

# Rare Cells: Focus on Detection and Clinical Relevance

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**Abstract** The study of rare-cell populations is assuming a growing importance to the advancement of medical diagnostics and therapeutics. In several clinical studies, counting rare cells can provide valuable information on the status of the patient; examples are the search for circulating tumor cells in peripheral blood, tumor stem cells, endothelial cells, hematopoietic progenitor cells and their subpopulations, antigen-specific T-cells, invariant natural killer T cells, and fetal cells in maternal circulation. The study of rare-cell populations is useful not only to understand disease mechanisms, but also to find novel targets. With multiparameter capabilities and a very high analysis rate, flow cytometry is at present the most potent technology to address rare-cell analysis. This chapter will describe the main issues of the pre-analytical phase, including the amount of blood to use, the use of pre-enriched populations, the number of markers to use, and the number of cells to acquire. Moreover, we will discuss the importance of excluding doublets and the

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use of a DUMP channel, along with the importance of using optimal methodologies in all phases, including collection of biological samples, adequate controls, and expert use of software and hardware.

**Keywords** Rare events • Antigen-specific T cells • Invariant natural killer cells • Polyfunctionality • Circulating endothelial cells

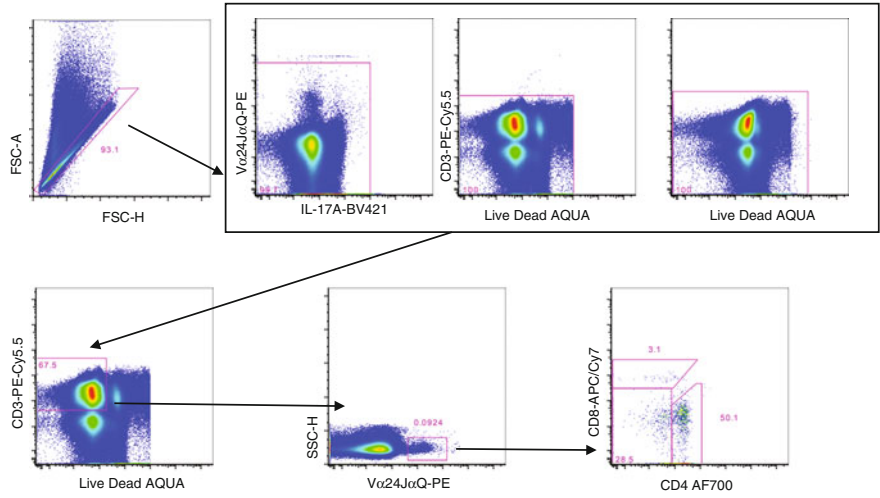
## 1 Introduction

The detection, count, and functional analysis of rare cells can give relevant information either to the basic scientist or to the clinician. The term “rare” typically refers to events with a frequency of 0.01% or less [1, 2]. Applications of rare-cell analysis include the detection in blood of tumors such as metastatic breast cancer cells [3] or neuroblastoma cells infiltrating the bone marrow [4], monitoring of minimal residual disease [5, 6], detection of stem cells and rare HIV-infected cells in peripheral blood [7], identification of antigen (Ag)-specific T cells [8] and invariant natural killer T cells [9, 10], and analysis of mutation frequencies in genetic toxicology [11]. This chapter will discuss the detection of some rare cell populations of interest for immunologists and oncologists, along with some crucial technical issues, along with the requirements for isolating and enumerating such cells in peripheral blood.

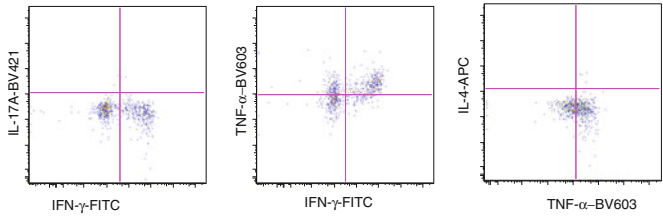
## 2 Ag-Specific T Cells

Ag-specific T cells are clearly fundamental in specific immune responses, and they form the basis of immunological memory. To know their frequency, phenotype, and functional capability can be essential to evaluate the immune status of an individual, to understand the mechanism(s) of an eventual immunopathological event, or eventually to predict immune protection. Considering that the repertoire of T cells can be theoretically unlimited (i.e., on the order of  $10^{14}$ ), the frequency of T cells specific for a single Ag in the peripheral blood (the most accessible tissue of the organism) is very low. Indeed, in the absence of acute infections, specific T-cell frequencies are typically much below 1% in either the repertoire of naïve (with a range of 0.2–60 cells/ $10^6$  naïve T cells) or memory cells [12].

