Deciphering neutrophil heterogeneity in human blood and tumors: Methods for isolating neutrophils and assessing their effect on T-cell proliferation

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Methods in Cell Biology
ISSN 0091-679X
https://doi.org/10.1016/bs.mcb.2024.10.010

Abstract

Neutrophils were historically considered a homogenous population of cells with functions limited to innate immunity against external threats. However, with the rise of immunotherapy, recent works have shown that neutrophils are also important actors in immuno-oncology. In this context, neutrophils appear as a more heterogenous population of cells. However, many reported neutrophil subpopulations, or neutrophils with various transcriptional states, lack functional characterization to confirm their suspected roles. Thus, we believe that functional assays remain essential to define the role of neutrophils in cancer. In this chapter, we present a T-cell proliferation assay based on the use of allogeneic T-cells to assess the suppressive capabilities of neutrophils isolated from human blood or tumor samples. Allogeneic T-cells are isolated in large quantities from the blood of non-cancerous donors and frozen in aliquots to be used in several experiments. This reduces variability by excluding other cancer-derived factors, which would be present if autologous T-cell were used and allows to isolate the effect of neutrophils on T-cell proliferation. Thawed T-cells have poor proliferative capacities and to initiate proliferation they require co-culture with mature dendritic cells that we generate from monocytes isolated from the same blood sample. Initially developed for lung cancer patients, our method to isolate low-density neutrophils (LDN) and normal-density neutrophils (NDN) can be used with any patient and adapted to other kind of samples (e.g., ascites, urine, …).

Abbreviations

AET	2-(2-aminoethyl) isothiourea dihydrobromide
CFSE	carboxyfluorescein succinimidyl ester
CMFDA	5-chloromethylfluorescein diacetate
DC	dendritic cell
DL	dispase-low
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FcR	Fc receptor
gMDSC	granulocytic myeloid-derived suppressor cell(s)
HS	human serum
IL	interleukin
LDN	low-density neutrophils
NDN	normal-density neutrophils
NK	natural killer
NSCLC	non-small cell lung cancer
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline

PolyIC	polyinosinic-polycytidylic acid
PVA	polyvinyl alcohol
RNAseq	RNA sequencing
RT	room temperature
TAN	tumor-associated neutrophils
TL	thermolysin-low
#	number of

1. Introduction

Historically, neutrophils have been characterized as a homogeneous, terminally differentiated cell population with a primary role in innate immunity against external threats (Ackerman, 1964; Cavaillon, 2011; Kay, 2016; LoBue, 1973; Perry, 1971). The first description of neutrophils in tumors (tumor-associated neutrophils or TAN) dates back to 1863, but their role in cancer was long overlooked (Balkwill & Mantovani, 2001). In recent decades, the rise of immunotherapy and the growing interest in immuno-oncology have driven research on neutrophils in cancer patients. These studies revealed that neutrophils exhibit a variety of roles in this context, which put into question their perceived homogeneity. For instance, a higher abundance of TAN has been correlated with reduced survival (Gentles et al., 2015), but they can also stimulate T-cell response (Eruslanov et al., 2014). Blocking their recruitment into non-small cell lung cancer (NSCLC) with a leukotriene B4 antagonist did not improve patients' survival (Jänne et al., 2014).

In the blood of cancer patients, one sign of neutrophil heterogeneity emerged with the identification of "low-density neutrophils" (LDN), distinct from the "classical" and denser "normal-density neutrophils" (NDN). Initially, LDN were described as a single population related to neutrophils but distinguished by their ability to suppress T-cell proliferation *in vitro* and were referred to as granulocytic myeloid-derived suppressor cells (gMDSC) (Gabrilovich, 2017). However, the view of immunosuppressive LDN and non-suppressive NDN has been repeatedly proven inaccurate since then (Pettinella et al., 2024; Pillay, Tak, Kamp, & Koenderman, 2013; Shaul et al., 2020; Vanhaver et al., 2023). Recent advancements, including single-cell RNA sequencing (RNAseq) and the identification of new surface markers in both blood and tumor, further confirm this heightened level of neutrophil heterogeneity (Salcher et al., 2022; Shaul et al., 2020; Vanhaver et al., 2023; Zilionis et al., 2019).

These descriptive findings, especially those based on transcriptomic characterization, often lack information regarding the function and activities of neutrophil subpopulations. In the absence of definitive transcriptional signature predicting neutrophil function, *in vitro* assessment of neutrophil suppressive capacities remains necessary to advance our understanding of their exact roles and mechanisms. Progress in these areas is crucial for advancing immunotherapy. This functional approach has allowed us to differentiate LDN of lung cancer patients into a mature, $CD45^{high}$ $CD16^{high}$ subpopulation with suppressive capacities and an immature $CD45^{low}$ $CD16^{low}$ subpopulation without suppressive capacities (Vanhaver et al., 2023). A previous study in head and neck cancer patients showed that the mature $CD16^{high}$ LDN were the most suppressive (Pettinella et al., 2024).

It is important to note that neutrophils possess characteristics that distinguish them from most mononuclear cells and need to be considered when studying them. They are short-lived and cryosensitive (Lionetti, Hunt, Lin, Kurtz, & Valeri, 1977; Trellakis et al., 2013), meaning that *in vitro* functional assays require fresh samples. The choice of anticoagulant during blood collection can significantly affect the number of neutrophils isolated (Cassetta et al., 2020; Freitas, Porto, Lima, & Fernandes, 2008). When studying subpopulations of neutrophils, the numbers can be both low and highly variable, which can limit the experiments that are possible. In the case of LDN, they constitute only a small fraction of the total leukocyte population, and the separation into $CD45^{high}$ and $CD45^{low}$ LDN subpopulations typically results in each subpopulation representing between 0.1% and 5% of the low-density fraction of the blood, the peripheral blood mononuclear cells (PBMC) (Vanhaver et al., 2023). Additionally, sample processing can influence experimental results, as neutrophils can be activated by various physical or chemical stresses (Gordon, Papazaharoudakis, Sadlon, Arellano, & Okada, 1994). Transcriptomic analysis is also hindered by their cryosensitivity and the limited amount of RNA that can be extracted from neutrophils, leading to underestimations of their presence during bulk RNAseq (Li, Cui, Nambiar, Sunwoo, & Li, 2019; Subrahmanyam et al., 2001). Consequently, single-cell RNAseq analysis that uses platforms incapable of isolating sufficient RNA from neutrophils, result in poor identification and characterization of neutrophils (Salcher et al., 2022). All of these characteristics underscore the need for standardized protocols and careful consideration when designing new experiments involving neutrophils.

In this chapter, we describe a method for assessing neutrophil suppressive capacities using a T-cell proliferation assay with allogeneic T-cells isolated

from non-cancerous donors (Sections 2.1–2.3). We opted for allogeneic T-cells rather than autologous ones to eliminate interference from other cancer-derived factors in the blood of cancer patients and therefore isolate the effect of the neutrophils (Hao, Li, & Hu, 2023; Zhang, Liu, Chen, Xu, & Xu, 2023). Those T-cells alone have poor proliferative capacities and need to be supplemented with mature dendritic cell (DC). Those mature dendritic cells are differentiated from monocytes obtained from the same non-cancerous donor as the T-cells (Sections 2.4 and 2.6). In Section 3, we detail how we obtain blood neutrophils subpopulations and TAN via fluorescence-activated cell sorting (FACS). Those neutrophils are then co-cultured with T-cells loaded with a fluorescent tracker dye (Section 4) and stimulated through CD3 and the co-stimulatory receptor CD28 (Section 5). We subsequently analyze the resulting variation in T-cell proliferation induced by the neutrophils through the tracker dye dilution, as detailed in Section 6. Part of the chapter also describes an alternative method for isolating neutrophils using magnetic beads (Section 3.1.3). An overview of the complete process is shown in Fig. 1.

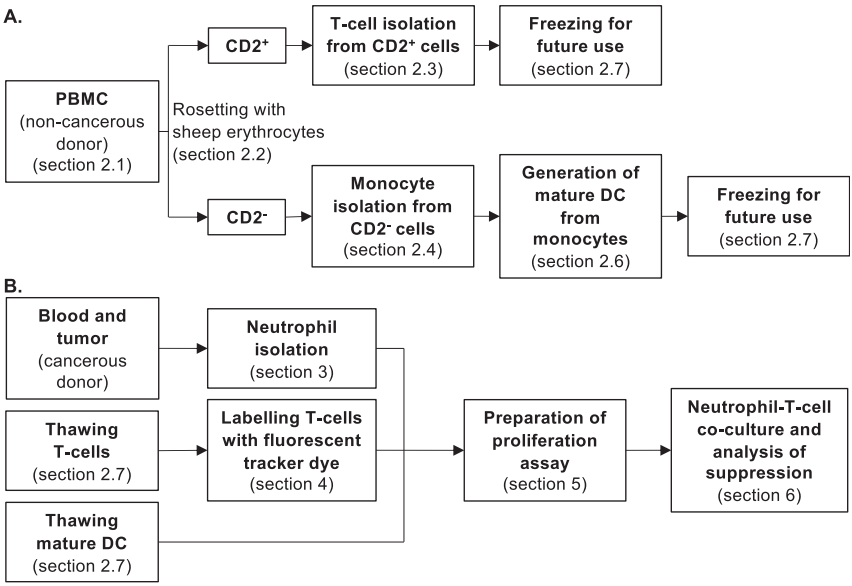

Fig. 1 Overview of the process. (A) T-cell and monocyte are isolated from non-cancerous donors (our lab (de Duve institute) uses blood of hemochromatosis patients). Mature DC are generated from the monocytes. T-cells and dendritic cells are frozen for future use in T-cell proliferation assays. (B) Neutrophils are isolated from cancer patients and used directly in T-cell proliferation assays with thawed allogeneic T-cell and mature DC.

2. T-cell isolation and mature DC generation

T-cell proliferation is the most commonly used assay to assess the suppressive capacities of human neutrophils. As described in the introduction, we use allogeneic T-cells from non-cancerous donors rather than autologous T-cells to reduce variability by excluding other cancer-derived factors and isolate the effect of neutrophils on T-cell proliferation. Those cells can then be frozen, for later use in separate experiments.

We obtain allogeneic T-cells from up to 500 mL of peripheral blood from hemochromatosis patients who need to be bled regularly as part of their routine treatment. This allows us to isolate T-cells in numbers large enough for repeated experiments. Blood can also be collected from healthy donors, but in smaller volume, leading to lower T-cell yield. To ensure that CD3-engagement is not induced during the isolation process, we isolate the T-cells by separating PBMC into $CD2^+$ and $CD2^-$ cells through rosetting with sheep erythrocytes (Section 2.2), followed by the depletion of $CD56^+$ NK cells from the $CD2^+$ fraction (Section 2.3). The T-cells are then frozen and stored in a liquid nitrogen freezer for prolonged storage. However, after thawing, T-cells barely proliferate in response to anti-CD3 and anti-CD28 stimulation. T-cell proliferation can be rescued by the addition of a small number of autologous mature DC, differentiated from the monocytes contained in the $CD2^-$ fraction (Sections 2.4 and 2.6).

