023). Of note, such a mitochondrial checkpoint appears to be a common mechanism that regulates not only adaptive immune responses elicited by DCs (Kepp et al., 2023; Zhao et al., 2023), but also cellular and organismal aging (Iske et al., 2020; Jiménez-Loygorri et al., 2024; Victorelli et al., 2023) as well as the ability of malignant cells to engage immune effectors upon treatment (Yamazaki et al., 2020), with an extra layer of negative regulation by apoptotic caspases (Han et al., 2020; Ning et al., 2019; Rodriguez-Ruiz et al., 2019; Rongvaux et al., 2014; White et al., 2014).

Importantly, iniDCs can also be harnessed to implement *in vitro* screenings for additional DC functions including phagocytic competence and cross-presentation proficiency, standing out as a convenient platform for the discovery of so-called immunogenic cell death (ICD) enhancers, i.e., molecules that improve the ability of dying cancer cells to elicit tumor-targeting immune responses by acting on DCs rather than malignant cells themselves (Galluzzi et al., 2024). Moreover, by offering access to a large and homogeneous amount of mature DCs, the iniDC platform enables a variety of *in vivo* assays, including prophylactic and therapeutic vaccination experiments in syngeneic mice optionally bearing specific genetic modifications or co-administrered with the pharmacological agent of choice (Galluzzi et al., 2024).

Perhaps the most important weakness of the iniDC platform is that (at least until now) it has only been implemented in mouse DCs. That said, while employing a Dox-inducible construct for the expression of the SV40

large T-antigen to conditionally immortalize human DC precursors (similar to the approach used to generated iniDCs) (Richter et al., 2013) would extend the *in vitro* screening potential offered by iniDCs to the human system, human DCs would remain incompatible with *in vivo* applications, with the partial (and hitherto underdeveloped) exception of humanized mice (Chuprin et al., 2023). Despite this (and potentially other) limitation(s), iniDCs stand out as preferential screening tools for the development of novel immunomodulatory agents with a broad range of applications.

Acknowledgments

We are indebted to Sebastian Thieme (University Clinic "Carl Gustav Carus" Dresden, Dresden, Germany) for the kind gift of iniDCs. O.K. is supported from Agence National de la Recherche (ANR) and Institut National du Cancer (INCa). G.K. is supported by the Ligue contre le Cancer (équipe labellisée); ANR—Projets blancs; AMMICa US23/CNRS UMS3655; Association pour la recherche sur le cancer (ARC); Cancéropôle Ile-de-France; Fondation pour la Recherche Médicale (FRM); a donation by Elior; Equipex Onco-Pheno-Screen; European Joint Programme on Rare Diseases (EJPRD); European Research Council Advanced Investigator Award (ERC-2021-ADG, ICD-Cancer, Grant No. 101052444), European Union Horizon 2020 Projects Oncobiome, Prevalung (Grant No. 101095604) and Crimson; INCa; Institut Universitaire de France; LabEx Immuno-Oncology ANR-18-IDEX-0001; a Cancer Research ASPIRE Award from the Mark Foundation; the RHU Immunolife; Seerave Foundation; SIRIC Stratified Oncology Cell DNA Repair and Tumor Immune Elimination (SOCRATE); and SIRIC Cancer Research and Personalized Medicine (CARPEM). This study contributes to the IdEx Université de Paris ANR-18-IDEX-0001. Views and opinions expressed are those of the author(s) only and do not necessarily reflect those of the European Union, the European Research Council or any other granting authority. Neither the European Union nor any other granting authority can be held responsible for them. The LG lab is/has been supported (as a PI unless otherwise indicated) by one NIH R01 grant (#CA271915), by two Breakthrough Level 2 grants from the US DoD BCRP (#BC180476P1, #BC210945), by a grant from the STARR Cancer Consortium (#I16-0064), by a Transformative Breast Cancer Consortium Grant from the US DoD BCRP (#W81XWH2120034, PI: Formenti), by a U54 grant from NIH/NCI (#CA274291, PI: Deasy, Formenti, Weichselbaum), by the 2019 Laura Ziskin Prize in Translational Research (#ZP-6177, PI: Formenti) from the Stand Up to Cancer (SU2C), by a Mantle Cell Lymphoma Research Initiative (MCL-RI, PI: Chen-Kiang) grant from the Leukemia and Lymphoma Society (LLS), by a Rapid Response Grant from the Functional Genomics Initiative (New York, US), by a pre-SPORE grant (PI: Demaria, Formenti), a Collaborative Research Initiative Grant and a Clinical Trials Innovation Grant from the Sandra and Edward Meyer Cancer Center (New York, US), by startup funds from the Department of Radiation Oncology at Weill Cornell Medicine (New York, US), by industrial collaborations with Lytix Biopharma (Oslo, Norway), Promontory (New York, US) and Onxeo (Paris, France), as well as by donations from Promontory (New York, US), the Luke Heller TECPR2 Foundation (Boston, US), Sotio a.s. (Prague, Czech Republic), Lytix Biopharma (Oslo, Norway), Onxeo (Paris, France), Ricerchiamo (Brescia, Italy), and Noxopharm (Chatswood, Australia).

Competing interests

O.K. is a scientific cofounder of Samsara Therapeutics. G.K. is on the Board of Directors of the Bristol Myers Squibb Foundation France. G.K. is a scientific cofounder of everImmune, Osasuna Therapeutics, Samsara Therapeutics and Therafast Bio. G.K. is in the scientific advisory boards of Hevolution, Institut Servier, Longevity Vision Funds and Rejuveron Life Sciences. G.K. is the inventor of patents covering therapeutic targeting of aging, cancer, cystic fibrosis and metabolic disorders. G.K.'s wife, Laurence Zitvogel, has held research contracts with Glaxo Smyth Kline, Incyte, Lytix, Kaleido, Innovate Pharma, Daiichi Sankyo, Pilege, Merus, Transgene, 9m, Tusk and Roche, was on the Board of Directors of Transgene, is a cofounder of everImmune, and holds patents covering the treatment of cancer and the therapeutic manipulation of the microbiota. G.K.'s brother, Romano Kroemer, was an employee of Sanofi and now consults for Boehringer-Ingelheim. The funders had no role in the design of the study; in the writing of the manuscript, or in the decision to publish the results. LG is/has been holding research contracts with Lytix Biopharma, Promontory and Onxeo, has received consulting/advisory honoraria from Boehringer Ingelheim, AstraZeneca, OmniSEQ, Onxeo, The Longevity Labs, Inzen, Imvax, Sotio, Promontory, Noxopharm, EduCom, and the Luke Heller TECPR2 Foundation, and holds Promontory stock options. The other authors have no conflicts of interest to declare.

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