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Technical note

Immuno-monitoring of CD8+ T cells in whole blood versus PBMC samples

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Abstract

The study of natural T cell responses against pathogens or tumors, as well as the assessment of new immunotherapy strategies aimed at boosting these responses, requires increasingly precise ex vivo analysis of blood samples. For practical reasons, studies are often performed using purified PBMC samples, usually cryopreserved. Here, we report on FACS analyses of peripheral blood T cells, performed by direct antibody staining of non-purified total blood. For comparison, fresh PBMC, purified by Ficoll, were analysed. Our results show that the latter method can induce a bias in subpopulation distribution, in particular of CD8+ T cells, and sometimes lead to inaccurate measurement of antigen specific CD8+ T cell responses. Direct analysis of total blood can be applied to longitudinal immuno-monitoring of T cell-based therapy. While the need to purify and cryopreserve PBMC for subsequent studies is obvious, the use of whole blood has the advantage of providing unbiased results and only small amounts of blood are used.

Keywords: Lymphocytes; Naïve; Tetramers; Flow cytometry

1. Introduction

Increasingly complex manipulations of the immune system to boost T cell responses are now being tested for the development of effective immunotherapies against infectious diseases or cancer in humans (e.g. vaccination or adoptive cell transfer strategies). This requires more and more precise analysis for careful immuno-monitoring of patients in order to determine the effects of these new strategies, in an effort to optimise and accelerate the development of successful strategies. The detection of T cell activity is often no longer sufficient, as precise characterisation of T cells is needed. In addition, the analysis of samples that have undergone manipulation such as culture of cells yields mostly subjective information. With improved tools available for analysis of cellular immune responses, it is now possible to perform detailed ex vivo analysis of natural immune responses as well as responses to immunotherapy. Ex vivo phenotypic analysis can provide information on the activation status of T cells, their differentiation status, and functional analysis on their

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effector mechanisms. For this purpose, direct analysis of fresh whole blood samples appears to be the method of choice as cells remain largely unmanipulated (Appay and Rowland-Jones, 2002).

Nonetheless, for practical reasons, studies are often performed using cryopreserved purified PBMC samples. Several T cell analyses comparing whole blood and purified PBMC procedures have shown that these two procedures can yield noticeably different results. For instance, variations in CD4/CD8 ratios have been reported, and it has been suggested that the whole blood procedure may be more accurate (De Paoli et al., 1984; Renzi and Ginns, 1987; Ashmore et al., 1989; Romeu et al., 1992). These studies were usually performed on whole lymphocyte populations (e.g. CD4, CD8, NK), but did not include subpopulations (e.g. naïve and antigen experienced cells). A more recent study demonstrated that assessment of chemokine receptor expression could also vary from one procedure to another (Berhanu et al., 2003).

We report here that the simple step of Ficoll purification of PBMC can induce a bias in subpopulation distribution, in particular of CD8+ T cells, and lead to inaccurate measurement of antigen specific CD8+ T cell responses. The main obstacle for consistent use of whole blood is in performing longitudinal analysis, due to the need for comparative data that may be best obtained by including samples from different dates in the same assay. However, our experience in the context of longitudinal immuno-monitoring of T cell based therapy suggests that immediate monitoring on fresh whole blood is entirely feasible.

2. Methods

2.1. Whole blood samples

Samples from HLA-A2+ healthy or vaccinated melanoma patients were obtained from volunteers attending the clinic. The relevant local Institutional Review Boards and Ethics Committees approved the study. Heparinised blood samples were used fresh within 4 h, and peripheral blood mononuclear cells (PBMCs) were separated from blood using Ficoll–Hypaque™ or NycoPrep™ according to the manufacturer's recommendations.

2.2. Reagents

HLA-peptide tetrameric complexes ("tetramers") were produced as previously described (Altman et al., 1996), and included the following MHC-I peptides: A2 Melan-A-ELAGIGILTV, A2 CMV pp65-NLVPMVATV, A2 EBV BMLF1-GLCTLVAML, A2 flu matrix-GILGFVFTL, B7 CMV pp65-TPRVTGGGAM and B8 EBV BZLF1-RAKFKQLL. Anti-CD8-PerCP (Peridinin chlorophyll protein) anti-CD38-APC (allophycocyanin), anti-Bcl-2-FITC (fluorescein isothiocyanate), anti-CD45RA-FITC, anti-CCR7-Alexa 648, anti-Granzyme A-FITC and anti-Granzyme B-Alexa 648 antibodies were purchased from Becton Dickinson Pharmingen (San Diego, CA).

