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# A protocol for phenotypic detection and enumeration of circulating endothelial cells and circulating progenitor cells in human blood

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## Abstract

Blood circulating endothelial cells (CECs) and circulating hematopoietic progenitor cells (CPCs) represent two cell populations that are thought to play important roles in tissue vascularization. CECs and CPCs are currently studied as surrogate markers in patients for more than a dozen pathologies, including heart disease and cancer. However, data interpretation has often been difficult because of multiple definitions, methods and protocols used to evaluate and count these cells by different laboratories. Here, we propose a cytometry protocol for phenotypic identification and enumeration of CECs and CPCs in human blood using four surface markers: CD31, CD34, CD133 and CD45. This method allows further phenotypic analyses to explore the biology of these cells. In addition, it offers a platform for longitudinal studies of these cells in patients with different pathologies. The protocol is relatively simple, inexpensive and can be adapted for multiple flow cytometer types or software. The procedure should take 2–2.5 h, and is expected to detect 0.1–6.0% viable CECs and 0.01–0.20% CPCs within blood mononuclear cell population.

## INTRODUCTION

A minute fraction of the human peripheral blood is made up by CPCs and CECs. Hematopoietic functional assays have greatly enhanced our understanding of CPC biology and allowed the identification of specific surface markers for these cells. Nevertheless, much needs to be characterized in terms of the function of these hematopoietic precursors in tissue neovascularization[1,2]. On the other hand, despite being identified four decades ago, the function of CECs is still obscure[3]. To assist the research on the biology of these rare cell populations, we describe here a protocol for the phenotypic identification of CECs and CPCs in the human blood. Under this protocol, we are conducting studies of blood cells from different sources, including umbilical cord blood, buffy coats, and healthy individual and cancer patient samples. The study of CECs and CPCs in patients has recently raised great interest, as these cells have been proposed as surrogatemarkers formore than a dozen pathologies, including heart disease and cancer. In addition, antiangiogenic therapy as well as therapeutic

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angiogenesis treatmentsmay directly and immediately affect CECs and CPCs because of their direct exposure to the infused agent. Validating a biomarker for pro- or antiangiogenic agents is critically important for the development of these emerging therapies[4]. Moreover, obtaining peripheral blood at multiple time points during treatment is a minimally invasive, routine procedure.

In 2001, CEC enumeration was proposed by the Bertolini group from Italy as a biomarker of response in mouse models of cancer[5]. In patients, evaluation of CEC kinetics and viability predicted the clinical benefit of “metronomic” chemotherapy for cancer[6]. Using multicolor flow cytometry, our group has established the surface phenotype of viable CECs (defined as $CD31^{bright}CD34^{+}CD45^{-}CD133^{-}$ cells) and CPCs (identified as $CD31^{+}CD34^{bright}CD45^{dim}CD133^{+}$ cells)[7,8]. This type of analysis is a departure from the techniques that used singlemarkers for CEC identification, and relies on specific cell surface phenotypes using a combination of only four markers to identify both viable CECs and CPCs. We have shown that in rectal cancer patients the number of viable CECs and CPCs is decreased by bevacizumab (a vascular endothelial growth factor (VEGF)-specific antibody, Genentech Inc.) administration alone[9,10]. In recurrent glioblastoma patients, we have demonstrated that the number of viable CECs correlated with radiographic (MRI) progression through therapy with a pan- VEGF receptor tyrosine kinase inhibitor—AZD2171 (AstraZeneca Pharmaceuticals)[11]. In the same study, we have also shown that CPCs had differential biomarker value, and predicted relapse of glioblastomas during drug interruptions[11]. Given the variety of surface markers, methodologies and protocols used by different groups to identify CECs[3,8], the different potential origins of these endothelial cells (tumor, systemic, bone marrow) and the currently unresolved issue of circulating endothelial progenitor cell (referred to as CEP or EPC) phenotype, standardization is needed to allow cross-studies comparisons. Based on our phenotypic analyses, we propose here a standard protocol for evaluation of viable CECs and CPCs.