2.1 PBMC isolation from whole blood

As described above, we use the blood obtained from hemochromatosis patients to obtain allogeneic cells for our proliferation assay. We first isolate the PBMC, and then the T-cells and monocytes, with the latter being used to generate mature dendritic cells.

a. The blood is collected at the local hospital. We receive from each hemochromatosis donor a blood bag containing around 500 mL of blood. The blood bag is centrifuged for 20 min at 3800*g* with full acceleration and breaking reduced by half. This separates the plasma (found at the top of the bag), the leukocytes (located at the interface) and the erythrocytes (found at the bottom of the bag) (Fig. 2). Remember to place the bag in a way that will allow the erythrocytes to settle away from the collection ports (Fig. 2).

b. Collect the plasma into a collection bag (Fresenius, Cat# PD11150). Our lab (de Duve Institute) uses a manual plasma extractor. The plasma

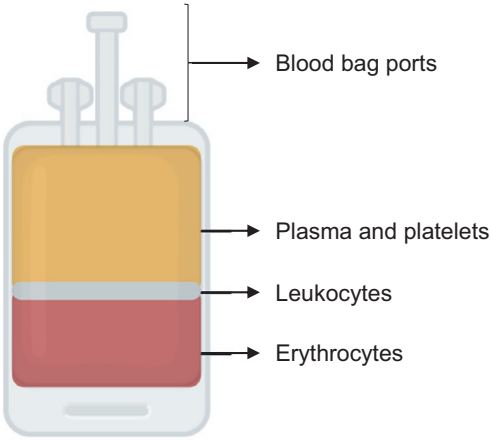

Fig. 2 Blood bag after centrifugation. The entire blood bag is centrifuged at 3800*g* for 20 min with full acceleration but breaking reduced by half. This allows the erythrocytes to settle at the bottom of the bag, with the leukocytes above and the plasma on top. The plasma is either discarded or collected to be treated and used as human serum (see Section 2.2.2 point d) and the leukocytes collected to isolate the PBMC by density gradient separation.

can be used for other application, including being treated for use as human serum (as described in Section 2.2.2 point d).

c. Collect the leukocytes, located above the erythrocytes, into a transfer bag (Terumo, Cat# BB*T015CM). You should obtain around 50 mL of white blood cells mixed with some platelets, plasma and red blood cell.

d. Dilute the content of the transfer bag 1:1 (v:v) with cold phosphate buffered saline (PBS) (ITW Reagents, Cat# A0964,9050) + 1 mM Ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher Scientific, Cat# 15575-038). EDTA is a Ca^{2+} chelating agent that prevents blood coagulation. Alternatively, whole blood can be directly used for the density gradient presented below, with a dilution of 4:1 (v:v) with cold PBS + EDTA 1 mM.

e. Separate the PBMC from the erythrocytes, neutrophils, and platelets by density gradient separation. To do so, add 15 mL of Lymphoprep (Stemcell Technologies, Cat# 07851) into a 50 mL conical centrifuge tube (VWR, Cat# 525-0610) at room temperature (RT). 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.

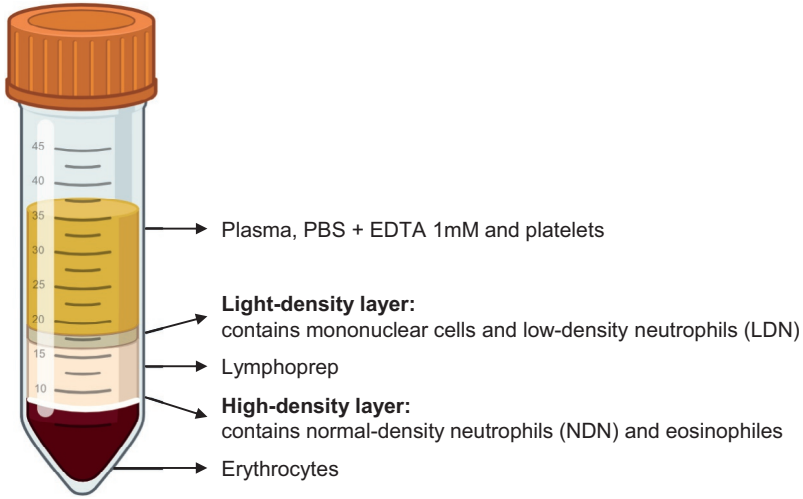

Fig. 3 Density gradient separation of peripheral blood. Blood leukocytes diluted with PBS + EDTA 1 mM (1:1; v:v) or whole blood diluted with PBS-EDTA 1 mM (4:1; v:v) is carefully layered onto Lymphoprep solution. Density separation is achieved after centrifugation at 900*g* for 20 min without acceleration or brakes. Shown is a sketch of how the blood components separate. *Note:* LDN are frequently found in the blood of cancer patients, depending on the protocol used, they are virtually absent or present in very low frequencies in the blood of healthy donors (Cassetta et al., 2020).

f. Following the density gradient separation, the PBMC settle in a thin layer below the plasma, and above the Lymphoprep and erythrocyte pellet (Fig. 3). Carefully collect the PBMC layer with a pipette and place it into a fetal bovine serum (FBS)-coated 50 mL conical centrifuge tube. The conical centrifuge tube is pre-coated with FBS (Thermo Fisher Scientific, Cat# A5256701) for at least 24 h at 4 °C. This prevents monocyte and DC from adhering to the plastic.

g. Wash the cells with cold PBS + 1 mM EDTA to dilute any Lymphoprep picked up with the PBMC layer and centrifuge at 400*g* for 10 min with maximum acceleration and brake. This allows the PBMC to settle in the pellet, under the diluted Lymphoprep.

h. Carefully aspirate the supernatant and as much of the residual platelets that settle in a white ring above the PBMC as possible. Remaining platelets could interfere in later steps with the DC generation (Singh et al., 2021; Tešić, Pekle Simonič, Roškar, Rožman, & Švajger, 2020) and with flow cytometry analyses by interacting with antibodies (own observations).

i. Repeat the washing steps with 50 mL of cold PBS + 1 mM EDTA.

j. Inspect the cell solution for platelets underneath a microscope if doubtful that all platelets have been successfully removed by aspiration. Should platelets remain, resuspend the PBMC in 50 mL of PBS + 1 mM EDTA and centrifuge them at 330*g* for 6 min and aspirate the supernatant. Platelets are too light to pellet at this speed.

k. Resuspend the PBMC in RPMI 1640 (Thermo Fisher Scientific, Cat# 52400025) at RT and count the cells with Tuerk's solution (Merck, Cat# 109277). Tuerk's solution stains cell nuclei and contains acetic acid that lyses erythrocytes. This allows to specifically count leukocytes even in the presence of residual platelets or erythrocytes. Retain 1×10^6 cells for flow cytometry assessment of the efficiency of cell isolation (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 interference with the T-cell receptor caused by CD3-binding beads. Erythrocyte rosetting separates $CD2^+$ and $CD2^-$ cells from PBMC. CD2 is an adhesion molecule present on T-cells and natural killer (NK) cells, which binds to the LFA3-homologue on sheep erythrocytes leading to high yield of T-cells (Ocklind, 1988). CD2/LFA-3 interaction causes an aggregation of NK and T-cells around the 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. 4). In the following section, we describe the preparation of sheep erythrocytes. Second, we describe the erythrocyte rosetting protocol.

2.2.1 Preparation of sheep erythrocytes

a. Use reagents at RT. We use sheep erythrocytes, 50% blood suspension in Alsever-buffer (Labor Dr. Merk, Cat# E-410). 2-(2-aminoethyl) isothiourea dihydrobromide (AET) is provided by Merck (Cat# A5879-100G).

b. Check the hematocrit (% erythrocytes) of the sheep erythrocytes first, using the provider's website: https://labormerk.com/produkte/blutprodukte.

c. Using the value of hematocrit, prepare the AET solution by dissolving **X** mg of AET in **Y** mL of milli-Q water (see Table 1). Add 1 M NaOH until the solution reaches a pH of 9. Control the pH value with a pH meter.

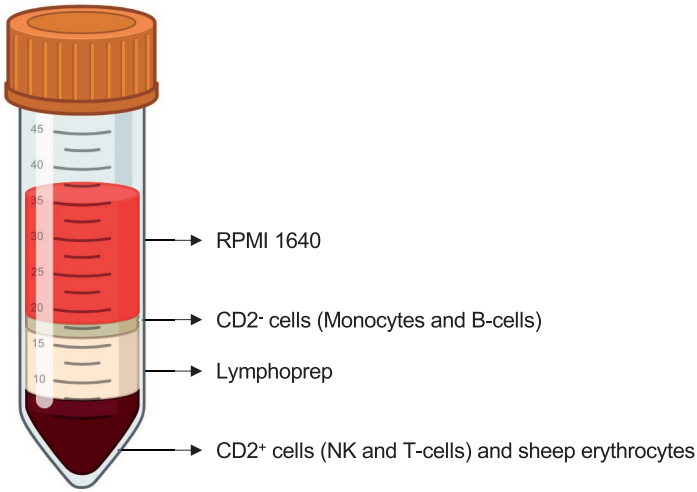

Fig. 4 Density gradient separation of $CD2^+$ and $CD2^-$ cells. Sheep erythrocytes express an LFA3-homologue that binds to human CD2 and thus causes the spontaneous aggregation of human T and NK cells with sheep erythrocytes (Ocklind, 1988). The sketch indicates how $CD2^+$ and $CD2^-$ cells separate by density gradient.

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

Hematocrit (%)	X: AET (mg)	Y: H_2O 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
21	1313	28.3	32.8
22	1375	29.7	34.3

d. Filter the AET suspension with a 0,22 µm Steriflip filter (Millipore, Cat# SCGP00525) and a syringe into a 50 mL conical centrifuge tube.

e. Resuspend sheep erythrocytes by shaking and 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 prepared in c. 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 of PBS. The erythrocyte pellet should be of dense consistency and require repeated gentle pipetting for complete resuspension.

h. Centrifuge at 910*g* for 8 min and wash with PBS. Repeat three times in total. The pellet's consistency should become less dense with each wash. Perform an additional washing step if the supernatant is 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. It is advised to test the prepared sheep erythrocytes on human 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 with sheep erythrocytes

a. Centrifuge the PBMC obtained at step k of Section 2.1 at 400*g* for 10 min. Aspirate the supernatant and resuspend them in RPMI 1640 at 10^7 cells/mL (or in at least 10 mL if there are less than 10^8 cells).

b. Add 1 mL sheep erythrocytes (preparation described in Section 2.2.1) for each 5×10^7 PBMC. Mix the cells well and incubate them at RT for 5 min, gently agitating the tube from time to time.

c. Carefully layer the PBMC and sheep erythrocytes suspension on 15 mL of Lymphoprep and perform a density gradient separation (as described in Section 2.1, step e).

d. During the centrifugation, prepare the erythrocytes lysis solution by mixing 12 mL of NH_4Cl at 160 mM (pH 7.4), 1.5 mL of RPMI 1640 and 1.5 mL of human serum (HS). This laboratory (de Duve Institute) uses allogeneic "serum" prepared with the plasma of 40 hemochromatosis patients. The plasma is pooled together and modified by addition of calcium and thrombin and then decomplemented by heating. A complete protocol is described in reference Baurain et al., 2000. Commercially available human serum can be used instead.

e. The $CD2^-$ cells settle in the light density layer, above the Lymphoprep (Fig. 4). Collect the $CD2^-$ cells into an FBS-coated 50 mL conical centrifuge tube. Wash and count them with Tuerk's solution. $CD14^+$ cells are obtained from the $CD2^-$ cells (as described in Section 2.4) and used to generate autologous mature dendritic cells (Section 2.6).

f. Come back to the 50 mL conical centrifuge tube still containing the pelleted sheep erythrocytes with the $CD2^+$ cells attached (Fig. 4). Carefully remove the remaining RPMI 1640 and the Lymphoprep and retain the pellet.

g. Resuspend the pellet in the 15 mL of erythrocytes lysis solution, prepared in d. and immediately centrifuge at 900*g* for 90 s.

h. Quickly aspirate the lysis solution and resuspend the pellet in 50 mL of RPMI 1640.

i. Centrifuge the tube at 400*g* for 10 min. Aspirate the supernatant and resuspend in 20 mL of RPMI 1640. Count the $CD2^+$ cells with Tuerk's solution.

j. Retain 5×10^5 $CD2^+$ and $CD2^-$ cells for purity assessment by flow cytometry (Section 2.5).

