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A method for high purity sorting of rare cell subsets applied to T_{DC}

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Abstract

T_{DC} are a recently described subset of polyclonal $\alpha\beta$ T-cells with dendritic cell properties. Because of their low number in peripheral immune compartments, isolation and characterization of T_{DC} with existing purification methods is technically challenging. Here we describe a customized gating strategy and a flow cytometry-based cell sorting protocol for isolation of T_{DC} . The protocol was developed because, despite very conservative gating for dead-cell and doublet exclusion, cells obtained with normal sorting procedures were enriched for T_{DC} but not pure. Re-sorting the output of the first round of sorting results in highly pure T_{DC} . Cells obtained with this method are viable and can be used for in vitro characterization. Moreover, this double-round sorting strategy can be universally applied to the isolation of other rare cell subsets.

Keywords

Flow cytometry; Sorting; T_{DC} ; Purification

1. Introduction

Flow cytometry and cell sorting are widely used techniques that make possible the identification and physical separation of cell subsets based on cell surface markers. Initially, single laser flow cytometers allowed discrimination between two populations of cells, but the advent of polychromatic cytofluorometry has rendered feasible the simultaneous analysis of many subsets (Herzenberg et al., 2002; Chattopadhyay and Roederer, 2012; Fung et al., 2010; Lugli et al., 2013). The ability to simultaneously detect multiple markers has revealed a high level of complexity among immune cells, resulting in the identification and characterization of many T cell and antigen-presenting cell (APC) subsets.

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Author Contributions

MK performed research, MK and JDA designed the research and wrote the paper.

Competing financial interests

The authors declare no financial interests.

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T_{DC} constitute a rare subset of haematopoietic cells recently identified by our group (Kuka et al., 2012). The name reflects their surface phenotype, which has markers of $\alpha\beta$ polyclonal T cells as well as those of conventional DC. The ability to isolate highly purified T_{DC} led to the finding that, in addition to their T-DC characteristics, T_{DC} have a distinct gene expression profile characterized by a cytotoxic signature. T_{DC} proliferate in response to TCR-mediated stimulation and give rise to memory cells during LCMV infection. In addition, T_{DC} upregulate costimulatory molecules and produce IL-12 when stimulated with TLR agonists. They can process and present antigen on MHC-II molecules, as well as proliferate in response, suggesting that their genetic duality is reflected in their functional properties. Even more interesting, T_{DC} follow the development routes of both DC and T cells. Indeed, while dependent on FLT3 signaling and expressing the conventional DC lineage marker Zbtb46 (Satpathy et al., 2012; Meredith et al., 2012), T_{DC} maturation also requires a thymus and positive selection. Although the physiological role of T_{DC} is still under investigation, their properties prompt interesting speculations about their relevance not only in infection but also in tumor immunity settings (Kuka et al., 2012).

Gene expression and functional studies with purified T_{DC} were technically challenging, due to the low frequency of this population in the spleen and lymph nodes. Isolation of cells by flow cytometry is usually preferable to other methods for studies involving rare cell populations. In particular, in the case of T_{DC}, isolation with techniques such as the use of magnetic beads is not possible because these cells co-express markers of different cell lineages. Here we show an optimized method for isolation of high-purity T_{DC} via a double-round cell sorting strategy.

2. Materials and methods

2.1. Mice

C57BL/6 (B6) mice were obtained from the NCI Frederick Animal Facility. The Animal Care and Use Committee of the National Cancer Institute approved all animal experimental procedures.