To phenotypically identify viable CEC and CPCs, the use of the following fluorescently labeled antibodies is recommended: CD31-FITC (a marker of endothelial cells and monocytes), CD34-APC (present on hematopoietic precursors and endothelial cells), CD133-PE (present on hematopoietic precursors), CD45-PerCP (a pan-hematopoietic marker) and VEGFR2 (KDR)-PE (present on endothelial cells, certain monocytes and hematopoietic precursors).

# MATERIALS

## REAGENTS

- Peripheral blood (see REAGENT SETUP) ! **CAUTION** Experiments involving human subjects must conform to institutional and national guidelines.

  Informed consent must be obtained.
- CD31-FITC (BD Pharmingen, cat. no. 555445)
- CD34-APC (BD Pharmingen, cat. no. 340441)
- CD133-PE (Miltenyi Biotec, cat. no. 130-080-801)
- CD45-PerCP (BD Pharmingen, cat. no. 340665)
- VEGFR2 (KDR)-PE (R&D Systems, cat. no. FAB 3578)
- Fluorescently labeled isotype-matched IgG1 antibodies (BD Pharmingen, cat. no. 555748)
- Fc-receptor (e.g., CD16/CD32) blocking antibody (Miltenyi Biotec, cat. no. 120-000-442)

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- To-Pro-3 iodide (Invitrogen, cat. no. T3605)
- Propidium iodide (PI) (Invitrogen, cat. no. P1304MP)
- ! **CAUTION** All fluorescent reagents are light-sensitive. Refrigerate in a dark place.
- ACK lysis buffer (Cambrex Bio Science, cat. no. 10-548E)
- 0.5% (w/v) BSA and 2 mM EDTA in 1× PBS ($Ca^{2+}$ and $Mg^{2+}$ free)
- 2% (v/v) paraformaldehyde ! **CAUTION** paraformaldehyde is toxic.

## EQUIPMENT

- EDTA collection tubes (e.g., Vacutainer; BD Biosciences, cat. no. 366450 or equivalent)
- Blue top "Tiger" tubes (8 ml BD Vacutainer CPT tubes; BD Biosciences cat. no. 362761) for mononuclear cells
- 15 ml polystyrene conical centrifuge tube (BD Falcon, cat. no. 352099 or equivalent)
- 5 ml polystyrene round-bottom test tube (BD Falcon, cat. no. 352054 or equivalent)
- 40 μm cell strainers (BD Falcon, cat. no. 352340)
- FACSCalibur flow cytometer (Becton Dickinson, cat. no. 342945), or equivalent
- CellQuest (Largo) software
- Excel spreadsheets (Microsoft Office)

## REAGENT SETUP

**Blood—**A minimum of 5 ml peripheral blood should be drawn by venipunctures and immediately transferred into EDTA collection tubes for plasma collection. For the protocol option using upfront mononuclear cell separation, collect blood in blue top "Tiger" tubes. These blood samples can be used for both CEC/CPC and plasma angiogenic protein evaluation, and should be drawn at various time points (depending on the half-life of the agent and the duration of treatment cycles). The samples should be kept on wet ice at all times and processed within 2–3 h of collection. ▲ **CRITICAL** The use of plasma EDTA is recommended to prevent platelet degranulation.

**! CAUTION** All samples should be handled in isolators and human materials discarded according to the institutional regulations.

**! CAUTION** Lengthy storage of samples or their maintenance at room temperature is not recommended as it may affect cell viability and phenotype.

# PROCEDURE

## Blood transport and storage

**1|** Blood specimens should be immediately transferred on wet ice to the laboratory. This is recommended as viability may affect surface marker expression and is a major issue for CEC/ CPC analyses.

▲ **CRITICAL STEP** Samples should be kept on ice at all times. Save a 3 ml aliquot in case the analysis needs to be repeated.

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### Immunostaining

**2|** Immunostaining should be performed following option A for whole blood samples or option B for mononuclear cell samples.

▲ **CRITICAL STEP** Immunostaining of the patient samples should be performed by trained personnel in designated laboratory areas.

#### (A) Whole blood sample preparation

**i.** Centrifuge fresh samples at 700*g* for 20 min at 4 °C with no brake.

**ii.** Remove gently the upper phase (plasma) with a 5 ml pipette into a separate tube and store in 0.25 ml aliquots.

**iii.** Resuspend the lower phase containing the blood cells using 10 ml of cold 1× PBS containing 0.5% (w/v) BSA and 1.5 mM EDTA.