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

Only T and NK cells express CD2 (Krensky et al., 1983). T-cells are further isolated from the $CD2^+$ fraction by depleting NK cells using anti-CD56 magnetic beads. CD56 is a cell adhesion glycoprotein that is a prototypic marker for NK cells and the rare NKT cells (Van Acker, Capsomidis, Smits, & Van Tendeloo, 2017). The purity of the isolated cells and the success of the separation are assessed by flow cytometry in Section 2.5.

a. The buffer contains PBS + EDTA 1 mM + 2% HS and is filtered through a 0,22 μm filter (Merck, Cat# S2GPT05RE).

b. Use $CD2^+$ cells isolated from PBMC by rosetting (Section 2.2.2). Centrifuge them at 400*g* for 10 min.

c. Using the cell count from Section 2.2.2 point h, resuspend the cells in 60 μL of buffer per 10^7 cells.

d. Per 10^7 cells, add 20 μL of Fc receptor (FcR) blocking reagent (Miltenyi Biotec, Cat# 130-059-901, RRID:AB_2892112) and 20 μL of magnetic anti-CD56 beads (Miltenyi Biotec, Cat# 130-050-401, RRID: AB_3076236).

e. Mix well and incubate for 20 min at 4 °C. Gently shake every 5 min during incubation.

f. Separate T-cells and NK cells using either manual or automatic magnetic columns. Follow the manufacturer's instruction if using manual separation. If the automatic AutoMACS separator (Miltenyi Biotec) is used, add 10 mL of buffer to the cell at the end of the incubation. Centrifuge at 400*g* for 10 min and resuspend the cells at 2×10^7 cells/mL in buffer supplemented with 15 μg/mL of gentamycin (Thermo Fisher Scientific, Cat# 15750060). Separate the cells in 5 mL aliquot into 15 mL conical centrifuge tubes (VWR, Cat# 525-0604). Sort the cells with the AutoMACS separator using the program possel_s. Collect the cells into 15 mL conical centrifuge tubes containing 1 mL of HS, keep the samples cool at all times using racks pre-cooled to 4 °C (e.g., Chill 15 Rack, Miltenyi Biotec, Cat# 130-092-952).

g. The $CD56^+$ NK cells are in the positive fraction, the T-cells in the negative eluate fraction. Retain both fractions, wash them by centrifugation at 400*g* for 10 min with RPMI 1640 and count the live cells with Trypan Blue (Merck, Cat# T8154-20ML). We usually obtain around 10^8 T-cells from one 500 mL blood bag.

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

i. Freeze the T-cells and store them in liquid nitrogen as described in Section 2.7. These T-cells are used in the proliferation assay (Sections 4–6).

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

In our experience, frozen T-cells, once thawed, lack myeloid cells that sustain T-cell proliferation *in vitro*. To supplement isolated T-cells (Section 2.3) with co-stimulatory cells, autologous mature DC are differentiated from monocytes. These are isolated from the $CD2^-$ fraction (Section 2.2.2) 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. The buffer contains PBS + EDTA 1 mM + 2% HS and is filtered through a 0,22 μm filter.

b. Use $CD2^-$ cells isolated from PBMC after rosetting (Section 2.2.2). Centrifuge them at 400*g* for 10 min.

c. Using the cell count from Section 2.2.2 point e, resuspend the cells in 60 μL of buffer per 10^7 cells.

d. Per 10^7 cells add 20 μL of FcR blocking reagent and 20 μL of magnetic anti-CD14 beads (Miltenyi Biotec, Cat# 130-050-201, RRID:AB_2665482).
e. Mix well and incubate for 20 min at 4 °C. Gently shake every 5 min during incubation.
f. Isolate monocytes by either manual or automatic magnetic columns. Follow the manufacturer's instruction if using manual separation. If the automatic AutoMACS separator is used, add 10 mL of buffer to the cell at the end of the incubation. Centrifuge at 400*g* for 10 min and resuspend the cells at 2×10^7 cells/mL in buffer supplemented with 15 μg/mL of gentamycin. Separate the cells in 5 mL aliquot into 15 mL conical centrifuge tubes. Sort the cells with the AutoMACS separator using the program possel_s. Collect the cells into FBS-coated 15 mL conical centrifuge tubes containing 1 mL of HS, keep the samples cool at all times using racks pre-cooled to 4 °C (e.g., Chill 15 Rack).
g. The $CD14^+$ monocytes are in the positive fraction. Wash the isolated cells by centrifugation at 400*g* for 10 min with RPMI 1640 and count them with Trypan Blue.
h. Retain 5×10^5 cells to assess the purity of the cells by flow cytometry (Section 2.5).
i. 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 each individual steps of the isolation process (Fig. 5). This laboratory (de Duve Institute) uses a BD LSRFortessa (BD Biosciences) and BD FACSDiva software (BD Biosciences) for acquisition. Analyses are done with FlowJo software (version 10.10.0 for MacOS, BD Biosciences). The staining protocol and fluorochrome used should be adjusted to your own system. T-cells should be $CD2^+$, $CD3^+$ and (in part) $CD8\beta^+$, and $CD14^-$, $CD33^-$ and $CD56^-$. NK cells should be $CD2^+$, $CD56^+$, $CD3^-$, $CD33^-$, $CD14^-$, and mostly $CD8\beta^-$. Monocytes should be $CD14^+$ and $CD33^+$, and $CD2^-$, $CD3^-$, $CD8\beta^-$ and $CD56^-$.

Samples:

i. Unstained control (PBMC)
ii. PBMC
iii. $CD2^+$ cells

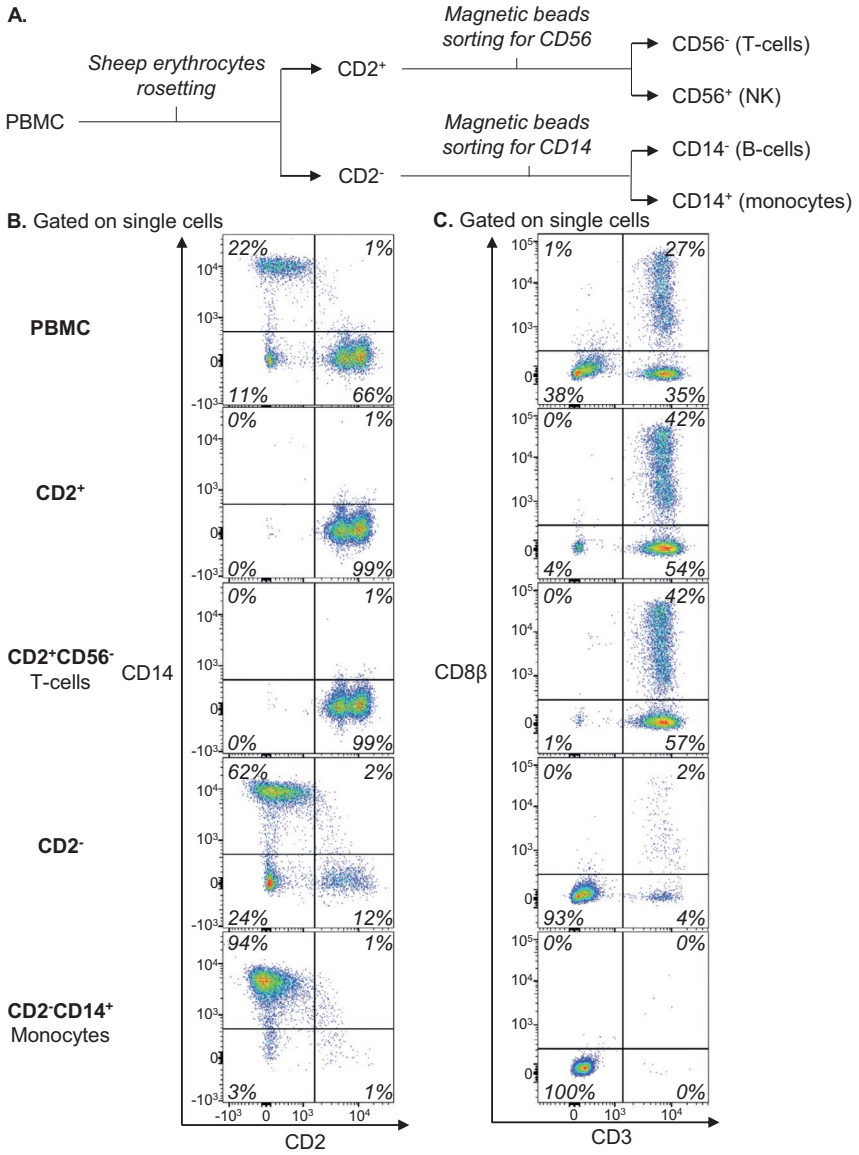

Fig. 5 Assessment of T-cell and monocyte purity by flow cytometry after isolation. (A) Diagram of the separation workflow to obtain autologous T-cells and monocytes from PBMC. (B) Analysis of the expression of CD2 (NK and T-cells) and CD14 (monocytes) by PBMC and subsequently isolated populations. (C) Expression of CD3 (T-cells) and CD8β (T-cells, and a fraction of NK (Baume, Caligiuri, Manley, Daley, & Ritz, 1990)) by PBMC and subsequently isolated populations.

iv. $CD2^{+}CD56^{+}$ NK cells
v. $CD2^{+}CD56^{-}$ T-cells
vi. $CD2^{-}$ cells
vii. $CD2^{-}CD14^{+}$ monocytes
viii. $CD2^{-}CD14^{-}$ cells

Antibody panel: anti-CD2/AF647, anti-CD3/AF488, anti-CD8β/PE, anti-CD14/PerCP, anti-CD33/BV711 and anti-CD56/BV510 (for detailed references see Table 2, antibody concentrations are optimized for our own settings, adapt to your own needs).

Procedure:

a. The staining buffer contains PBS + EDTA 1 mM + 2% HS and is filtered through a 0,22 μm filter. FcR blocking mix is composed of staining buffer + 33% FcR blocking reagent.

Table 2 Stains references and concentration used: The concentrations given in this table have been optimized for our flow cytometer (BD LSRFortessa) and should be titrated and adapted to your own setup.

Target antigen	Fluorochrome	Clone	Staining concentration (μg/mL)[a]	Reference
CD1a	BV421	HI149	0,50	BD Biosciences Cat# 563938, RRID:AB_2744316
CD2	AF647	LT2	5,00	Bio-Rad Cat# MCA1194A647, RRID:AB_324865
CD3	AF488	HIT3a	10,00	BioLegend Cat# 300320, RRID: AB_493691
CD3	PE	UCHT1	0,13	BD Biosciences Cat# 555333, RRID:AB_395740
CD3	BV510	HIT3a	0,50	BD Biosciences Cat# 564713, RRID:AB_2738909

Table 2 Stains references and concentration used: The concentrations given in this table have been optimized for our flow cytometer (BD LSRFortessa) and should be titrated and adapted to your own setup.—cont'd

Target antigen	Fluorochrome	Clone	Staining concentration (μg/mL)[a]	Reference
CD8β	PE	2ST8.5H7	0,30	BD Biosciences Cat# 641057, RRID:AB_1645747
CD8α	APC	RPA-T8	0,25	BD Biosciences Cat# 555369, RRID:AB_398595
CD11c	PE-Cy7	B-ly6	2,00	BD Biosciences Cat# 561356, RRID:AB_10611859
CD14	PerCP	MφP9	0,25	BD Biosciences Cat# 340660, RRID:AB_400071
CD15	PE	HI98	0,15	BD Biosciences Cat# 555402, RRID:AB_395802
CD16	BV421	3G8	1,00	BD Biosciences Cat# 562874, RRID:AB_2716865
CD19	BV510	SJ25C1	1,00	BD Biosciences Cat# 562947, RRID:AB_2737912
CD33	BV711	WM53	1,88	BD Biosciences Cat# 563171, RRID:AB_2738045
CD45	AF700	HI30	12,50	BioLegend Cat# 304024, RRID: AB_493761
CD56	BV510	NCAM16.2	0,50	BD Biosciences Cat# 563041, RRID:AB_2732786

Continued

Table 2 Stains references and concentration used: The concentrations given in this table have been optimized for our flow cytometer (BD LSRFortessa) and should be titrated and adapted to your own setup.—cont'd

Target antigen	Fluorochrome	Clone	Staining concentration (μg/mL)[a]	Reference
CD83	APC	HB15e	0,19	BD Biosciences Cat# 561960, RRID:AB_10894952
CD137	BV421	4B4-1	0,66	BioLegend Cat# 309820, RRID: AB_2563830
CD206	FITC	19.2	0,13	BD Biosciences Cat# 551135, RRID:AB_394065
Fixable viability dye	eFluor 780	NA	1/400 dilution	Thermo Fisher Scientific Cat# 65-0865-14
HLA-DR	PE-Cy7	L243	0,50	BioLegend Cat# 307616, RRID: AB_493588
Sphero Rainbow Calibration Particles	NA	NA	2500 particles/well	BD Biosciences Cat# 559123, RRID:AB_2869258

[a]Or dilution ratio if the stock concentration was not available.

b. Pellet the cells by centrifugation at 400 g for 10 min. Aspirate the supernatant and resuspend the cells with 50 μL of FcR blocking mix per 5×10^5 cells. Seed 50 μL per sample into a non-sterile 96-wells conical-bottom plate (Thermo Fisher Scientific, Cat# 249570). Add 50 μL of either staining buffer (in unstained control) or antibody mix, at twice the staining concentration, to the cells. Incubate at 4 °C for 20 min. Wash twice with 200 μL of staining buffer by centrifugation at 400*g* for 5 min. Resuspend in 200 μL of staining buffer to transfer the cells into a non-sterile 96-wells round-bottom plate (Corning, Cat# 353910) or any alternative suitable for flow cytometry acquisition.

c. Proceed with flow cytometry analysis with FlowJo software. The $CD2^+CD56^-$ fraction usually contains 95–99% $CD2^+CD3^+$ T-cells. The $CD2^-CD14^+$ fraction usually contains more than 90% $CD2^-CD33^+CD14^+$ monocytes.