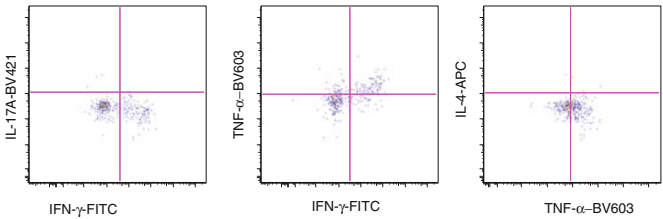
Two main approaches can be used to detect Ag-specific T cells (Fig. 1). First, recombinant MHC-peptide multimers recognized by a specific T-cell receptor (TCR) allow one to identify the total pool of T cells with a distinct specificity. The multimerization of peptide-MHC complexes that increases the relative binding avidity and the use of combinatorial color-coded tetramers (enabling the simultaneous use of several tetramers to detect a greater number of different antigen specificities) are the major advantages of this approach [13, 14]. Second, it is



Gated on CD4+ iNKT cells



Gated on CD8+ iNKT cells



**Fig. 1** Gating strategy to analyze the production of cytokines by different types of iNKT cells. Lymphocytes were identified by physical parameters, doublets were removed by using FSC-H versus FSC-A, then iNKT cells were characterized by the expression of CD3 and invariant TCR (TCR Vα24JαQ). In this subset, three populations were identified according to CD4 and CD8 expression. The percentage of CD4+ and CD8+ iNKT cell producing IFN-γ, TNF-α, IL-17A, and IL-4 was quantified. A minimum of 10 million cells per sample were acquired

possible to analyze either the production of cytokines or the expression of activation markers expressed by Ag-specific T cells after specific in vitro stimulation by peptides, intact proteins, or peptide pools that cover the whole sequence of a protein, and thus cover almost all possible T-cell epitopes [15].

Most cytometry-based approaches are limited to the simultaneous screening of up to 100 distinct T-cell specificities in one sample, but recently MHC multimers labeled with individual DNA barcodes have allowed the screening of >1000 peptide specificities in a single sample and detection of low-frequency CD8 T cells specific for virus- or cancer-restricted antigens. This means, for example, that it is possible to detect specific T-cell populations recognizing neopeptides in tumor-infiltrating lymphocytes or in peripheral blood [16]. Finally, a peptide-MHC dodecamer as a “next-generation” technology has been reported. This alternative technique is able to detect two- to five-fold more antigen-specific T cells in both human and murine CD4+ and CD8+  $\alpha\beta$  T-cell compartments compared to the equivalent tetramers [17].

Interest in the evaluation and quantification of Ag-specific responses derives from the fact that a new era for vaccine development has now arisen, one that generates cellular immune responses instead of the classical antibodies. Thus, the repertoire of specific T cells (in terms of cytokine production, proliferation, and killing capability) could be useful to predict vaccine response [18].

As an example of the analysis of the antiviral capabilities of T cells, the magnitude and duration of anti-smallpox immunity has been studied. Antiviral antibody responses remained stable up to 75 years after vaccination, whereas antiviral T-cell responses declined slowly, with a half-life of 8–15 years [19]. Then, it was shown that the duration of immunity following smallpox infection was remarkably similar to that observed after smallpox vaccination, with antiviral T-cell responses that declined slowly over time and antiviral antibody responses that remained stable for decades after recovery from infection [20].

The evaluation of the production of different cytokines by Ag-specific T cells could mirror not only the response to a given pathogen, but also the effect of different treatments. For example, it has been shown that cytotoxic granule release dominates gag-specific CD4+ T-cell response in different phases of HIV infection [21] and that CD4+ gag-specific T lymphocytes are unaffected by CD4-guided treatment interruption and therapy resumption [8].