**iv.** Centrifuge samples at 700*g* for 20 min at 4 °C with no brake.

**v.** Remove the upper phase and discard it; resuspend the cell pellet by gentle pipetting or vortex, and transfer 2.5 ml into a separate tube and keep on ice.

**vi.** Add FcR-blocking agent in the tube at a concentration of 1 μg $ml^{-1}$, and incubate the samples on ice for 10 min.

▲ **CRITICAL STEP** Fc-blocking is an important step that prevents the unspecific binding of antibodies, which is common for many cell types, including B lymphocytes, NK cells, granulocytes, monocytes, macrophages and platelets.

**vii.** Distribute 500 μl of samples into one isotype control and three sample tubes and add the following antibodies: (i) 4 μl of IgG-FITC, 5 μl of IgG-PE, 10 μl of IgG-PerCP and 3 μl of IgG-APC (for control); (ii) 4 μl of CD31-FITC, 5 μl of IgG-PE, 10 μl of CD45-PerCP and 3 μl of IgG-APC; (iii) 4 μl of CD31-FITC, 5 μl of CD133-PE, 10 μl of CD45-PerCP and 3 μl of CD34-APC; and (iv) 4 μl of CD31-FITC, 5 μl of VEGFR2-PE, 10 μl of CD45-PerCP and 3 μl of CD34-APC. During the first run, it is important to run single color controls to set up the compensation matrix. Thereafter, tubes 1 and 2 can be used to confirm compensation settings. Note that a master-mix for each combination of antibodies can be prepared if multiple samples are immunostained at the same time. Alternatively, premixed combinations of antibodies can be ordered from the manufacturing company (BD Pharmingen). For additional studies of CEC/CPC phenotype, prepare extra tubes. For example, CD14-FITC (4 μl per sample) can be used in combination with VEGFR2-PE, CD45-PerCP and CD34-APC to identify subsets of monocytic cells. Viability can be tested using nuclear dyes such as the DNA intercalator PI (replace the PE antibody) or monomeric cyanine dyes (To-Pro-3, replace the APC antibody).

▲ **CRITICAL STEP** If using fluorescence-labeled antibodies from other manufacturers, the concentration should be optimized by testing serial concentrations to achieve similar staining patterns.

**viii.** Vortex briefly and incubate for 30 min at 4 °C or on ice in the dark.

**ix.** Add 9 ml of ACK lysing buffer (to lyse red blood cells (RBCs)), vortex briefly and incubate at room temperature (18–25 °C) for 3 min. ▲ **CRITICAL STEP** Lysis should not be performed for more than 3–5 min as it may affect cell viability. However, it is important that the RBC lysis is complete, to avoid difficulties in enumeration of CECs and CPCs.

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**i.** Wash twice with 9 ml of cold regular 1× PBS and centrifuge at 250*g* at 4 °C with brake for 5 min to remove the supernatant. ▲ **CRITICAL STEP** If assessing viability, after washing suspend the pellet in 1 ml of 1× PBS containing 20 μm $ml^{-1}$ of viability dye and gently mix the suspension. After approximately 20 min of incubation at 4 °C in the dark, run the samples on the flow cytometer in their staining solution. Use single color control tubes with the viability dye only. All samples should be kept cold during acquisition and should be run within 1 h after the addition of the dye; otherwise, cells should be fixed.

**ii.** Resuspend the cell pellet in 500 μl of 1× PBS and filter the samples through 40 μm cell strainer into 5 ml BD Falcon tubes and label the tube with the staining combination information.

▪ **PAUSE POINT** The tubes should be held at 4 °C (or on ice) in the dark before acquisition on the flow cytometer. If the samples are not run immediately through the flow cytometer, samples should be fixed in 2% paraformaldehyde to ensure optimal results.

▲ **CRITICAL STEP** Samples should not be exposed to light as bleaching of the fluorophores may occur.