2.2. Reagents

Phosphate-buffered saline (PBS) 7.4 (1X), RPMI 1640 medium with L-glutamine, HEPES 1M buffer solution, and L-Glutamine 200 mM solution were purchased from Gibco, Life Technologies. BenchMark fetal bovine serum (FBS) and ACK Lysing Buffer were purchased from Gemini and Lonza, respectively. 2-mercaptoethanol was purchased from Sigma-Aldrich, gentamicin sulfate 50 mg/ml from Cellgro, and Sheath Fluid 8X Concentrate from Biosure. Purified rat anti-mouse CD16/CD32 (Mouse Fc Block) clone 2.4G2 (BD) and Alexa Fluor700-conjugated anti-mouse TCR β chain clone H57 were obtained from BD Pharmingen. PE-conjugated anti-mouse TCR β chain clone H57, PerCP-Cy5.5-conjugated anti-human/mouse CD45R (B220), APC-conjugated anti-mouse CD11c clone N418, and eFluor450-conjugated anti-mouse MHC Class II (I-A/I-E) clone M5/114.15.2 were purchased from Ebioscience. LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit was purchased from Life Technologies, RNeasy Micro Kit from Qiagen, and SuperScript. III One-Step RT-PCR System with Platinum® Taq DNA Polymerase from Invitrogen. A FacsAria II (BD) instrument with a blue, red, and violet laser was used for cell sorting.

2.3. Preparation of single-cell suspensions and staining

Murine spleens were disrupted and filtered through a 40 μ m strainer to obtain single-cell suspensions. Splenocytes were resuspended in PBS containing 2% FBS, and red blood cells lysed with ACK lysing buffer. After lysis cells were filtered again to get rid of all cell clumps and resuspended at a concentration of 200–300 million/ml. ~50 million splenocytes

were stained in a volume of 200 μ l in 96-well round-bottom plates. 5 μ g/ml Mouse Fc Block antibody was added to cell pellets prior to staining with fluorochrome-conjugated antibodies.

2.4. Cell sorting

Before cell sorting, splenocytes were filtered again through a 40 μ m strainer to eliminate all remaining cell clumps and resuspended at a concentration of 20–30 million/ml. Splenocytes were sorted through a 70 μ m nozzle at a sheath pressure of 70 psi and a drop drive frequency of 90–95 kHz. A highly pure sorting modality (4-way purity sorting for FACS Aria) was chosen for both rounds of sortin. The flow rate during the first-round sorting was approximately 10,000 events/second. Sorted cells were collected in 5 ml polypropylene tubes containing 1 ml collection medium (RPMI supplemented with 50% FBS, 100 μ g/ml gentamicin, 4 mM L-glutamine, 20 mM HEPES, 50 μ M 2-mercaptoethanol). The second sort was performed with cells in the collection medium used in the first sort. Cells were not centrifuged, washed, or stained again to avoid loss of material. The same gates were used for both rounds of sorting. Because of the low number of events and the fact that the cells were highly diluted, the second sort flow rate was variable but it rarely reached 10,000 ev/sec. After the second-round sorting, cells were concentrated by centrifugation. Depending on the purpose of the experiment, cells were washed with either PBS or culture medium (RPMI supplemented with 10% FBS, 100 μ g/ml gentamicin, 4 mM L-glutamine, 20 mM HEPES, 50 μ M 2-mercaptoethanol).

2.5. RNA isolation and RT-PCR

RNA was isolated with the RNeasy Microkit (Qiagen). All manufacturer's instructions, including optional treatment with DNase, were followed. For cDNA and RT-PCR the SuperScript III One-Step Platinum Taq DNA Polymerase kit (Invitrogen) was used. Primers for amplification of *Actb* mRNA are the following: 5'-GTGGGCCGCCCTAGGCACC-3' (forward) and 5'-CTCTTTGATGTCACGCACGAT-3' (reverse).