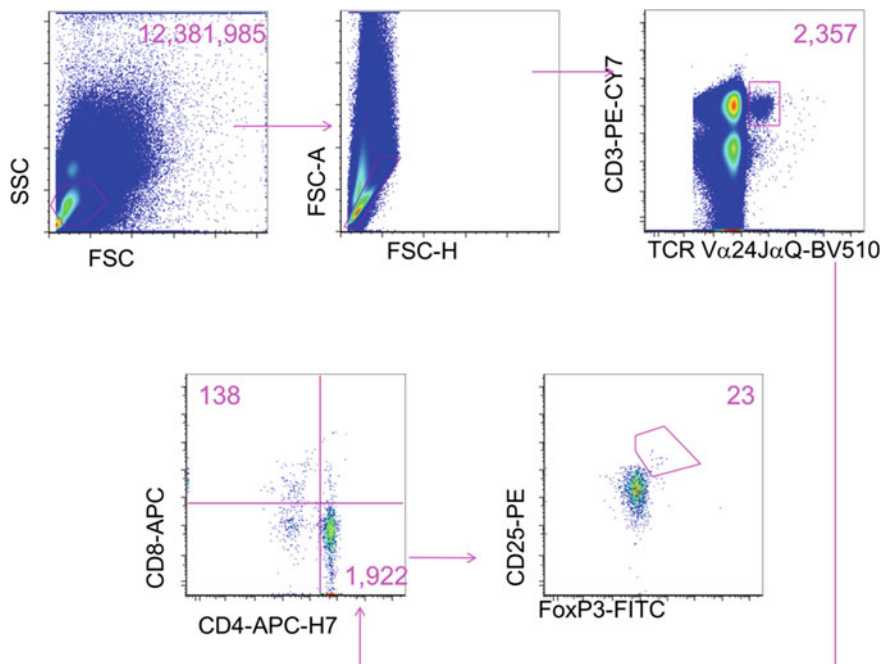
Cytomegalovirus (CMV) has an enormous impact on the overall immune profile of each individual. Preserving both CD8+ T-cell memory and a long-term control of CMV requires considerable effort from the host immune system. One hallmark of CMV infection is the maintenance of large populations of CMV-specific memory CD8+ T cells (that can be as high as 10% of peripheral CD8+ T cells)—a phenomenon termed “memory inflation.” Recent data suggest that memory inflation is associated with impaired immunity in the elderly [22]. Finally, novel features of *M. tuberculosis* antigen-specific T-cell differentiation have been discovered, which reveals pathways that limit and promote immune control of infection [23].

### 3 Invariant Natural Killer T (iNKT) Cells

iNKT cells are innate-like lymphocytes characterized by the expression of markers typical of T lymphocytes (CD3, CD4, CD8) and NK markers (CD161, CD56), but they are uniquely identified by the expression of an invariant V $\alpha$ 24J $\alpha$ 18 TCR, and they recognize as cognate antigens self and foreign lipids presented by CD1d [24, 25]. In humans, iNKT cells have a low frequency in peripheral blood, as they represent 0.1–0.001% of T cells. iNKT cells are considered to be a bridge between innate and adaptive immunity because they can rapidly produce cytokines after stimulation and are able to mediate both protective and regulatory immune functions. However, on the basis of CD4 and CD8 expression, mature iNKT cells can be divided into functionally distinct subsets, i.e., CD4<sup>+</sup>CD8<sup>−</sup>, CD4<sup>−</sup>CD8<sup>−</sup>, and CD4<sup>−</sup>CD8<sup>+</sup> [26, 27]. Each subset of iNKT cells can release large amounts of Th1 [interferon (IFN)- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$ ], Th2 [interleukin (IL)-4, IL-12, and IL-13] [28] and Th17 (IL-17, IL-22) cytokines [29]. Different subsets of iNKT cells have different immunological properties: CD4<sup>+</sup> iNKT cells release Th1, Th2, and Th17 cytokines, while CD8<sup>+</sup> and CD4<sup>−</sup>CD8<sup>−</sup> cells exhibit Th1 phenotypes and cytotoxic activity [30] (Fig. 1). Moreover, a regulatory phenotype of iNKT cells has also been described, based upon the production of IL-10 and transforming growth factor- $\beta$ , and on the expression of classical markers of regulatory T cells such as CD4<sup>+</sup>, CD25<sup>bright</sup>, and FoxP3<sup>+</sup> [31, 32] (Fig. 2).

iNKT cell defects can predispose an individual to autoimmune diseases because of the failure of immune regulation [33]. For example, NKT cells play a key regulatory role in type 1 diabetes. The absence of NKT cells correlates with exacerbation of type 1 diabetes, whereas an increased frequency and/or activation of NKT cells prevents autoimmunity against beta cells [34]. Similarly, the reduced frequency of iNKT cells in peripheral blood from patients with systemic lupus erythematosus supports the idea of a protective role for these cells in the immunopathology of SLE [35]. In addition, a low number of iNKT cells has been found in blood from patients with rheumatoid arthritis (RA), compared to blood from healthy individuals, and low iNKT cell frequencies were associated with an active form of the disease [36]. Studies on iNKT cells in patients affected by multiple sclerosis (MS) then showed the presence of different defects, but yielded contradictory results, mainly because of non-stringent methods used for the identification of these cells and the limited analysis of the distribution and/or function of their subsets [26]. Recently, we reported that the percentage of iNKT cells from patients affected by different forms of multiple sclerosis are similar, but patients affected by progressive forms are characterized by high level of Th1 and Th17 iNKT cells [9].