### (B) Mononuclear cell sample preparation

**i.** For this procedure, collect the blood sample in blue top “Tiger” tubes.

**ii.** Centrifuge the CPT tubes with blood samples at 1,600*g* with no brake for 25 min at 16–20 °C.

**iii.** Collect approximately 2 ml fuzzy layer of plasma containing mononuclear cells and located just above the gel barrier inside the tube.

**iv.** Add FcR-blocking agent in the tube at a concentration recommended by the manufacturer (e.g., 20 μl per $10^7$ cells when using Miltenyi Biotec FcR-blocking agent), and incubate the samples on ice for 10 min.

**v.** Distribute 500 μl of samples into four tubes and add the following antibodies: (i) 4 μl of IgG-FITC, 5 μl of IgG-PE, 10 μl of IgG-PerCP and 3 μl of IgG-APC; (ii) 4 μl of CD31-FITC, 5 μl of IgG-PE, 10 μl of CD45-PerCP and 3 μl of IgG-APC; (iii) 4 μl of CD31-FITC, 5 μl of CD133-PE, 10 μl of CD45-PerCP and 3 μl of CD34-APC; and (iv) 4 μl of CD31-FITC, 5 μl of VEGFR2-PE, 10 μl of CD45-PerCP and 3 μl of CD34-APC. Note that a master-mix for each combination of antibodies can be prepared if multiple samples are immunostained at the same time. Alternatively, premixed combinations of antibodies can be ordered from the manufacturing company (BD Pharmingen). For additional studies of CEC/CPC phenotype, prepare extra tubes. For example, CD14-FITC (4 μl per sample) can be used in combination with VEGFR2-PE, CD45-PerCP and CD34-APC to identify subsets of monocytic cells. Add 1× PBS as needed to make the necessary volume (500 μl). For additional studies of CEC/CPC phenotype, prepare extra tubes. For example, CD14-FITC (4 μl per sample) can be used in combination with VEGFR2-PE, CD45-PerCP and CD34-APC to identify subsets of monocytic cells. Viability can be tested using nuclear dyes such as the DNA intercalator PI (replace the PE antibody) or monomeric cyanine dyes (To-Pro-3, replace the APC antibody).

▲ **CRITICAL STEP** If using fluorescence-labeled antibodies from other manufacturers, the concentration should be optimized by testing serial concentrations to achieve similar staining patterns.

**vi.** Vortex briefly and incubate for 30 min at 4 °C or on ice in the dark.

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**vii.** Wash twice with 9 ml of cold regular 1× PBS and centrifuge at 250*g* at 4 °C with brake for 5 min to remove the supernatant.

▲ **CRITICAL STEP** If assessing viability, after washing suspend the pellet in 1 ml of 1× PBS containing 20 μm $ml^{-1}$ of viability dye and gently mix the suspension. After approximately 20 min of incubation at 4 °C in the dark, run the samples on the flow cytometer in their staining solution. Use single color control tubes with the viability dye only. All samples should be kept cold during acquisition and should be run within 1 h after the addition of the dye; otherwise, cells should be fixed.

**i.** Resuspend the cell pellet in 500 μl of 1×PBS and filter the samples through 40 μm cell strainer into 5 ml cytometry tubes and label the tube with the staining combination information.

▪ **PAUSE POINT** Hold the tubes at 4 °C (or on ice) in the dark before acquisition on the flow cytometer. If the samples are not run immediately through the flow cytometer, samples should be fixed in 2% paraformaldehyde to ensure optimal results.

▲ **CRITICAL STEP** Samples should not be exposed to light as bleaching of the fluorophores may occur.

## Flow cytometric analysis

**3|** Set the gate on the mononuclear populations, to avoid RBC, platelet, cell debris and neutrophil contamination. The use of CPT tubes simplifies the analyses as only mononuclear cells are recovered. Collect 100,000–150,000 events in the mononuclear cell gate.