2.6 Generation of mature DC

Pure T-cells showed poor proliferative capacity after thawing as remaining myeloid cells that sustain T-cell proliferation do not survive the thawing 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 DC from monocytes over 7 days and the staining protocol used to confirm the success of the maturation process. During the first 5 days, the monocytes, exposed to IL-4 and GM-CSF, differentiate into DC. Those monocyte-derived DC are then exposed to polyinosinic-polycytidylic acid (PolyIC) and TNFα, in addition to IL-4 and GM-CSF for 2 days to provoke maturation. The use of mature DC, rather than monocyte, eliminates the risk of neutrophils orientating those differentiated cells toward a suppressive phenotype during our proliferation assay (Section 5). The DC population generated with our method is not homogeneous, as evidenced by the heterogeneous expression of CD1a. $CD1a^+$ and $CD1a^-$ mature DC induce similar level of T-cell proliferation (Ito et al., 1999). It is however important to note that the $CD1a^+$ subset of mature DC has been shown to produce more IL-12 upon stimulation by LPS or CD40L and skew $CD4^+$ T-cells maturation towards a Th1 phenotype (Cernadas, Lu, Watts, & Brenner, 2009).

a. The DC complete medium for this section is RPMI 1640 + 10% FBS + 1,5 mM GlutaMAX (Thermo Fisher Scientific, Cat# 35050061) + 100 U/mL penicillin +100 μg/mL streptomycin (Merck, Cat# P4333). The staining buffer contains PBS + EDTA 1 mM + 2% HS and is filtered through a 0,22 μm filter. The FcR blocking mix is composed of staining buffer + 33% FcR blocking reagent.

b. Use fresh $CD14^+$ monocytes isolated as described in Section 2.4. Use the cell count from Section 2.4, step g to resuspend the cells at 10^6 cells/mL in DC complete medium pre-warmed to 37 °C.

c. Gently seed 1 mL (=10^6 cells) per well into a 12-well tissue culture plate (Corning, Cat# 3513). We usually plate around 6×10^7 cells.

d. Gently add 1 mL of DC complete medium supplemented with 200 ng/mL GM-CSF (final concentration: 100 ng/mL) (sargramostim (Leukine), Partner Therapeutic, NDC# 71837-5843) and 120 IU/mL IL-4 (final concentration: 60 IU/mL which corresponds to 30 ng/mL in our case) (Miltenyi Biotec, Cat# 130-094-117). As an optional control, add, in 1 well, 1 mL of DC complete medium without IL-4 or GM-CSF to keep undifferentiated monocytes. Incubate the cells at 37 °C, 5% CO_2 for 2 days.

e. After 2 days, gently add 2 mL of DC complete medium supplemented with 100 ng/mL GM-CSF and 120 IU/mL IL-4. In the optional control well, add 2 mL of DC complete medium without cytokines. Incubate the cells at 37 °C, 5% CO_2 for 3 days.

f. After 3 more days of incubation, start the DC maturation by directly adding maturation cytokines to the wells. In each well, containing 4 mL of medium, add cytokines so as to obtain a "final concentration" of 50 ng/mL GM-CSF, 60 IU/mL IL-4, 10 ng/mL TNFα (Thermo Fisher Scientific, Cat# 200-01A-1MG) and 50 μg/mL PolyIC (Merck, Cat# P1530-100MG) (except for the undifferentiated control). Incubate the cells at 37 °C, 5% CO_2 for 2 days.

g. On the seventh day of incubation, visually inspect cell morphology using light microscopy. Most of the cells should be semi-adherent to the plastic. Only a very small fraction of the cells will adopt a more fusiform morphology and strongly adhere to the bottom of the well.

h. Harvest the cells in FBS-coated 50 mL conical centrifuge tubes using 5 mL pipettes. Wash gently each well with 1 mL of RPMI 1640 to collect the remaining semi-adherent cells that will easily detach. The adherent, fusiform cells should not be collected.

i. If several tubes are required to collect all the cells, centrifuge them for 10 min at 400*g* and pool the cells together in one FBS-coated 50 mL conical centrifuge tube. Resuspend them in 20 mL RPMI 1640 and count them with Trypan Blue.

j. Resuspend the cells at 10^6 cells/mL in RPMI 1640. Transfer 10^6 mature DC into an FBS-coated 15 mL conical centrifuge tube for phenotypic assessment by flow cytometry. Freeze the rest and store them in a liquid nitrogen freezer as described in Section 2.7.2. Collect the undifferentiated monocytes in a second FBS-coated 15 mL conical centrifuge tube.

k. To control the phenotype of the cells, pellet the 10^6 mature DC and monocytes by centrifugation at 400*g* for 10 min. Aspirate the supernatant and resuspend the cells in 100 μL of FcR blocking mix. Seed 50 μL per sample into a non-sterile 96-wells conical-bottom plate. Add 50 μL of either staining buffer or antibody mix, at twice the staining concentration, to the cells. Incubate at 4 °C for 20 min. Wash twice with 200 μL of staining buffer by centrifugation at 400*g* for 5 min. Resuspend in 200 μL of staining buffer to transfer the stained cells into a non-sterile 96-wells round-bottom plate and proceed with flow cytometry analysis.

Samples

i. Unstained control
ii. Stained sample
iii. Undifferentiated control

Antibody panel: anti-CD1a/BV421, anti-CD3/AF488, anti-CD11c/PE-Cy7, anti-CD14/PerCP, anti-CD19/BV510 and anti-CD83/APC (for detailed references see Table 2, antibody concentrations are optimized for our own settings, adapt to your own needs).

l. Depending on your own flow cytometry systems, adjust colors and compensation controls accordingly. This laboratory (de Duve Institute) uses a BD LSRFortessa and BD FACSDiva software for acquisition. Analyses are done with FlowJo software.

m. The monocytes, in the optional undifferentiated control well, are $CD3^-$ $CD19^-$, $CD14^+$, $CD11c^+$, $CD1a^-$ and $CD83^-$. The DC, differentiated from monocytes by exposure to GM-CSF and IL-4, lose the expression of CD14. Their maturation, induced by TNFα and PolyIC, is associated with the expression of CD83 (Subrahmanyam et al., 2001). All cells in the mature DC condition should be $CD14^-$ and more than 85% usually are $CD83^+$. A proportion of mature DC should be $CD1a^+$, their frequency varies from donor to donor independently of the maturation (Cernadas et al., 2009). See Fig. 6 for an example.

2.7 Freezing and thawing of cells

To reduce variability between experiments, we use the same batch of T-cells and mature DC obtained in Sections 2.3 and 2.6. They are stored in a liquid nitrogen freezer and, when neutrophil samples are available, the required amount of mature DC and T-cell are thawed. Here, we describe the freezing and thawing protocols.

2.7.1 T-cells

a. Prepare freezing serum 1–2 h before use. This solution is composed of HS + 20% dimethyl sulfoxide (DMSO) (Santa Cruz Biotechnology, Cat# sc-358,801) and is stored at 4 °C until use.

b. Centrifuge the T-cells at 400*g* for 10 min and aspirate the supernatant.

c. Using the cell count from Section 2.3, step g, resuspend the cells in cold RPMI 1640 at 5×10^6 cells/mL.

d. Dilute the cell suspension 1:1 with freezing serum for a final concentration of $2{,}5 \times 10^6$ cells/mL.

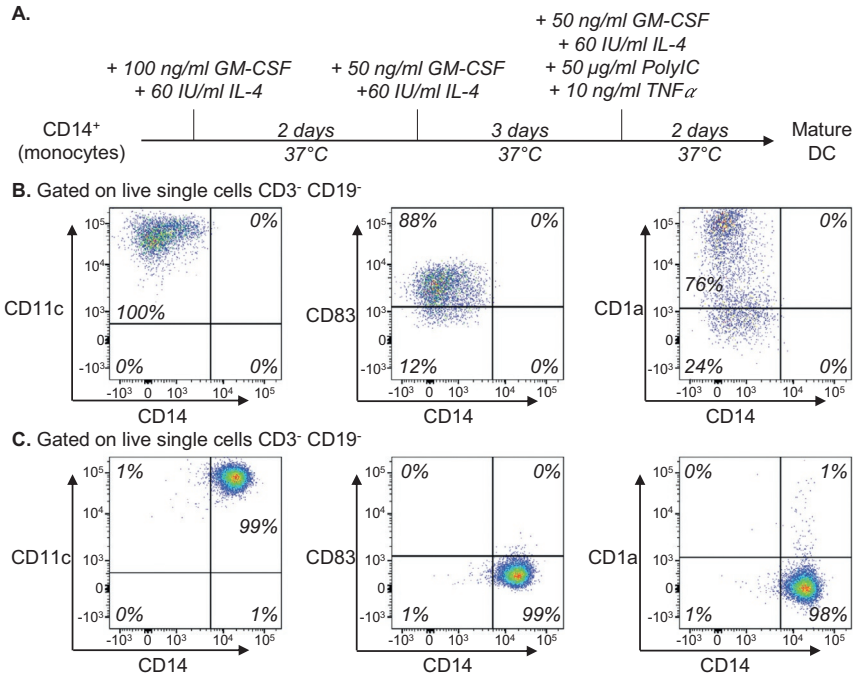

Fig. 6 Monocytes differentiation into mature DC. (A) Workflow of the DC differentiation protocol. (B) Phenotype of mature DC after 7 days of culture with cytokines. All cells should be $CD3^-$ and $CD19^-$. (C) Phenotype of undifferentiated monocytes cultured for 7 days in medium without the maturation cytokines. All cells should be $CD3^-$ and $CD19^-$.

NOTE: The number of T-cells frozen in each vial should be adapted to your needs. In our experience, $2{,}5 \times 10^6$ cells/vial is sufficient to perform most experiments. The 10^8 T-cells obtained in Section 2.3 can thus be distributed into 40 vials, which can then be used in 40 separate proliferation assays.

e. Quickly transfer 1 mL to each cryovial (Thermo Ficher Scientific, Cat# 377267).

f. Transfer the vials to −80 °C. We advise keeping the freezing process as temperature-controlled as possible. To achieve that, we use the CoolCell FTS30 Cell Freezing Container (Azenta Life Sciences, Cat# BCS-170).

g. Transfer the cells into liquid nitrogen after 24 h.

h. Thaw the T-cells on the morning of the proliferation assay. Rest the cells at 37 °C, 5% CO_2 for at least 4 h. Use a 15 mL conical centrifuge tube and a 48-well tissue culture plate (Corning, Cat# 3548). T-cells, after thawing, should be plated at a cell density of $2\text{-}3 \times 10^6$ cells/mL/cm^2.

i. For thawing the cells, prepare 12 mL of thawing medium: RPMI 1640 + 5% HS + 100 U/mL penicillin + 100 μg/mL streptomycin + 7.5 U/mL (or 3,75 μg/mL) DNase I (Stemcell Technologies, Cat# 7469) + 50 IU/mL IL-2 (aldesleukine (Proleukine), Novartis, CNK 1185-958) warmed at 37 °C.

j. Thaw the cells by holding the cryovial into a warm water bath until only a small ice pellet remains. Transfer them into a 15 mL conical centrifuge tube and add, drop-wise, 10 mL of warm thawing medium. Use this medium to rinse the cryovial.

k. Centrifuge the cells at 400*g* for 10 min. Aspirate the supernatant and resuspend the cells in 1 mL thawing medium. Transfer the cells into a 48-well plate and incubate at 37 °C, 5% CO_2, for at least 4 h.