The frequency and activity of iNKT cells was studied in acute and chronic infections, including that by HIV, where iNKT cells can constitute a considerable viral reservoir [37]. In the early phases of HIV infection, CD4<sup>+</sup> iNKT cells are rapidly depleted in the gut after primary infection [38], but are preserved in gut-associated lymphoid tissue (GALT) characterized by the presence of a Th2



**Fig. 2** Gating strategy to analyze iNKT cells with regulatory potential. Lymphocytes were identified by physical parameters, doublets were removed by using FSC-H versus FSC-A, then iNKT cells were characterized by the expression of CD3 and invariant TCR (TCR Vα24JαQ). In this subset, three populations were identified according to CD4 and CD8 expression. Among CD4 + iNKT cell, the expression of CD25 and FoxP3 allowed the identification of iNKT cells with regulatory potential. In pink, the number of cells present in the gate or in the quadrant

iNKT subset [39]. Circulating iNKT cells are functionally impaired, express high levels of PD-1 [40], and can produce IFN- $\gamma$  [40]. Moreover, they present an inverse correlation between the expression of CD161 and cytokine secretion [41]. In addition, a successful antiretroviral therapy is able to induce a rapid recovery of NKT cells [42], but the reconstitution of the CD4+ iNKT cell subset remains delayed [43]. Recently, we found that in patients with low CD4/CD8 ratio after successful antiretroviral therapy the different subset distributions (CD4+ and CD8+ iNKT cells), the persistent level of activation among iNKT cells, the amount of IL-17-producing CD4+ iNKT cells, and that of IFN- $\gamma$ -producing CD8+ iNKT cells reflected what happens in the whole T-cell compartment [10].

iNKT cell number and activation have been intensely investigated in patients suffering from different cancers. Contradictory results emerged, dependent on the type of tumor and the methods used to identify these cells [44]. For instance, reduced iNKT cell frequency and function have been observed in patients with hematologic cancers or in those with different solid tumors. In accordance with these observations, reduced iNKT cell frequency was correlated to poor overall

survival in patients with acute myeloid leukemia or head and neck squamous cell carcinoma. Interestingly, elevated iNKT cell frequency in some tumors is thought to be a positive prognostic indicator. Concerning activation and functionality, upon activation, iNKT cells can exhibit potent cytotoxic functions to promote the killing of tumor cells such as acute myeloid leukemia [45].

## 4 Circulating Endothelial Cells and Their Precursors

In 1934, non-hematological cells were found in the blood of cancer patients for the first time [46]. Almost six decades later, in 1991, an endothelial identity was attributed to these cells [47]. Since then, circulating endothelial cells (CECs) have been studied in several pathologic conditions that have in common the presence of vascular injury [48–50].

CECs are mature endothelial cells detaching from the intima monolayer in response to endothelial damage [51]. Endothelial dysfunction can take place during the development and the progression of different cardiovascular disorders. Thus, in order to find an early biomarker using a non-invasive technique, monitoring endothelial activity is assuming an important role in clinical practice [52–54]. Indeed, although CECs are rare in healthy individuals, they could be easily counted in the blood of patients with cardiovascular-related complications, suggesting that they may be taken as indicator of disease severity [55].

CECs are also crucial in the neovascularization of tumors both at primary or at metastatic sites [56–59]. Indeed, it has been found that CEC count increased in cancer patients and could correlate with tumor progression [60, 61], and that their number decreased after surgical removal of the tumor and chemotherapy [60]. Kinetics and in vitro viability of CECs are promising predictors of the response to treatment with anti-angiogenic agents in patients with advanced breast cancer or colorectal cancer [62, 63]. CECs would also reveal tumor growth and disease progression at an early stage, in view of the known fragility of the tumor vasculature [64].

If CECs are believed to be mature endothelial cells that have been released into the circulation from an area of disrupted vessels, circulating endothelial precursor or progenitor cells (EPCs) are bone marrow-derived cells that contribute to vasculogenesis—including tumor-associated vasculogenesis [65]. EPCs, which take part in postnatal vasculogenesis, are recruited from the bone marrow under the stimulation of growth factors and cytokines and reach the sites of neovascularization in both physiological and pathological conditions such as malignancies where they contribute to the “angiogenic switch” and tumor progression. The presence of circulating EPCs in the bloodstream of patients with hematological malignancies has been demonstrated and correlated with a poor prognosis [66].

Despite the number of studies performed, investigation of endothelial circulating cells and their progenitors is technically challenging and conflicting results are frequently reported owing to discrepancies in terms of terminology and protocols

used for the detection of these cells, leading to ambiguous conclusions in clinical practice [55]. The huge problem in finding these cells is that a unique marker or a combination of markers that identify circulating endothelial cells and their progenitor has not been yet identified. At present, the most common markers used for this purpose are DNA, CD34, CD45, CD133, CD31, CD146, and CD309. In particular, CECs are defined as events that are DNA+, CD34+, CD45<sup>dim</sup>, CD31+CD133<sup>-</sup>, CD309+. EPCs are defined as DNA+, CD34+, CD45<sup>dim</sup>, CD31+, CD133+, CD309± [67–70].