3. Results and Discussion

The frequency of murine T_{DC} is around 0.04% in spleen and 0.06% in lymph nodes (Kuka et al., 2012). This rare subset of cells can be defined with a minimum set of four cell surface markers: TCR β , CD11c, MHC-II, and B220 (Fig. 1). One gating strategy is to select CD11c⁺MHC-II⁺ cells from total splenocytes and within this gate select cells that are TCR β ⁺B220⁺ (Fig. 1A). Alternatively, it is possible to first gate on TCR β ⁺B220⁺ cells and then on CD11c⁺MHCII⁺ (Fig. 1B). In the latter case the expected frequency of T_{DC} is around 0.1–0.2% of T cells. Whereas both these gating strategies can be used for surface marker characterization, the second strategy was preferable for cell sorting because the initial gating on TCR β ⁺ or TCR β ⁺ cells allowed for visualization of a higher number of events and a higher sorting purity in our experience. The combination of fluorochromes conjugated to the staining antibodies was chosen based on parameters such as brightness, quenching, and photo-bleaching. For cell sorting purposes very bright fluorochromes with a clear distinction between the negative and positive peaks are recommended. Moreover, isolation of T_{DC} involves hours of sorting, thus it is important that fluorochromes maintain their intensity throughout this long process. Our recommended fluorochrome combination for an instrument with blue, red, and violet lasers is PE (anti-TCR β), eFluor450 (anti-MHC-II), APC (anti-CD11c), and PerCP-Cy5.5 (anti-B220). In particular, we recommend carefully choosing the anti-TCR β conjugated antibodies. We found that the Alexa Fluor700-conjugated antibody to TCR β does not discriminate very well between TCR β ⁺ and TCR β cells, even if the two peaks seem distinct. As a result, many cells in the TCR β ⁺ gate are actually DC, not T_{DC}. This, for example, can be assessed by comparing the frequency of

T_{DC} obtained with Alexa Fluor700- or PE-conjugated anti-TCR β (Fig. 1B and C). In addition, most of the “false” T_{DC} have TCR levels that are lower than conventional T cells and high levels of CD11c and MHC-II, the latter being similar to conventional DC but not to T_{DC} (Fig. 1D).

A critical point in designing the sorting protocol was exclusion of dead cells and of cell conjugates. T cells and DC interact and form conjugates in the presence of antigenic stimulation, and although these conjugates are usually present only in settings involving cell activation, a few might be found in cells from unmanipulated mice (Bousso, 2008; Lim et al., 2011; Revy et al., 2001). Because T_{DC} bear both T cell and DC markers, it was important to exclude conjugates and ensure that one is working with a homogeneous single cell population. Therefore, we adopted a very conservative dead-cell and doublet-exclusion gating strategy (Fig. 2A). Gates were designed to perform a three-way sort. First, TCR β ⁺ cells were selected and separated into T_{DC} or T cells based on the expression of CD11c and MHC-II. The third population to be sorted, DC, was identified as the CD11c⁺MHC-II⁺ expressing subset in the TCR β ⁺ gate (Figure 2A). A “purity” sorting modality was chosen for the first round sorting; however, users might consider other sorting modes depending on whether they want to favor yield or purity. Unfortunately, because the frequency of T_{DC} in the spleen is very low, both the yield and the purity of sorted T_{DC} after the first round was suboptimal. Whereas the theoretical number of T_{DC} in a spleen of 50×10^6 cells should be about 2×10^4 cells, typically the average number of cells that was obtained after the first-round sorting was about 6000 cells/spleen. Of these, only an average of 43% were T_{DC} (Fig. 2B), whereas the purity of simultaneously sorted DC and T cells was >95% (Fig. 2A). We found that even a very small number of contaminating DC or T cells could lead to misinterpretation of results obtained with sorted T_{DC}. For this reason, we devised a cell sorting strategy that would minimize contamination from these two cell subsets. Attempts to improve the purity of T_{DC} by lowering the event rate to 10,000 events/sec, and the choice of high purity sorting filters, failed to achieve acceptable purity. Therefore, a two-round sorting strategy was designed that allowed us to isolate T_{DC} at a level of purity approaching 100%. This strategy consisted in subjecting the output from the first round to a second round of sorting. After the second round of sorting, a yield of about 1000 pure T_{DC}/spleen (Figure 2A and B) was obtained when the procedure was performed under optimal conditions. Although cells were stained only prior to sorting with a lived/dead discrimination marker, viability data obtained through forward and side scatter correlated with those of the live/dead marker, and cell viability after the second-round was similar to that of the first-round (Figure 2C). It should be noted that after the second round of sorting, the purity of T_{DC} was close to 100% when we consider the viable cells. A small percentage of dead cells contaminated the sorting output, and these cells were negative for all markers present in our staining combination (Supplementary Figure). They may represent dead T_{DC} or other subsets, or simply debris or bubbles detected as events due the slow rate of cell acquisition. In any case, one should take into account this small percentage of contaminating cells that was beyond our ability to optimize with this technique.