### ? TROUBLESHOOTING

## Data analysis

**4|** When using the whole blood method (Step 2A), set the gate on lymphocytes and monocytes (mononuclear cells; Fig. 1a). The use of "Tiger" tubes allows for upfront separation of mononuclear cells (Fig. 2a), but gating is recommended to exclude any contamination with non-mononuclear cells. Identify viable CECs as $CD31^{bright}$, $CD34^{dim}$ and $CD45^{-}$ cells; these cells are $CD133^{-}$. Identify CPCs as $CD34^{bright}$, $CD133^{+}$ and $CD45^{dim}$ cells; these cells are $CD31^{+}$ (Figs. 1b–d and 2b–d). Calculate the number of viable CECs and the number of CPCs as percent of the total number of mononuclear cells, and average the values obtained from CECs from tubes 2–4 and for CPCs from tubes 3–4. Upon acquiring the data, the changes in CEC/CPC number can be analyzed as a function of time and for correlation with outcome or tumor response measurements performed during treatment (e.g., tumor volume measured on MRI scans, tumor diameter or cross-section area on MRI or CT scans, FDG uptake by PET scan, etc.). Percent expression of the cell number can be log-transformed to calculate power. To characterize patterns of changes of these cell counts over time, one should construct a plot for each cell to graphically assess the change and perform longitudinal data analysis.

▲ **CRITICAL STEP** Save the settings for inter-channel compensation and analysis and use for all samples, but set the gate individually for each sample on the mononuclear cell populations to exclude any neutrophil, platelet, RBC or cell debris contamination.

**^TIMING—**Proceed with sample preparation for flow cytometry within 2 h of blood collection

For immunostaining, the procedure described in Step 2A (Steps i–ix) will take approximately 2 h, whereas the one in Step 2B (Steps i–viii) will take approximately 90 min

The flow cytometry procedures (Steps 3 and 4) will take approximately 15 min for four tubes once a compensation matrix has been established.

### ? TROUBLESHOOTING

**Step 2A(ix):** If the sample does not become red-transparent, centrifuge at 300*g* at 4 °C with brake for 5 min to remove the supernatant and repeat the lysing step for 1–2 min at room temperature.

**Step 2B(vii):** If the pellet is colored red, it indicates that RBC lysis was insufficient and may interfere with cytometric analysis. Repeat ACK lysis in between the two washing steps.

**Step 3:** It is important to run single color controls or alternating color controls to confirm that the compensation matrix is accurate. Although previous cytometer settings can be reused, minor compensation adjustments may need to be performed. If analysis of tube 2 (CD31-FITC, IgG-PE, CD45-PerCP, IgG-APC) suggests a change in settings, run single color controls to reset the compensation matrix. If the fluorescence signal is missing in any channel or is inadequate (e.g., too dim), consider running samples on an alternate machine to determine if the problem is related to antibody staining or hardware. However, for optimal results, the same machine should be used to run all samples within a study. Nonetheless, if you identify an instrument malfunction, use another cytometer or fix the samples in 2% paraformaldehyde and analyze later. If the machine is functioning but the problems persist, repeat staining procedure using a different aliquot.

## ANTICIPATED RESULTS

Using our protocol, two populations were detectable in numbers that allowed kinetic analyses by flow multiple time points in patients. One population consisted of $CD31^{bright}CD34^{dim}CD45^{-}CD133^{-}$ cells (referred to as viable CECs in our reports, ranging typically from 0.1% to 6.0% of blood mononuclear cells) and the other was represented by $CD31^{+}CD34^{bright}CD133^{+}CD45^{dim}$ CPCs (0.01–0.20% of blood mononuclear cells)[8]. Data should be considered as valid if the s.d. is no more than 0.20% for CECs (in tubes 2–4 or in duplicates of these tubes) and 0.01 for CPCs (in tubes 3 and 4). These cells have a typical endothelial and hematopoietic progenitor phenotype, respectively. We and others have discovered promising clinical correlations between the concentration of these two populations and the effect of antiangiogenic therapy and/or tumor response in phase 1/2 clinical trials of antiangiogenic therapy. Specifically, we and others have found that bevacizumab can decrease the number of viable CECs and CPCs in rectal and breast cancer patients[7,9,10,12], and the decrease in viable CECs in breast cancer patients predicted a better outcome[12]. In recurrent glioblastoma patients treated with AZD2171, we found that viable CECs and CPCs have differential predictive value: viable CEC number correlated with tumor radiographic progression (evaluated by MRI), whereas the number of CPCs correlated with progression after periods of drug interruption[11].