2.7.2 Mature DC

a. Prepare freezing serum 1–2 h before use. This solution is composed of HS + 20% dimethyl sulfoxide (DMSO) and is stored at 4 °C until use.

b. Centrifuge the mature DC (obtained at Section 2.6) at 400*g* for 10 min.

c. Using the cells count from Section 2.6 step i, resuspend cells in cold RPMI 1640 without serum at 5×10^5 cells/mL.

d. Dilute the cell suspension 1:1 with freezing serum for a final concentration of 2.5×10^5 cells/mL.

NOTE: We recommend adapting the number of mature DC frozen in each vial to obtain one sample of mature DC for each vial of frozen T-cell frozen at Section 2.7.1. However, 2.5×10^5 cells/vial should be considered a minimum.

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 use the CoolCell FTS30 Cell Freezing Container.

g. Transfer the cells into liquid nitrogen after 24 h.

h. Thaw the mature dendritic cells on the morning of the proliferation assay. Rest the cells at 37 °C, 5% CO_2 for at least 4 h before use. Use an FBS-coated 15 mL conical centrifuge tube and a 24-well ultra-low attachment plate (Corning, Cat# 3473).

i. For thawing the cells, prepare 12 mL of pre-warmed thawing medium: RPMI 1640 + 10% FBS + 100 U/mL penicillin + 100 μg/mL streptomycin + 7.5 U/mL (or 3,75 μg/mL) DNase I.

j. Thaw the cells by holding the cryovial into a warm water bath until only a small ice pellet remains. Transfer them into an FBS-coated 15 mL conical centrifuge tube and add, drop-wise, 10 mL of warm thawing medium. Use this medium to rinse cryovial.

k. Centrifuge the cells at 400*g* for 10 min. Aspirate the supernatant and resuspend the cells in 1 mL thawing medium supplemented with 50 ng/mL GM-CSF, 60 IU/mL IL-4, 50 μg/mL PolyIC and 10 ng/mL TNFα. Transfer the cells into the 24-well ultra-low attachment plate and incubate at 37 °C, 5% CO_2 for at least 4 h.

3. Neutrophil isolation

The aim of the protocol presented is to compare the suppressive capacities of neutrophils isolated from blood (Section 3.1) or from tissue (Section 3.2). Tissues require slightly different preparation steps, e.g., tumors or tumor biopsies need to be dissociated. All isolation methods require density gradient separation steps to isolate LDN from NDN.

The neutrophils are sorted from the samples by flow cytometry (Section 3.3). The same panel of markers and gating strategy for all samples are used, except for the anti-CD206 marker for macrophage, which is replaced by an anti-CD3 antibody in blood samples. Moreover, this panel of markers allows for the separation of immature $CD45^{low}$ and mature $CD45^{high}$ subpopulations present within the LDN of the blood (Vanhaver et al., 2023).

3.1 Neutrophil preparation from blood

Neutrophils are most commonly isolated from the blood of cancer patients. The LDN are isolated from the mononuclear layer following a density gradient separation. However, the sample sizes obtainable with blood are usually small, ranging from 20 to 100 mL, and the low number of LDN that can be isolated from it limits the number of experiments. Moreover, they are composed of, at least, two sub-populations with opposite effect on T-cell proliferation: mature LDN $CD45^{high}$ and immature LDN $CD45^{low}$ (Pettinella et al., 2024; Vanhaver et al., 2023). These two LDN subpopulations are analogous to the immature and mature neutrophil populations previously observed in the blood of patients with head and neck cancer and melanoma (Gondois-Rey et al., 2021; Lang et al., 2018). We separate them by using two markers (e.g., CD45, CD16, or CD10) instead of one. We usually obtain between 20,000 and 200,000 of each LDN sub-population from 42 mL of blood (see Table 3 for an example of the yield obtained from one lung cancer patient). In contrast, NDN are mature cells isolated from the high-density layer of the blood and present in high number, allowing greater flexibility in experiments. The blood should be collected by venipuncture

Table 3 Example of neutrophils yields from the blood and tumor of one lung cancer patient.

From 42 mL of blood			From the tumor	
Light density fraction		**High density fraction**	**Light density fraction**	**High density fraction**
23 × 10^6 PBMC		**68,5 × 10^6 NDN**	**10^7 cells sorted**	
LDN $CD45^{high}$	LDN $CD45^{low}$	NDN	TAN	No neutrophils
109,000	258,000	Stopped at 500,000	252,000	/

NDN sorting was stopped after reaching 500,000 cells as we do not need more cells. If necessary, several millions NDN can be sorted as they represent more than 90% of the cells present in the high density fraction.

into EDTA tubes (Sarstedt, Cat# 01.1605.001) and treated immediately. Circadian variation in neutrophils function and frequency should be considered (Aroca-Crevillén, Adrover, & Hidalgo, 2020) and the time of collection should always be recorded. Our experiments are always performed with blood collected between 8 am and noon.

a. Dilute whole blood 4:1 (v:v) with PBS + 1 mM EDTA at RT. Do not use cold PBS + 1 mM EDTA as the temperature shift may activate neutrophils (Gordon et al., 1994).

b. Perform density gradient separation on the diluted blood as previously described in Section 2.1, step e.

c. Following the density gradient separation, the light-density layer, containing PBMC and LDN, settles in a thin layer below the plasma, and above the Lymphoprep solution (Fig. 3). Carefully collect only the light-density layer with a pipette and place it into an FBS-coated 50 mL conical centrifuge tube.

d. Once the light-density layer is removed, with a pipette, collect the high-density layer located below the Lymphoprep solution and above the erythrocytes pellet (Fig. 3). Transfer into a 15 mL conical centrifuge tube. Pick up, at most, 5 mL.

3.1.1 Light-density layer (LDN fraction)

a. Wash the light-density layer with PBS + 1 mM EDTA and centrifuge at 380*g* for 10 min.

b. The cells settle in the pellet. Carefully remove the supernatant and as much of the white platelet ring covering the cell pellet as possible.

c. Repeat the washing steps with 50 mL PBS + 1 mM EDTA twice. Resuspend the cells a third time and centrifuge them at 330*g* for 6 min if platelets remain.
d. Proceed as fast as possible with neutrophil staining protocol in Section 3.3.

3.1.2 High-density layer (NDN fraction)

a. Prepare a solution of 1% polyvinyl alcohol (PVA) in advance:
  i. Add 1% PVA (Merck, Cat# P1763-250G) to a 0,9% NaCl solution in sterile distilled water at room temperature and mix vigorously.
  ii. Microwave the solution for 30 s and mix. Repeat until the PVA is fully dissolved and the solution transparent. Let the solution cool at RT before use.
  iii. Store at RT, protected from light.

NOTE: Use a large, heat-resistant, bottle to prevent the solution from boiling over.

b. Prepare three saline solutions in advance to lyse the erythrocytes in later steps:
  i. Stock solution: 20% NaCl in distilled water (m:m), sterilized.
  ii. Lysis solution: Dilute the 20% NaCl stock in sterile distilled water down to 0,2% NaCl (m:m).
  iii. Reconstitution solution: Dilute the 20% NaCl stock in sterile distilled water down to 1,2% NaCl (m:m).
c. Filter the PVA solution with a 0,22 µm Steriflip filter and syringe and dilute the high-density layer of the blood in the 1% PVA solution at a 1:2 ratio (v:v).
d. Carefully mix the PVA solution and the cells by repeatedly turning the tube upside-down.
e. Incubate 30 min at RT. The erythrocytes settle at the bottom of the tube.
f. Pick up the PVA fraction, above the erythrocytes, and transfer into a new 15 mL conical centrifuge tube. Discard the erythrocytes.
g. Add PBS + EDTA 1 mM to the PVA fraction up to the 15 mL mark and centrifuge the tube 10 min at 380*g*.
h. Aspirate the supernatant. Lyse the remaining erythrocytes with 3 mL of 0,2% NaCl lysis solution by resuspending the cells carefully but thoroughly. After 50–60 s, reconstitute osmolarity with 7 mL of 1,2% NaCl reconstitution solution. Add 5 mL of PBS + EDTA 1 mM + 2% HS at RT and centrifuge at 380*g* for 10 min.

i. Wash with 15 mL of PBS + EDTA 1 mM + 2% HS and centrifuge at 380*g* for 10 min.
j. Proceed as fast as possible with neutrophil staining protocol for isolation by FACS (Section 3.3).

3.1.3 Alternative method to isolate NDN from the high-density layer without FACS

As some assays require negative selection of the neutrophils, we have developed an alternative protocol to isolate NDN from the high-density fraction of the blood without use of a FACS sorter. We employ antigen-specific depletion with magnetic beads to eliminate contaminating cells present in the PVA fraction (Section 3.1.2, step f). Instead of osmolysis, erythrocytes are eliminated by glycophorin A (CD235a) beads. Moreover, eosinophils are targeted using biotinylated anti-Siglec-8 and anti-CCR3 (CD193) antibodies, and residual contaminating T-cells with a biotinylated anti-CD3 antibody. This magnetic sorting approach allows us to avoid the stresses induced by osmolysis and fluorescence-activated cell sorting. With this protocol, millions of NDN can be isolated at a high purity (between 95% and 99%) suitable for proliferation assay. The large number of isolated NDN opens up possibilities for other analysis requiring larger number of cells, such as proteomic analysis. In cases where neutrophils do not need to be warmed back at 37 °C for co-culture, the isolation protocol should be performed at 4 °C using cold buffers. The protocol described below is specifically tailored for proliferation assays and is thus performed at room temperature.

a. Pick up the PVA solution containing NDN obtained at step f of Section 3.1.2.
b. Count the cells:
   i. With Tuerk's solution to determine the number of leukocytes.
   ii. With Trypan Blue to determine the total number of cells, including erythrocytes.
c. The staining buffer contains PBS + EDTA 1 mM + 2% HS and is filtered through a 0,22 µm filter.
d. Centrifuge at 380*g* for 10 min.
e. Resuspend in staining buffer with 20% FcR blocking reagent in 70 µL per 10^7 leukocytes. Incubate 5 min at RT.
f. Per 10^7 leukocytes, counted with Tuerk's solution, add 10 µL of anti-Siglec-8 biotinylated antibody (Miltenyi Biotec, Cat# 130-098-712, RRID:AB_2653435), 10 µL of anti-CD193 biotinylated antibody

(Miltenyi Biotec, Cat# 130-097-066, RRID:AB_2655903) and 10 μL of anti-CD3 biotinylated antibody (BioLegend, Cat# 300404, RRID: AB_314058). Those antibodies bind to the eosinophils and T-cells and the biotin will be targeted for magnetic sorting in subsequent steps.

g. Mix by carefully pipetting up and down. Incubate 10 min at RT.

h. Wash with 10 mL of staining buffer and centrifuge at 380*g* for 10 min.

i. Resuspend the cells in 60 μL of staining buffer per 10^7 cells. Incubate for 5 min at RT.

j. Per 10^7 cells, including erythrocytes, add 20 μL of anti-CD235a MicroBeads (Miltenyi Biotec, Cat# 130-050-501, RRID:AB_3076160), which attach to the erythrocytes, and 20 μL anti-Biotin MicroBeads (Miltenyi Biotec, Cat# 130-105-637, RRID:AB_2811216), which bind to the eosinophils and T-cells labeled with the biotinylated antibody (step f). Incubate 15 min at RT.

k. Separate NDN from erythrocytes and eosinophils using either manual or automatic magnetic columns. Follow the manufacturer's instruction if using manual separation. If the automatic AutoMACS separator is used, add 10 mL of staining buffer to the cells at the end of the incubation. Centrifuge at 400*g* for 10 min and resuspend the cells at 20×10^6 cells/mL in staining buffer supplemented with 15 μg/mL of gentamycin. Separate the cells in 5 mL aliquot into 15 mL conical centrifuge tubes. Sort the cells with the AutoMACS separator using the program Depl_05. Collect the cells into FBS-coated 15 mL conical centrifuge tubes containing 1 mL of FBS.

l. The NDN are collected in the negative eluate fraction. Wash them with staining buffer by centrifugation at 380*g* for 10 min and count the cells with Tuerk's solution.

m. Retain 5×10^5 cells to assess the purity of the cells by flow cytometry using the same staining protocol described for fluorescence-activated cell sorting (Section 3.3). Neutrophils usually represent more than 95% of the live cells.

n. Use the neutrophils immediately, they cannot be frozen and have a short life span *ex vivo*. For T-cell co-culture assay, refer to Section 5.3.