## 5 Circulating Tumor Cells

In cancer patients, circulating tumor cells (CTCs) can be found in the peripheral blood at very low concentration, ranging from 1 to 10 cells per 10 mL [71]. CTCs are released into the blood stream from primary tumors or from metastatic deposits, and could be useful for a better understanding not only of the phase of the disease and the efficacy of the treatment, but also of the biology of the metastatic process [72]. Strong evidence for CTCs as prognostic markers was first documented in patients suffering from breast cancer [73], and they are now under investigation in several other types of tumors [70]. Three well-known families of antigens can be present on CTCs, either alone or in different combination, *i.e.*, epithelial, mesenchymal, and/or stemness molecules, and can be considered useful markers for the identification of such cells.

A recent publication has described the presence and heterogeneity of a new CTC population that includes cells positive for epithelial cellular adhesion molecule (EpCAM), for cytokeratins (Cks), and also for the pan-hematopoietic marker CD45 [74]. Thus, these cells have characteristics of both epithelial and hematopoietic elements. Interestingly, it has been pointed out that they are not true cancer cells, but rather are tumor-associated macrophages [74]. So, evaluating the role of and eventual changes in circulating cells of the macrophagic lineage, which are often associated with tumors, play a crucial role in their metabolism [75], and can be identified by anti-CD68 mAbs, is under consideration as possible prognostic factors for cancer patients.

In any case, the choice of markers that are characteristic for CTC subpopulations is a really complicated issue; unfortunately, several publications have focused on just one or two markers, creating a heterogeneous amount of (often contrasting) data. Other studies have established which is the gene profile of CTCs. However, from the point of view of a clinician, the interpretation of genetic data is particularly difficult and not feasible in practice. For this reason, in clinical studies the real utility of CTCs for decisions regarding treatment is still under evaluation.

During the past few years, the use of CTCs as a real-time liquid biopsy has received attention [71]. Liquid biopsy is a new diagnostic concept, *i.e.*, a test done



on a sample of blood to look for cancer cells from a tumor that are circulating in the blood or for pieces of tumor-cell DNA that are in the blood. A liquid biopsy may be used to help find cancer cells at an early stage [76], even in the absence of an evident primary tumor.

## **6 Analysis of Rare Events by Flow Cytometry: The First Step**

Flow cytometry is at present the most potent technology to find rare cells, and the so called “next-generation” instruments with very high speed and sensitivity are already allowing an easy detection and analysis of such cells. In order to identify those elements, some practical suggestions have to be kept in mind.

The first step in planning an experiment that involves the estimation of rare cells is to establish the quantity of biological material required. For example, should the endpoint of the study be the evaluation of cytokine production by invariant natural killer T (iNKT) cells after in vitro stimulation, some pre-analytical considerations should be taken into account. In order to define circulating iNKT cells, several markers must be used, including those for recognizing CD3, CD4, CD8, and invariant TCR, as well as those for cell viability. Different cytokines such as TNF- $\alpha$ , IFN- $\gamma$ , IL-4, and IL-17 could be of interest. So a minimum of nine markers are required. If the study considers patients who are severely immunocompromised, like HIV+ individuals before undergoing antiretroviral therapy, the possible low number of lymphocytes must be taken into account. As a consequence, the amount of blood required to detect a reasonable number of rare cells producing one or more cytokines can be as high as 50 mL, since either resting or stimulated cells have to be analyzed [9, 10].

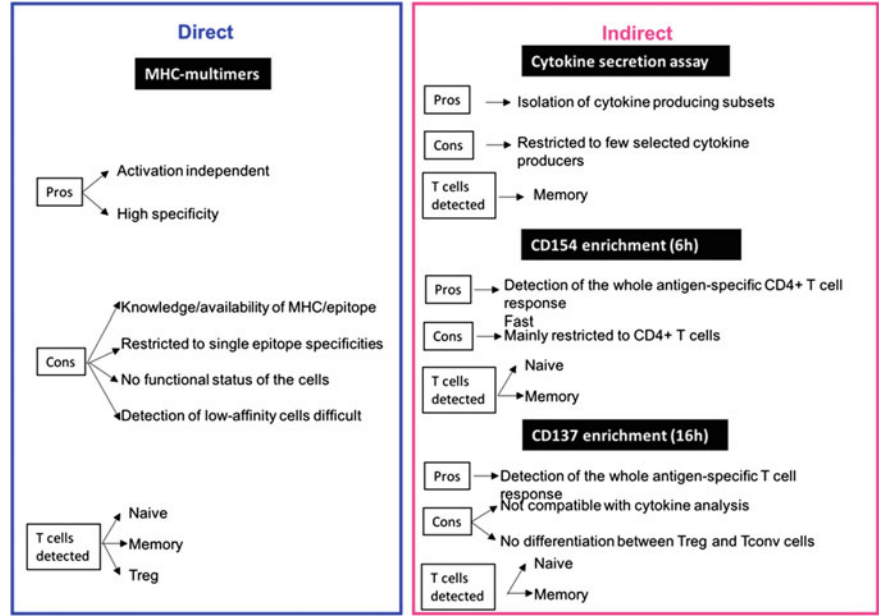
### **6.1 *Enrichment and Choice of Markers***