Our findings also indicate that comparative evaluations between treatment time points in clinical studies, and equally important, comparative evaluations between different studies using distinct markers for CEC evaluation in patients are impossible and misleading without a good understanding of the phenotypic characteristics and a standardization of the protocol. Ultimately, the validity of any of these cell populations—detected with various techniques—as a prognostic or treatment biomarker remains to be proven in large clinical studies.

In summary, the proposed flow cytometric protocol uses four different markers to phenotypically detect and distinguish endothelial cells from mature or progenitor hematopoietic ($CD45^{+}$) populations. These distinct populations can be identified based on their



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levels of expression of the surface markers ("fluorescence signal brightness") despite phenotypic overlap with different hematopoietic lineage-committed populations. We hope that our cytometry protocol and the insights obtained into the phenotype of CECs and CPCs provide data to the research community that will assist further research studies in this area, as well as validation of these cell populations as biomarkers.

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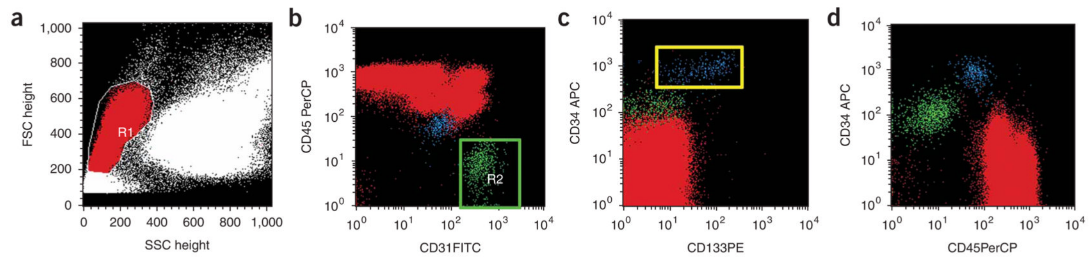


**Figure 1.**

Multicolor flow cytometric analyses of human mononuclear cells in whole blood samples. (**a–d**) Staining for CD31, CD34, CD133 and CD45 of a whole blood sample and gating on mononuclear cellular events on the forward-side scatter plot (in red in **a**) allow identification of two distinct populations of interest: $CD31^{bright}CD45^{-}CD34^{+}CD133^{-}$ (green rectangles) and $CD133^{+}CD34^{bright}CD31^{+}CD45^{dim}$ progenitor cells (blue, in yellow rectangles in **b–d**).

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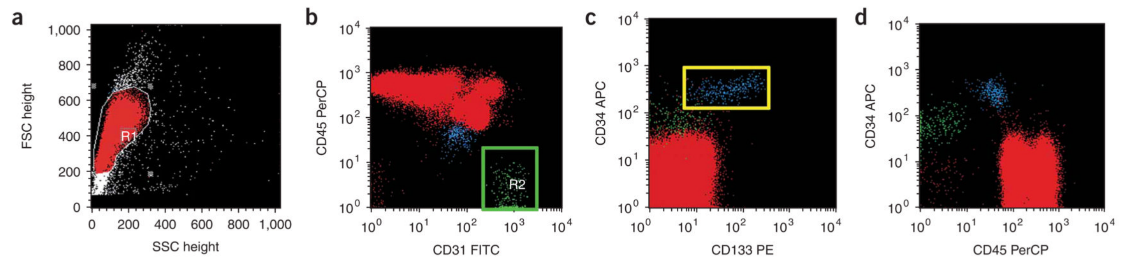


**Figure 2.**

Multicolor flow cytometric analyses of human mononuclear cells separated from whole blood samples. (**a–d**) Staining for CD31, CD34, CD133 and CD45 of a whole blood sample and gating on mononuclear cellular events on the forward-side scatter plot (in red in **a**) allow identification of two distinct populations of interest: $CD31^{bright}CD45^{-}CD34^{+}CD133^{-}$ (green rectangles) and $CD133^{+}CD34^{bright}CD31^{+}CD45^{dim}$ progenitor cells (blue, in yellow rectangles in **b–d**).