The double-round sorting strategy described in this paper allows for isolation of highly pure and viable cells. T_{DC} obtained with this method can be used for a variety of applications (Kuka et al., 2012). In particular, gene expression studies were performed with as few as 1000 sorted T_{DC}. An example shown here was performed with mRNA from 1400 freshly sorted ex vivo cells or from 1400 sorted cells cultured overnight. mRNA was retrotranscribed to cDNA and amplified with gene-specific primers in a onestep procedure that resulted in minimal loss of material. *Actb* mRNA amplification was performed with mRNA from 100 T_{DC}, T, and DC equivalents (Fig. 3), the results demonstrating that such gene expression studies can be performed with fresh or cultured T_{DC}.

The limitations of this protocol reside in the low number of cells obtained after two-round cell sorting. Even for studies requiring a very small number of cells (e.g. 1000), it is necessary to sort cells from 2–3 spleens from naïve mice. Indeed, the user should keep in mind that the time necessary to obtain 500–700 T_{DC} starting from 50–70 million cells (e.g. one naïve spleen) is between 1 and 1.5 hours. The problem of small numbers of T_{DC} per animal can be ameliorated by expanding them in vivo by the introduction of tumor cells that secrete FLT3L (Kuka et al., 2012). However, because total splenocyte numbers increase as well, this will not decrease the time it takes to sort a given number of cells.

Whereas iterative rounds of flow cytometry-based sorting have been used in the past to achieve high purity in single-cell cloning studies (Dangl et al., 1982; Weichel et al., 1985), the application of this strategy to the isolation of rare subsets of primary cells can be a useful aid to functional studies in different fields. In addition, the strategy we propose is cost-effective. An alternative method for pre-enrichment of T_{DC} is positive selection with anti-CD11c-coated magnetic beads. Besides considerations of cost (a parameter that will vary based on the sorting charges applied at different institutions and on whether the lab is equipped with the necessary reagents and magnets), this method might not result in optimal enrichment. In fact, the first-round sort results in an enrichment of T_{DC} to about 43% (Figure 2C), whereas enrichment with beads could be no better, and no doubt considerably less, than 4–8%, their fractional representation in the CD11c⁺ population (Figure 1A).

In conclusion, we believe that this double-round sorting method can be easily applied to other cell types as well, and therefore be useful for the characterization of more rare subsets whose purity after one round of sorting is suboptimal.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Gating strategies for identification of T_{DC}

A–B, Two alternative gating strategies for identification of T_{DC} from B6 splenocytes are shown. **C,** Identification of "false" T_{DC} when anti-mouse TCRβ conjugated to Alexa Fluor700 was used. **D,** Expression levels of different markers on T_{DC}, T, or DC were compared after usage of PE-conjugated or Alexa Fluor700-conjugated anti-mouse TCRβ antibodies.

Fig. 2. Doublet exclusion gating and sorting strategy

A, The sorting strategy for T, DC, and T_{DC} is depicted. Discrimination between live and dead B6 splenocytes was performed by Aqua Live/Dead staining. Two sequential doublet exclusion gatings were performed on live (Aqua Live/Dead-negative) cells. T_{DC} are identified as CD11c⁺MHC-II⁺ cells gated on TCRβ⁺B220⁻ splenocytes. DC (gated on TCRβ⁺B220⁻ splenocytes) or T cells (gated on TCRβ⁺B220⁻ splenocytes) are also depicted. **B**, Absolute numbers (left) and frequencies (right) of cells collected from the T_{DC} gate are depicted. The mean + SEM of at least nine independent experiments is shown. **C**, Frequencies of live (Aqua Live/Dead-negative or forward/side scatter-positive) cells collected from the T_{DC} gate are depicted. The mean + SEM of ten independent experiments is shown.

Fig. 3. RT-PCR analysis

Actb mRNA amplification was performed with mRNA from 100 T_{DC}, T, and DC equivalents. mRNA was isolated from freshly sorted cells or cells cultured overnight.