On the basis of the experimental endpoint(s) (e.g., phenotyping, functional assays), the rare population of interest may be enriched or not, and the number of markers that are needed to unambiguously identify a rare cell population needs to be clearly defined. For example, as discussed above, the accurate quantification of CECs and their progenitors (EPCs) is a matter of debate: which combination of markers is the most adequate, or what is needed for an eventual pre-analytical enrichment (by density gradient, buffy coat, and/or magnetic enrichment). Giving a look to the immune system, quantitative pre-enrichment of target cells via magnetic cell separation, which allows rapid processing of large samples ( $10^6$ – $10^9$  cells) [77, 78] is an excellent approach to increase the relative number of rare antigen-specific T-cell frequencies. The enrichment could be performed by known markers that characterize

the rare-cell population, or by using tetramers, or by performing a specific enrichment of cytokine-secreting cells (for example, those that produce IFN- $\gamma$  or IL-4) [79].

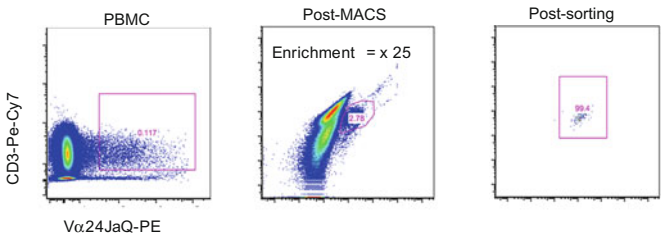
Rare antigen-specific CD4 T cells could also be pre-enriched by using anti-CD154 mAb. This live-cell assay could be applied to detect antigen-specific CD4+ T cells with diverse cytokine profiles. By including fluorescently conjugated CD154-specific antibody during stimulation, the assay is fully compatible with intracellular cytokine staining and can be used for cultures as long as 24 h [80]. Finally, another molecule can be used for enrichment of activated CD4+, CD8+, or CD1d+ T cells, namely CD137 (4-1BB), a member of the TNFR superfamily, which has been shown to be expressed following 16–24 h of stimulation [81]. The combined analysis of CD137 and CD154 following a short-term (6 h) stimulation might be optimal to detect in parallel conventional T cells and regulatory T cells (Tregs) reacting against the same antigen [82] (Fig. 3).

The enrichment can have negative effects if rare cells are lost, or, on the contrary, positive effects if unwanted cells are removed [67, 69, 83]. Another strategy to facilitate the detection of rare cells is to increase the number of antigen-specific T cells by in vitro expansion methods, even if the expansion of a single T cell is affected not only by its functional status (e.g., naïve, memory, anergic), but also by the presence of other reactive or accessory cells. Therefore, it is difficult to obtain the frequency of a given cell population in the original samples from the frequencies obtained after prolonged in vitro culture. Similarly, the phenotype and function of the expanded cells may be significantly altered by culture conditions [9, 12] (Fig. 4).