3.2 Neutrophil preparation from solid tissue

Samples from tumors or tumor biopsies are rare, difficult to obtain and usually small, which limits the expected neutrophil yield. Additionally, solid

tissue samples require the most manipulation for neutrophil isolation, as the tissue must be gently dissociated. Neutrophils are isolated from the light-density fraction following density gradient separation. A staining for CD45 is necessary, to facilitate leucocytes discrimination from non-immune stromal, cancer or epithelial cells that may be present.

a. We obtain samples from a hospital in very close proximity, and process tumor samples within 30 min after resection. The specimens are placed in a sterile flask without buffer during transport. If such a short delay between resection and processing is not possible or if processing cannot be performed immediately, the tumor should not be left completely dry and 5 mL of PBS + EDTA 1 mM at RT can be added at this step. However, we do not recommend delays longer than 1 h between tumor resection and processing as it may influence the survival and activation state of the neutrophils.

b. Place the tumor and any liquid that may be present in the flask into a sterile Petri dish underneath a flow hood. Add 5 mL of PBS + EDTA 1 mM at RT (except if it was already added at step a). Cut the tumor into very small pieces (1–2 mm) using a sterile scalpel. The pieces should be small enough to not clog a 10 mL pipette.

c. Place the tumor pieces and fluid into a gentleMACS C Tube (Miltenyi Biotec, Cat# 130-093-237). Rinse the Petri dish with a small amount of PBS + EDTA 1 mM at RT and add it to the gentleMACS C Tube to reach 10 mL. If the total volume exceeds 10 mL, separate the tumor and fluid between several C tubes.

d. Add DNase I at 7,5 U/mL (or 3,75 μg/mL) and Liberase TL and DL (thermolysin- and dispase-low, both Merck, respectively Cat# 05401020001 and LIBDL-RO) at 0.5 and 0.26 UI/mL respectively.

e. Dissociate the tumor cells using the pre-set program "h_tumor_01" on the gentleMACS Dissociator (Miltenyi Biotec, Cat# 130-093-235). Repeat the "h_tumor_01" program a second time.

f. Incubate the tumor cells in the gentleMACS C Tube under agitation for 45 min at 37 °C.

g. Repeat the mechanical dissociation by using twice the dissociator's pre-set program "h_tumor_01."

h. Transfer the tumor cells into an FBS-coated 50 mL conical centrifuge tube. Rinse the dissociation tube with 10 mL of FBS at RT and add this to the tumor cells in the conical centrifuge tube to neutralize enzymatic activity. Pellet the cells by centrifugation at 380*g* for 10 min.

i. Resuspend the tumor cells with 30 mL PBS + EDTA 1 mM at RT and pass them through a 70 µm sterile filter (Greiner Bio-One, Cat# 542070). Wash the filter with 20 mL of PBS + 1 mM EDTA and centrifuge at 380*g* for 10 min.
j. Repeat the previous step with a 40 µm sterile filter (Greiner Bio-One, Cat# 542040).
k. Resuspend the cells in warm RPMI 1640 to a total volume of 35 mL.
l. Perform density gradient separation on the cell suspension as previously described in Section 2.1, step e.
m. Following the density gradient separation, the light density fraction of the tumor settles in a thin layer between the RPMI 1640 and the Lymphoprep, while the pellet contains a few erythrocytes as well as some stromal and tumoral cells and small debris (Fig. 7). Carefully remove only the light density fraction with a pipette and place it into an FBS-coated 50 mL conical centrifuge tube.
n. Fill the centrifuge tube with PBS + 1 mM EDTA at RT up to the 50 mL mark and centrifuge at 380*g* for 10 min.
o. The cells settle in the pellet. Carefully remove the supernatant.
p. Repeat twice the washing steps with 50 mL PBS + 1 mM EDTA and centrifugation at 380*g* for 10 min.
q. Proceed as fast as possible to neutrophil staining protocol in Section 3.3 to continue with neutrophils isolation by FACS.

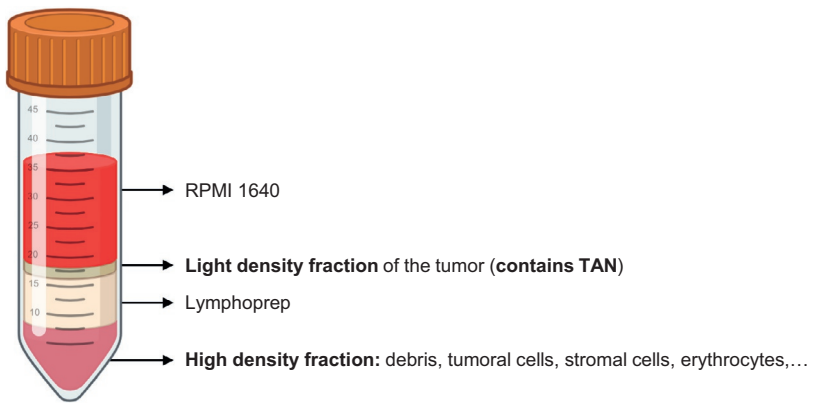

Fig. 7 Density gradient separation of tumoral cell suspension. In our experience, TAN settle in the light density fraction with tumoral cells, lymphocytes, macrophages, … We have never observed TAN in the high-density fraction which mainly contains debris and erythrocytes.

3.3 Neutrophil isolation by fluorescence-activated cell sorting

Different markers can be used to distinguish neutrophils and segregate their immature and mature subpopulations. We present here the staining protocol that we use to label the single cell suspensions obtained from the light and high density fractions of the blood as well as the tumor. We then describe the gating strategies used for FACS sorting in our research group (de Duve Institute) as an example (Fig. 8).

a. The staining buffer contains PBS + EDTA 1 mM + 2% HS and is filtered through a 0,22 μm filter.

b. Count the cells and prepare 100 μL of staining mix, at twice the staining concentration, per 10^7 cells.

c. Adapt the panel to in-house requirements for machine settings and compensation matrices. Shown below is the panel used in our research group (de Duve Institute) as an example.

Samples:

i. Unstained control

ii. Full stain

Antibody panel for blood samples: Viability dye (eFluor 780), Lineage stain (anti-CD19/BV510 and, anti-CD56/BV510), anti-HLA-DR/PE-Cy7, anti-CD3/AF488, anti-CD14/PerCP, anti-CD15/PE, anti-CD16/BV421, anti-CD33/BV711 and, anti-CD45/AF700 (for detailed references see Table 2, antibody concentrations are optimized for our own settings, adapt to your own needs).

Antibody panel for tissue samples: Viability dye (eFluor 780), Lineage stain (anti-CD3/BV510, anti-CD19/BV510, anti-CD56/BV510), anti-HLA-DR/PE-Cy7, anti-CD14/PerCP, anti-CD15/PE, anti-CD16/BV421, anti-CD33/BV711, anti-CD45/AF700 and, anti-CD206/FITC (for detailed references see Table 2, antibody concentrations are optimized for our own settings, adapt to your own needs).

d. Resuspend the cells in 100 μL of staining buffer + 33% FcR blocking reagent per 10^7 cells. Incubate for 5 min at RT.

e. Add 100 μL of antibody mix, at twice the staining concentration, per 10^7 cells (total: 200 μL per 10^7 cells) and incubate at RT in the dark for 20 min.

f. Wash the cells twice by centrifugation at 380*g* for 10 min with staining buffer.

g. Resuspend the cells at 500 μL per 10^7 cells or according to the sorter's cell density specifications in staining buffer.

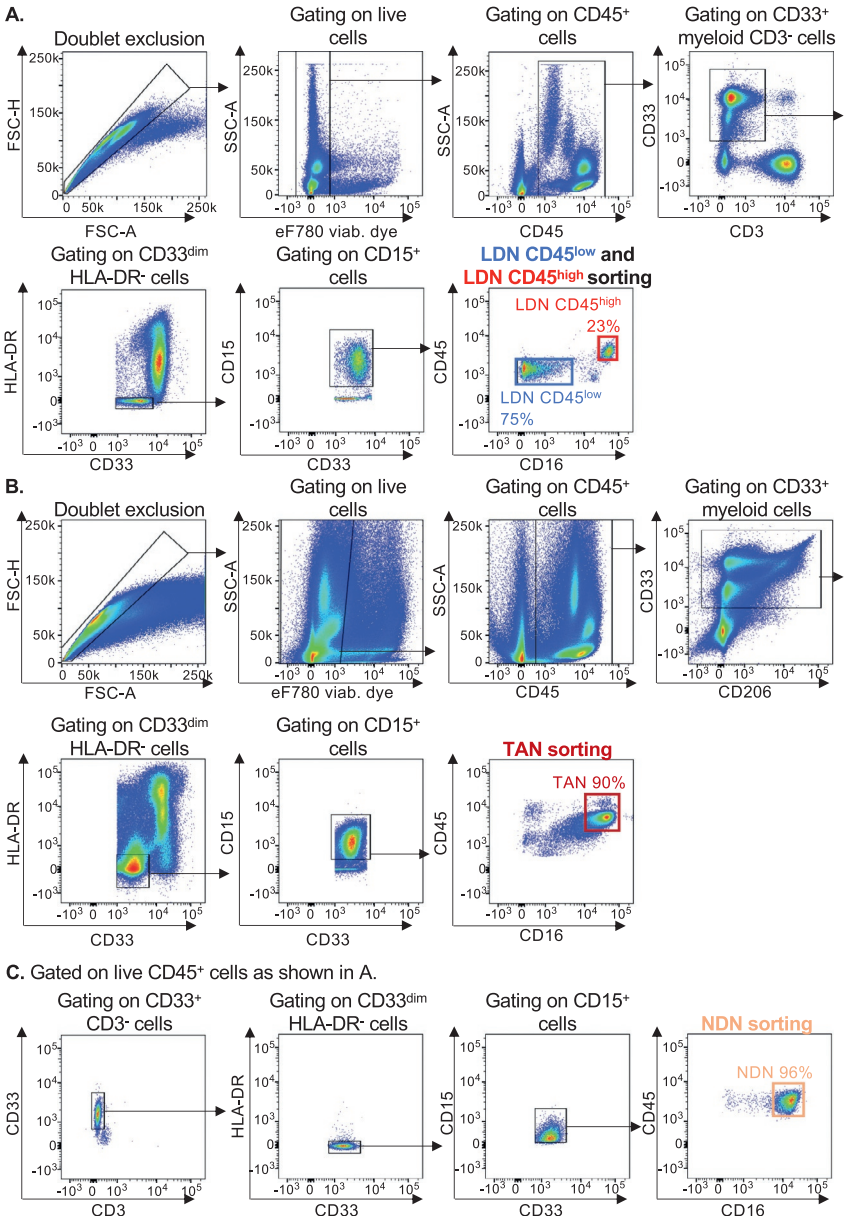

Fig. 8 Neutrophils gating and sorting strategy. Gating strategy employed in our laboratory (de Duve Institute) to isolate (A) LDN CD45^{high} and LDN CD45^{low} from PBMC and (B) TAN from the tumor. (C) NDN represent the vast majority of live CD45^{+} cells present in the high-density fraction. We are aware that the gating strategy used so far excludes a minor fraction of NDN/TAN displaying lower CD16 and CD45 expression.

h. This laboratory (de Duve Institute) uses a BD FACS Aria III (BD Biosciences) on the lowest speed setting (0.5) with the 100 μm nozzle (BD Biosciences, Cat# 643942), and BD FACSDiva software. The samples are maintained at RT during sorting.

i. For the gating strategy refer to Fig. 8.