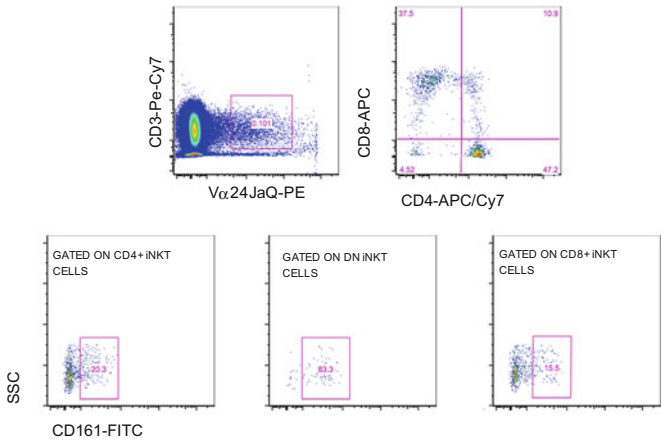


**Fig. 3** Enrichment methods for the detection of rare antigen-specific T cells

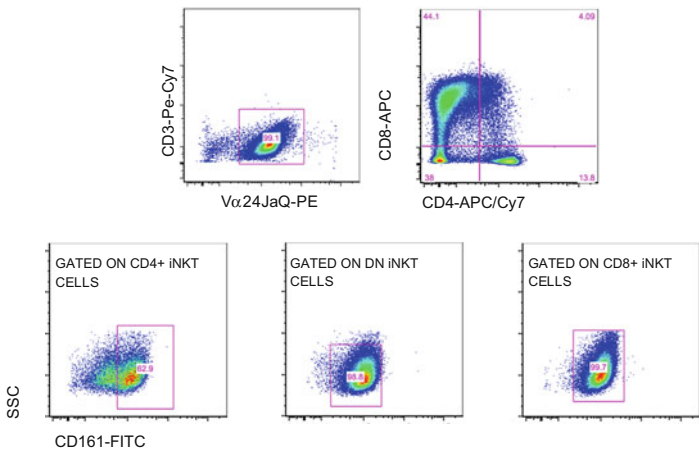
iNKT cell purification from PBMC



Pre-macs, pre-sorting phenotype



Phenotype after 24 d expansion



**Fig. 4** The phenotype of freshly isolated iNKT cells (upper panel) and that of in vitro expanded iNKT cells may significantly be altered by culture conditions. The middle panel reports the phenotype of these cells just before isolation, while the lower panel shows the phenotype after 24 days of in vitro culture. Note the expression of CD161, well evident in almost all cultured cells

Unfortunately, quite often the lack of well standardized methods for rare-cell detection influences the decision regarding the number of markers and type of antibody and fluorochrome that are necessary for the identification of the population of interest, as well as the rate and speed of acquisition. Depending on the technical characteristics of flow cytometers, which have a varying number of fluorescence channels, sensitivity, and speed, the first thing to decide is always where (in which channel, and by which fluorochrome) the most important marker allowing the identification and characterization of such populations should be detected. For example, in the case of iNKT cells, mAbs recognizing V $\alpha$ 24JQ invariant T-cell receptor allows the unique identification of these cells. Having done that, the panel has to be built following the general rule that the brightest fluorochrome must be used for the marker with the weakest expression. Last but not least, attention should be paid to compensation and acquisition of fluorescence-minus-one (FMO) controls [84–86].

## 6.2 *Number of Acquired Events*

Poisson statistics defines the probability that a given number of events will occur in a fixed interval of time/space, assuming that these events would occur with a known average rate and independent of the time elapsed from the previous event [87]. This sort of statistics is crucial for analyzing rare cells, since it can be applied to count randomly distributed cells in a certain volume. If we enumerate a total of  $N$  events, of which  $R$  meet a certain criterion (i.e., they are positive,  $P$ ), obviously, the proportion of  $P$  events is defined as  $R/N$ . The probability of any single event's being positive is between 0 and 1, and this is related to the random manner in which cells are selected for analysis. It is possible to define the variance,  $\text{Var}$ , as  $\text{Var}(R) = NP(1-P)$ . The standard deviation is the square root of  $\text{Var}$  [88], and the coefficient of variation (CV) is equal to  $1/\text{square root of Var}$  [89].

Let's consider a practical situation, the case of human PBMCs stained with a mAb recognizing iNKT cells (e.g., a population that express V $\alpha$ 24 $\beta$ Q TCR and CD3). If the frequency of these cells is 1 in 10,000 (0.01%),  $P = 0.0001$ . Good experimental practice suggests keeping the CV below 5%; thus, acquiring one million events gives a CV of 9.99, which is not acceptable, while 10 million events gives a CV of 3.16 (Table 1).

It must be taken into account that sometimes the number of acquired events cannot be high enough to respect this rule, for example because of the amount of blood that researchers can obtain from a patient. We can consider the case in which 1 million peripheral-blood T cells are stimulated with an antigen that activates less than 1 cell in 1000; T-cell activation can be analyzed by polychromatic flow cytometry, which allows the recognition of four functions per cell. Thus, among responding cells, up to 16 populations exist, likely with different frequencies. Clearly each subpopulation contains a few cells that typically must be absent in the

**Table 1** The number of acquired events for a cell population with final frequency 0.01%

Acquired events ( <b>N</b> )	100,000	1,000,000	4,010,000	10,000,000
Positive ( <b>R</b> )	10	100	401	1,000
Proportion ( <b>P</b> )	0.0001	0.0001	0.0001	0.0001
Variance ( <b>Var</b> )	10	100	401	1,000
Standard deviation (SD)	3.16	10.00	20.02	31.62
Coefficient of variation ( <b>CV</b> ) %	31.62	10.00	<b>4.99</b>	<b>3.16</b>

Note that to obtain a CV of 3.16 it is suggested to acquire 10 million events; acquiring 4,010,000 events gives a CV of 4.99

control sample (that with no stimulation). The number of positive cells is much lower than that suggested by a rigid statistical approach, but it is the general opinion that the events can be considered positive if alternative explanations for the presence of positive events in that channel can be excluded. There must be no noise due to dead cells or fragments, and cell activation must be really due to the antigenic stimulation and not to any in vivo pre-activation of T cells. In principle, there is no reason to fix a threshold for the number of events below which any frequency must be considered “negative” [90]. So, if adequate negative controls are set, “positivity” can be determined after comparison of the measurements (i.e., positive minus negative, namely positive minus the background), using standard statistical tools to compare the frequencies. For example, assuming that from the technical point of view the experiment is well performed, if T cells from “n” unvaccinated controls show no activation after stimulation with the adequate peptides, while T cells from “n” vaccinated individuals do, even extremely low frequencies can be taken as positive. The same logic can be applied in several other cases when negative controls are well chosen.

### 6.3 Sample Concentration and Flow Rate

Because it is crucial to acquire a high number of events for detection of rare-cell populations, the concentration in the sample and the flow rate are critical parameters, which can typically shorten acquisition time. However, particular attention has to be paid to the fact that increasing the flow rate results in an increase of coincidence, and thus in a higher CV, if flow cytometers use hydrodynamic focusing (the system used at present in most commercially available flow cytometers).

### 6.4 Thresholds, Gating, and DUMP Channel

Maximizing the signal-to-noise ratio is fundamental to distinguish the signal of the population of interest from the background. Fixing a threshold could help in this,

along with the use of a gating strategy that removes from the analysis dead cells (identified by a viability marker, such as amine reactive dyes, sold in different fluorochromes), that excludes doublets/aggregates/debris, and that uses a “DUMP” channel containing antibodies that identify cells of no interest. Furthermore, one has to monitor the parameter “time of acquisition” to remove the event bursts caused by clogs or other possible transient problems during the acquisition.

Of note, two other factors to consider to optimize the sensitivity of an assay are the cleanliness of the instrument and the integrity of the sample. It is important to make sure that the instrument and fluids used are clean and free of particles that could contribute falsely to the rare population.

## 6.5 *Data Analysis*

Finally, the analysis of data benefits from powerful hardware (in terms of gigabytes of random access memory, RAM), because depending on how many events and parameters have been acquired, data files tend to be quite huge (if not enormous). To minimize the file size, parameters that are not needed can be turned off, and thresholds can be used.

The high-throughput nature of flow cytometry, combined with the increasing capacity to measure more parameters at once, is generating massive and high-dimensional datasets on a routine daily basis. These data can no longer be adequately analyzed using the classical, mostly manual, analysis techniques and therefore require the development of novel computational techniques, as well as their adoption by the broad community. Computational flow cytometry provides a set of tools to analyze, visualize, and interpret large amounts of cell data in a more automated and unbiased way [91, 92].

In order to inspect data before the analysis, there are several software programs (based on principal component analysis, PCA) that use visualization techniques as alternatives to the traditional two-dimensional dot plots. The first paper on the use of PCA for analyzing flow cytometry data was published in 2007; this approach was applied to eight-color cytofluorimetric analysis on the virgin and memory T-cell compartments in donors of different age (young, middle-aged, and centenarians) [93]. For the first time, it was shown how to use a novel bioinformatic approach to analyze large datasets generated by polychromatic flow cytometry and to obtain the rapid identification of key populations that best characterize a group of subjects.

To date, several tools like SPADE, FlowMap, FlowSOM, viSNE, PhenoGraph, Scaffold map, and DREMI-DREVI are available. These approaches are mainly dimensionality reduction- or clustering-based techniques (reviewed in [92]). Concerning the identification of very rare cell types, there could be some issues related to the fact that they could be mistaken for noise by many clustering algorithms. To identify all relevant populations, it might be necessary to do an exhaustive gating, resulting in strong over-clustering, and then select only those

features related to a phenotype. With the traditional clustering algorithms, it is recommended to ensure that only relevant markers are used for clustering. Markers that vary little or that indicate properties not relevant for cell-type identification (for example, activation markers) are best left out, as these will only contribute noise to the similarity calculation. For example, in a recent paper different software programs were compared, i.e., SPADE, t-SNE, and FlowSOM, for the analysis of splenocytes of a wild-type C57BL/6 J mouse. Only very rare cell types, such as natural killer T (NKT) cells (which constitute only 0.3% of the dataset), were not distinguished, whereas relatively rare cell types such as neutrophils (which constitute 0.7% of the dataset) were distinguished by all three methods [92]. Interestingly, such a problem in the identification of rare events is not encountered with SWIFT (a tool present in the software Matlab), which is an automated gating technique specifically developed to identify rare populations [94].

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Single Cell Analysis

Contemporary Research and Clinical Applications

Robinson, J.P.; Cossarizza, A. (Eds.)

2017, XIII, 266 p. 84 illus., 70 illus. in color., Hardcover

ISBN: 978-981-10-4498-4