- **i.** Blood LDN contain a mix of suppressive $CD45^{high}$ and non-suppressive $CD45^{low}$ neutrophils which should be sorted separately.
- **ii.** Historically, gMDSC were described as $CD15^{+}$ HLA-DR^{-} myeloid cells. Thus, we defined TAN as $CD45^{+}$ $CD33^{dim}$ $CD15^{+}$ HLA-DR^{-} cells isolated from tumor tissue. However, it is now known that TAN can adopt an antigen presenting phenotype and express HLA-DR (Pylaeva et al., 2022; Singhal et al., 2016; Wu et al., 2024). Ideally, HLA-DR^{+} TAN and HLA-DR^{-} TAN should be analyzed separately for their effect on T-cell proliferation.
- **iii.** Blood NDN represent relatively homogenous population.

j. Collect the neutrophils in FBS at RT. Refer to Table 3 for an example of typical yields obtainable from blood and tumor from one lung cancer patient.

k. Neutrophils cannot be frozen and have a short life span *ex vivo*, maintain the collection tube at 37 °C for the shortest amount of time possible and avoid temperature variation.

4. Labeling of T-cells with fluorescent tracker dye

T-cell proliferation can be assessed in various ways. The most popular methods are labeling T-cells with radioactive thymidine or with fluorescent tracker dyes. We propose to use the latter. The T-cells are labeled with tracker dyes such as carboxyfluorescein succinimidyl ester (CFSE) or 5-chloromethylfluorescein diacetate (CMFDA) at the beginning of the experiment. Similar chemical properties mean these dyes are highly suitable to track cell proliferation (Zolnierowicz, Ambrozek-Latecka, Kawiak, Wasilewska, & Hoser, 2013). 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 (Lantz, Lemus, Lange, & Karol, 2001; Sebastià, Cristòfol, Martín, Rodríguez-Farré, & Sanfeliu, 2003). Second, the dyes covalently bond to several cytosolic proteins, including some with low turnover rates (Sebastià et al., 2003). 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, multiple

rounds of division can be monitored by flow cytometry through the dilution of the tracker 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). In our experience, the best results are obtained after 4 days of incubation, or about five rounds of divisions. At this point, the dye dilution results in nicely separated peaks and proliferating cells are not starved by nutrient depletion.

a. Use T-cells isolated in Section 2.3 and thawed at least 4 h in advance as described in Section 2.7.1.

b. T-cell complete medium consists of RPMI 1640 + 5% HS + 100 U/mL penicillin + 100 μg/mL streptomycin + 50 IU/mL IL-2. It is used at 37 °C.

c. Dilute CMFDA (Thermo Fisher Scientific, Cat# C2925) at 0,5 μM in warm pure RPMI 1640. Optimize to your own specific flow cytometry settings.

NOTE: Preliminary experiments in our lab indicate that CellTrace Violet (Thermo Fisher Scientific, Cat# C34557) lead to more defined separations between peaks and, thus, could be a suitable replacement for the CMFDA.

d. Count the T-cells with Trypan Blue. Transfer the necessary number of cells in a 15 mL conical centrifuge tube. We recommend using at least 10^6 T-cell for the staining steps. Add pure RPMI 1640 up to the 15 mL mark and pellet the cells by centrifugation at 380*g* for 10 min. Discard the supernatant and resuspend the cells in warm diluted CMFDA at a concentration of 1×10^6 cells/mL.

NOTE: It is essential to use pure RPMI 1640 as CMFDA reacts with the proteins present in the serum.

e. Mix gently and incubate for 20 min at 37 °C, 5% CO_2 and in the dark.

f. Fill the conical centrifuge tube with T-cell complete medium and wash the labeled T-cells once by centrifugation at 380*g* for 10 min. Resuspend the cells in 1 mL of T-cell complete medium and count them with Trypan Blue. Add complete medium to adjust the cell concentration to 6×10^5 cells/mL. Rest the cells at 37 °C, 5% CO_2 until seeding for the proliferation assay (Section 5.3).

5. Preparation of proliferation assay

5.1 Preliminary notes

a. T-cell complete medium consists of RPMI 1640 + 5% HS + 100 U/mL penicillin + 100 μg/mL streptomycin + 50 IU/mL IL-2. It is used at 37 °C. No supplementation with arginine, asparagine and tryptophan

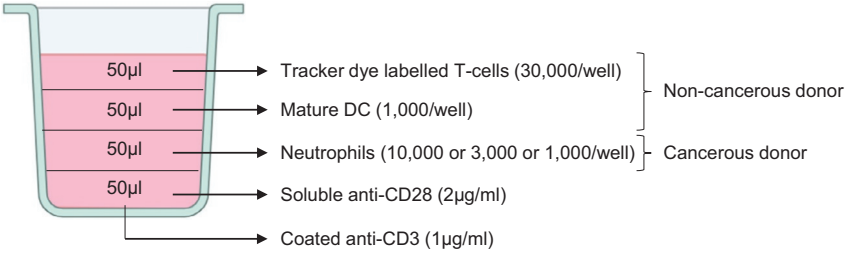

Fig. 9 Well design for the proliferation assay in a flat-bottom 96-wells tissue culture plate.

is required since RPMI 1640 already contains concentrations of the amino acids exceeding physiological conditions. Low amounts of IL-2 are added to prevent the T-cells from starving of the cytokine, and to avoid an IL-2-deprivation mediated apoptosis of the T-cells (Duke & Cohen, 1986).

b. The proliferation assay is assembled from components prepared at different times. The wells of the assay contain 200 µL in total (Fig. 9):
  - **i.** *T-cells*: labeled with a tracker dye, 30,000 per well (Section 4)
  - **ii.** *Mature DC*: 1000 per well (Section 2.7.2)
  - **iii.** *Anti-CD3 antibodies*: coated at 1 µg/mL in PBS for 2 h (Section 5.2.1)
  - **iv.** *Anti-CD28 antibodies*: soluble, 2 µg/mL final concentration (Section 5.2.2)
  - **v.** *Neutrophils:* Freshly isolated (Section 3). We add 3 different numbers of neutrophils, (10,000, 3000 and 1000) to the T-cell. This allow us to obtain three different neutrophils to T-cells ratios (Section 5.3.2) which are used to show a "dose-dependent" effect of the neutrophils (Section 6.2)

c. The T-cells are stimulated through anti-human CD3 (clone OKT3, BioLegend, Cat# 317326) and anti-human CD28 (clone CD28.2, BD Biosciences, Cat# 555725) antibodies. These antibodies target the CD3/TCR complex and the co-stimulatory molecule CD28, respectively. The anti-CD3 antibody is immobilized to the tissue culture plate (protocol described in Section 5.2.1) as T-cells require the stable cross-linking of TCR and prolonged dwell times for activation. The anti-CD28 antibody is added in solution.

d. Control wells consist of the following:
  - **i.** *Unstimulated control*: T-cells cultured in wells without anti-CD3 coating and soluble anti-CD28, with mature DC and without neutrophils

ii. *Stimulated control*: T-cells cultured in wells with anti-CD3 coating, and soluble anti-CD28, with mature DC and without neutrophils

NOTE: In those control wells, T-cell complete medium replaces the soluble anti-CD28 and the neutrophil suspension.

5.2 T-cell stimulation

5.2.1 Anti-CD3 stimulation

a. Coat the wells of the flat-bottom 96-wells tissue culture plate (Corning, Cat# 3596) by seeding 100 µL/well of anti-CD3 diluted at 1 µg/mL in sterile PBS. Use sterile PBS only for control wells without stimulation. Incubate the plate 2 h at 37 °C. Keep sterile.

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

5.2.2 Anti-CD28 stimulation

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

5.3 Cells plating

5.3.1 Mature dendritic cells

a. Count the mature DC thawed and rested in Section 2.7.2. Harvest 10^5 mature DC into an FBS-coated 15 mL conical centrifuge tube and wash by centrifugation at 400*g* for 10 min. Resuspend the cells in 5 mL of T-cell complete medium (20,000 cells/mL). Seed the cells at 1000 cells/well, i.e., 50 µL/well.

5.3.2 FACS isolated neutrophils

a. Add T-cell complete medium to the suspension of neutrophils to fill the tube. Centrifuge at 380*g* for 10 min.

b. Resuspend the cells in T-cell complete medium at 2×10^5 cells/mL using the count provided by the cell sorter.

NOTE: If you used magnetic sorting to isolate NDN use the cell count of Section 3.1.3, point l.

c. Add directly 50 µL of this suspension for the 1:3 neutrophils to T-cells ratio and dilute it further with T-cell complete medium for the 1:10 and 1:30 neutrophils to T-cells ratios. Use T-cell complete medium instead of neutrophils in control wells.

5.3.3 T-cells

a. Seed tracker dye-labeled T-cells (Section 4) at 30,000 cells/well, i.e., 50 μL/well. T-cells should be plated last.

b. Once everything is plated, centrifuge the plate at 200*g* for 1 min to initiate the assay and incubate at 37 °C, 5% CO_2 for 4 days.

6. Neutrophil-T-cell co-culture and analysis of T-cell proliferation

6.1 Proliferation analysis by flow cytometry

a. After 4 days, inspect the wells visually by light microscopy. You should see numerous clumps of T-cells in the stimulated wells and none in the non-stimulated wells. This inspection is also the occasion to check for bacterial contamination of the wells that might occur when working with *ex vivo* isolated cells.

NOTE: The following procedures are performed under non-sterile conditions and at RT.

b. The staining buffer contains PBS + EDTA 1 mM + 2% HS and is filtered through a 0,22 μm filter and used at 4 °C.

c. With a multichannel pipette, transfer the T-cells into a non-sterile 96-wells conical-bottom plate that will be used for staining. Centrifuge the plate at 400*g* for 5 min at 4 °C. Discard the supernatant.

d. Rinse the wells of the 96-wells flat-bottom co-culture plate with 200 μL of staining buffer and, then transfer the buffer to the corresponding wells of the 96-wells conical-bottom staining plate to rinse the pelleted cells. Centrifuge the staining plate at 400*g* for 5 min at 4 °C. Discard the supernatant.

e. Resuspend the cells in 50 μL per well of staining buffer + 10% FcR blocking reagent and incubate for 5 min at 4 °C.

f. In the meantime, prepare the antibody mix in staining buffer, at twice the staining concentration. Prepare 50 μL per well.

Antibody panel: Viability dye (eFluor 780), anti-CD3/PE, anti-CD8α/APC, anti-CD137/BV421 and, Sphero Rainbow Calibration Particles (for detailed references see Table 2, antibody concentrations are optimized for our own settings, adapt to your own needs).

NOTE: The Rainbow Particles are added as normalization constant at 2500 particles/well.

g. Add 50 μL of antibody mix, at twice the staining concentration, per well and mix carefully. Incubate at 4 °C in the dark for 20 min.

h. Centrifuge the plate at 400*g* for 5 min at 4 °C, discard the supernatant and wash the cells with 200 μL of staining buffer. Repeat this step for a second wash.

i. Centrifuge the plate at 400*g* for 5 min at 4 °C for a third time, discard the supernatant and use 200 μL of staining buffer to transfer the stained cells into a non-sterile 96-wells round-bottom plate or any alternative suitable for flow cytometry acquisition.

j. Proceed to flow cytometry acquisition according to your in-house machine specifications and settings. This laboratory (de Duve Institute) uses a BD LSRFortessa flow cytometer and BD FACSDiva software.

6.2 Data analysis

Our laboratory (de Duve Institute) uses FlowJo software for flow cytometry data analysis, Excel (Microsoft) for further analysis and presentation of numeric results and GraphPad Prism (GraphPad Software) for graphical representation and statistical testing. FlowJo's incorporated proliferation modeling tool and the associated proliferation indexes (BD Biosciences, 2019) are commonly used for analysis. However, as we assess both $CD4^+$ and $CD8^+$ T-cells proliferation, we obtain irregular proliferation peaks, which can make manual analysis, as described hereunder, necessary. Alternatively, $CD4^+$ and $CD8^+$ T-cells can be gated and analyzed separately. The anti-CD137 staining allows us to assess the effect of neutrophils on T-cells' activation separately from their effects on proliferation (Dawicki & Watts, 2004; Hirsch et al., 2024; Wolfl et al., 2007).

6.2.1 Flow cytometry analysis (with FlowJo software)

a. Use compensation controls acquired to create compensation matrix.

b. Gate successively for the lymphocytes (FSC-A/SSC-A), single cells (FSC-A/FSC-H), live T-cells (viability dye/CD3), and the proliferating cells (CMFDA/histogram). Finally, using CMFDA dilution, gate on each proliferation peak separately, with Peak 0 corresponding to the cells that did not divide, Peak 1 to cells that divided once and so on (Fig. 10). We usually obtain 6–7 peaks, corresponding to 5–6 division cycles. Use the unstimulated control to gate on $CD137^+$ and $CD137^-$ T-cells. An additional step to gate on the $CD8^+$ T-cells can be inserted (CD8/CD3), to separately assess their proliferation peaks and CD137 expression.

c. Create a gate for the Rainbow Particles (Fig. 10).

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

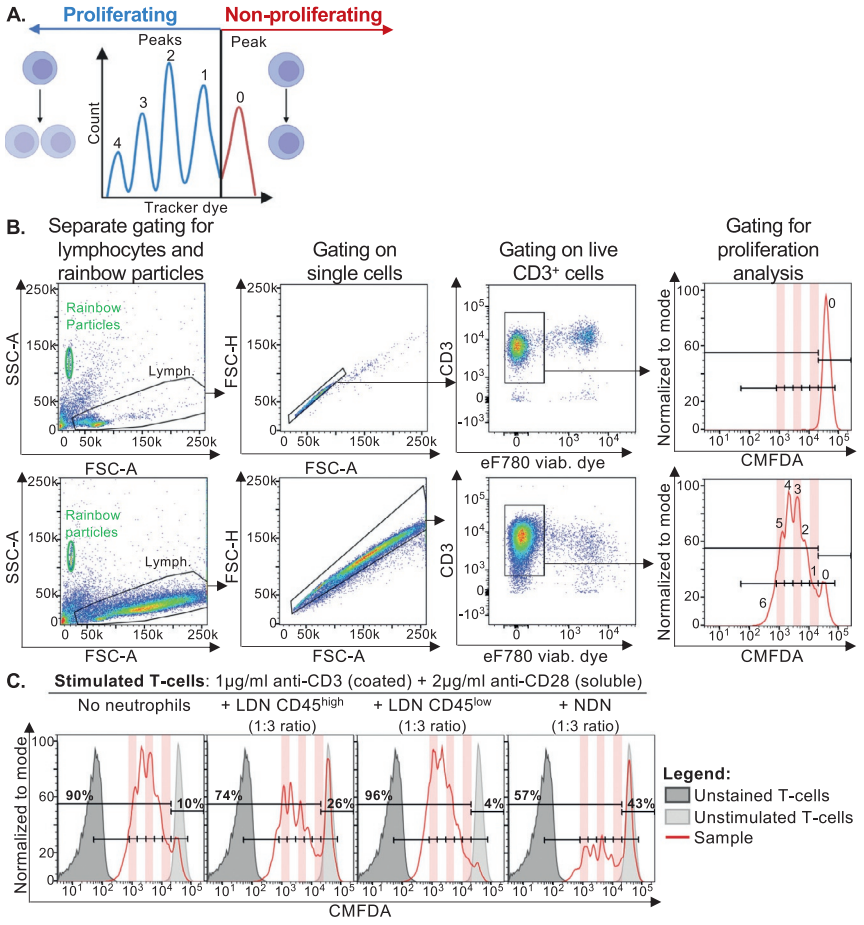

Fig. 10 Example of flow cytometry analysis of proliferation assay results. (A) Principle of the proliferation assay. T-cells are labeled with a tracker dye (e.g., CFSE or CMFDA). The intensity of the dye decreases by a factor of two with each division of proliferating cells. Non-proliferating cells retain high concentration 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, anti-CD8 and anti-CD137 antibodies. First, lymphocytes are selected according to their cell size and morphology. Next, single cells and then live $CD3^+$ cells are selected. Finally, proliferation is assessed by tracker dye dilution. Extra steps can be inserted to focus on $CD8^+$ or $CD8^-$ cells specifically. (C) Example of the flow cytometry analysis of a proliferation assay with suppressive LDN $CD45^{high}$ and NDN and non-suppressive LDN $CD45^{low}$, isolated from lung cancer patient blood, compared to a stimulated control.

e. Using FlowJo's table editor, extract numerical data for the following parameters for each sample (e.g., as an Excel file):
  i. *Particles count:* Number of Rainbow Particles
  ii. *Live T-cells count*: Number of live T-cells
  iii. Number of cells present in each proliferation peak
  iv. Percentage of $CD137^+$ T-cells

6.2.2 Proliferation assay data analysis (with Excel)

A variety of analyses are possible. We present here the ones that are used in our laboratory (de Duve Institute).

a. Determine the absolute number of (#) live T-cells present at the end of the coculture by normalization to the 2500 Rainbow Particles added per sample.
  i. $\text{Absolute\#live T} - \text{cells} = \frac{\text{Live T}-\text{cells count}}{\text{particles count}} \times 2{,}500$
  ii. T-cell recovery: Use the absolute # live T-cells to determine the fold change in the number of T-cells present at the end of the co-culture compared to T-cells stimulated in absence of neutrophils:

$$\text{T} - \text{cell recovery} = \frac{\text{Absolute\#live T} - \text{cells in the well}}{\text{Absolute\#live T} - \text{cells in stimulated control}}$$

b. Calculate:
  i. $\#\text{cells at the end of the culture} = \#\text{cells in Peak 0} + \#\text{cells in Peak 1} + \ldots$
  ii. $\#\text{divided cells} = \#\text{cells in Peak 1} + \#\text{cells in Peak 2} + \ldots$
  iii. $\#\text{cells at start of the culture} = \#\text{cells in Peak 0} + \frac{\#\text{cells in Peak 1}}{2^1} + \frac{\#\text{cells in Peak 2}}{2^2} + \frac{\#\text{cells in Peak 3}}{2^3} + \ldots$
  iv. $\#\text{divisions} = \left(\frac{\#\text{cells in Peak 1}}{2^1}\right)\text{x1} + \left(\frac{\#\text{cells in Peak 2}}{2^2}\right)\text{x2} + \left(\frac{\#\text{cells in Peak 3}}{2^3}\right)\text{x3} + \ldots$
  v. $\#\text{cells that went into division} = \#\text{cells at start of the culture} - \#\text{cells in Peak 0}$

c. The values calculated in point b use the number of events read in each proliferation peak which varies with the number of events acquired in each well. To allow comparison between condition within the same experiment, calculate the following indexes:

i. *Division index*: the mean number of divisions achieved by each T-cell present at the start of the culture

$$\text{Division index} = \frac{\#\text{divisions}}{\#\text{cells at the start of the culture}}$$

ii. *Expansion index*: the fold expansion of the culture

$$\text{Expansion index} = \frac{\#\text{cells at the end of the culture}}{\#\text{cells at the start of the culture}}$$

iii. *Proliferation index*: the mean number of divisions achieved by each T-cell that went into division

$$\text{Proliferation index} = \frac{\#\text{divisions}}{\#\text{cells that went into division}}$$

iv. *Precursor frequency (%)*: the percentage of initial cells that divided

$$\text{Precursor frequency} = \frac{\#\text{cells that went into division}}{\#\text{cells at the start of the culture}} \text{ x } 100$$

The percentage of $CD137^+$ T-cell can be used to compare T-cell activation between conditions or between dividing and non-dividing cells.

Comparing different experiments:

a. To compare results from separate experiments, set the value for the stimulated controls (T-cells, with stimulation, without neutrophils) as 1.

b. Normalize all other conditions to this control. This way, a normalized value of 1.5 indicates a 50% increase of the index compared to the stimulated control.

6.2.3 Graphical presentation (with Prism GraphPad)

Through the three different neutrophils to T-cells ratios used in the coculture (Section 5.3.2), we showcase the "dose-dependent" effect of the neutrophils on T-cell proliferation. Fig. 11 show the normalized division indexes corresponding to the different neutrophil sub-populations obtained from one patient. In our experience, this value is the one the most affected by neutrophils (Vanhaver et al., 2023).

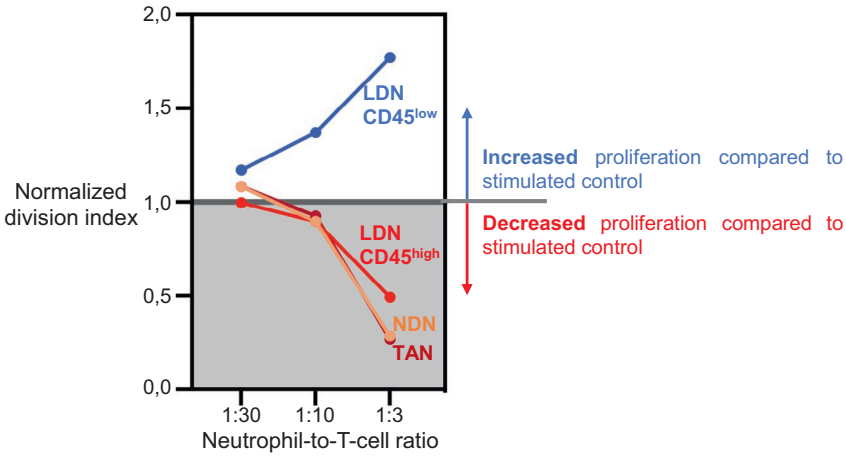

Fig. 11 Analysis of proliferation assay results. Flow cytometry data is exported into Excel files, or similar, to calculate the relevant values (e.g., division index, expansion index or proliferation index) as described in Section 6.2. The "dose-dependent" effect of neutrophils on T-cell proliferation is best assessed through the variation of the division index normalized on the value obtained from the stimulated control (without neutrophil). This figure shows the normalized division indexes obtained with three neutrophils to T-cells ratios in co-culture with suppressive LDN CD45^{high}, NDN and TAN, and non-suppressive LDN CD45^{low}, all isolated from the same patient. Graph created with GraphPad Prism version 10.2.0 for Mac (Dotmatics).

7. Concluding remarks

The method presented here describes how to assess the suppressive capacities and the heterogeneity of human neutrophils using T-cell proliferation as a readout. The reliable comparison of the function of neutrophils isolated from different sources and in different diseases, requires the neutrophils themselves to be the only variable within the assay. Our protocol for isolating T-cells and mature DC allows us to freeze around 40 aliquots of each cell type isolated from one blood bag of a non-cancerous donor. By thawing those cells when needed, we can then assess the effects of neutrophils isolated from 40 cancer patients on the same T-cell and mature DC. When we start to run out of frozen aliquots, we isolate a new batch of cells and compare their proliferative capacities, both in absence and in presence of neutrophils, to the previous batch. This allows to confirm that the newly isolated T-cells are able to proliferate, as some batches fail to proliferate in stimulated control conditions, even in presence of mature DC. This also allows us to demonstrate that T-cells isolated from different patients behave

similarly in presence of neutrophils, which, in our experience, has always been the case. With this protocol, we showed the heterogeneity of the LDN population and suppressive capacities of NDN isolated from the circulation of lung cancer patients. Furthermore, many additional functional assays can be derived from this basis.

Acknowledgments

This chapter follows work supported by grant #1610119 from the Région Wallonne Program WALINNOV (Belgium), Convention IMMUCAN. Pierre van der Bruggen was supported by the Fonds de la Recherche Scientifique (FNRS, Belgium): grant PDR T.0226.23 and grants CDR J.0212.24 and J.0209.20. This work was also supported by the Foundation against Cancer, Belgium: grant 2022-186. Nicolas Delhez and Christophe Vanhaver were supported by the Région Wallonne through a FRIA Fellowship of the Fonds de la Recherche Scientifique (FNRS, Belgium). We thank the de DUVE Institute (Belgium) and European COST action 20117 Mye-InfoBank for their continuous support.

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