2.3. Flow cytometry analysis

The use of whole blood to perform lymphocyte labelling with fluorochrome-conjugated antibodies is a straightforward procedure that requires no additional labour compared to the analysis of purified PBMC (red blood cell lysis versus PBMC density gradient separation or thawing). Titrated tetramers (PE conjugated) were added to 200–400 μl of heparinised whole blood (or 5×10^5 to 10^6 PBMC) and incubated for 15 min at 37 °C. Conjugated monoclonal antibodies specific for extracellular markers were added and incubated for 15 min at room temperature. To lyse red blood cells, 3 ml of FACS™ lysing solution (Becton Dickinson, San Jose, CA) (diluted 1/10 in water) were added to the blood, followed by 10–15 min at room temperature, and centrifugation (5 min at 1500 rpm) to discard the supernatant. Cells were then washed (with PBS, 0.5% BSA, 0.5 mM EDTA), and stored in Cell Fix™ buffer (Becton Dickinson, San Jose, CA) at 4 °C until analysis. For intracellular staining (i.e. Bcl-2 and Granzyme), after RBC lysis and washing, 300 μl of FACS™ permeabilising solution (Becton Dickinson, San Jose, CA) (diluted 1/10 in water) were added to the cells; tubes were vortexed and left for 15 min at room temperature in the dark. Following a washing step, conjugated monoclonal antibodies specific for intracellular markers were added and incubated in the dark for 15 min, before additional washing and resuspension in Cell Fix™ buffer. For PBMC staining, 5×10^5 to 10^6 cells were stained as described above without the red blood cell lysis step (unless specified). When low frequency tetramer positive cell populations were analysed from PBMC, a CD8+ T cell enrichment step was performed using MACS beads (Miltenyi, Koln, Germany) before staining. Samples were analysed on a BD CANTO. The fluorescence intensity of calibrating beads was verified in preset gates every 3 weeks to ensure stable FACS instrument performance over time. Before every data acquisition, compensation of the instrument was checked using freshly stained lymphocytes and parameters adjusted to fit fixed gates.

3. Results and discussion

3.1. Bias towards over-representation of the naïve CD8+ T cell subset in purified PBMC versus whole blood

In the periphery, CD8+ T cells exist in multiple subsets endowed with distinct characteristics and functions in the immune responses. Naïve cells are unprimed and have no effector function but constitute a diverse precursor pool, waiting to encounter foreign antigens, to become memory/effector T cells. In contrast, antigen primed CD8+ T cells constitute a heterogeneous population with various differentiated subsets that can be distinguished according to the expression of various cell surface markers (Sallusto et al., 1999; van Lier et al., 2003; Appay and Rowland-Jones, 2004). For instance, naïve cells can be identified as expressing both CD45RA and CCR7.

Assessing the nature of T cell responses implies examining such characteristics precisely. We have performed a comparative analysis of T cells detected either directly in whole blood or in fresh Ficoll-purified PBMC populations by flow cytometry. We found noticeable phenotypic differences in CD8+ T cells between the two methods. The analysis of naïve versus antigen experienced CD8+ T cell subsets revealed a significant increase in the percentage of CD45RA+/CCR7+ cells (i.e. naïve) in purified PBMC compared to whole blood (Fig. 1A). Such bias of the CD8+ T cell subset distribution towards more naïve cells in PBMC was consistent (observed in more than 90% of samples analysed) although relatively modest (1.25 times the mean increase in naïve cell percentage within the CD8+ T cell population) (Fig. 1B). This may be due to a selective trapping of antigen experienced CD8+ T cells with the rest of the leucocytes or erythrocytes during separation by density gradient centrifugation. Slight differences in density between naïve and antigen experienced CD8+ T cell populations (which are more granular; Fig. 1C) might also account for this bias.

A similar bias in purified PBMC was less obvious for CD4+ T cells; the percentage of naïve cells remained relatively stable from whole blood to PBMC (Fig. 1D). This is in line with a selective effect on CD8+ T cells (i.e. loss in PBMC) as previously reported (De Paoli et al., 1984; Renzi and Ginns, 1987). In addition to Ficoll (Ficoll–Hypaque™), we also used Nycodenz (NycoPrep™) to purify PBMC; no major differences between the two gradient solutions were observed as the percentage of naïve CD8+ T cells was higher in both cases compared to whole blood (Fig. 1E). To rule out a possible effect (i.e. loss of naïve CD8+ T cells) of the red blood cell (RBC) lysis buffer treatment used in the whole blood procedure, we compared the percentage of naïve CD8+ T cells in purified PBMC treated with this buffer or left untreated (i.e. normal purified PBMC procedure), as well as within the treated RBC fraction obtained from the density gradient PBMC separation (i.e. lower red fraction in which some lymphocytes remain, not separated) (Fig. 1F). Treatment with the RBC lysis buffer had no major impact; the percentage of naïve CD8+ T cells in both treated or untreated PBMC was higher than in whole blood. In contrast, the lymphocytes that remained in the RBC fraction presented a significantly lower percentage of naïve CD8+ T cells, thus indicating an enrichment of antigen experienced CD8+ T cells in this fraction, and accounting for their under-representation in purified PBMC. Interestingly, these differences may help to explain, at least to some extent, observations of better chemokine receptor expression using different procedures (Berhanu et al., 2003). For instance, if chemokine receptors are more expressed on naïve cells, their overall expression may appear better in purified PBMC, and inversely, for receptors more expressed by antigen experienced CD8+ T cells, the overall assessment may appear better in whole blood.

3.2. Potential impact on the analysis of antigen specific CD8+ T cells

The CD8+ T cells, which are important for the control of viral diseases and tumour growth, are fully armed antigen specific cells. The development of tetramer technology has provided a means to directly identify and characterise these cells and represents a revolution in the field of T cell immunology. Their precise analysis in the context of immunotherapy trials aimed at boosting T cell activity is obviously fundamental, but changes in CD8+ T cell subset distribution according to the analysis procedure may influence the eventual result. We performed comparative staining of antigen specific CD8+ T cells, identified using tetramers, from whole blood and purified PBMC.

In general, the results were satisfactory with relatively similar percentages of tetramer positive cells detected under both conditions (Fig. 2A). This may be expected as a 1.25 times enrichment in naïve cells would only have a limited influence on tetramer positive cell frequency considering the small percentage that tetramer positive populations can represent within the whole CD8+ T cell pool.

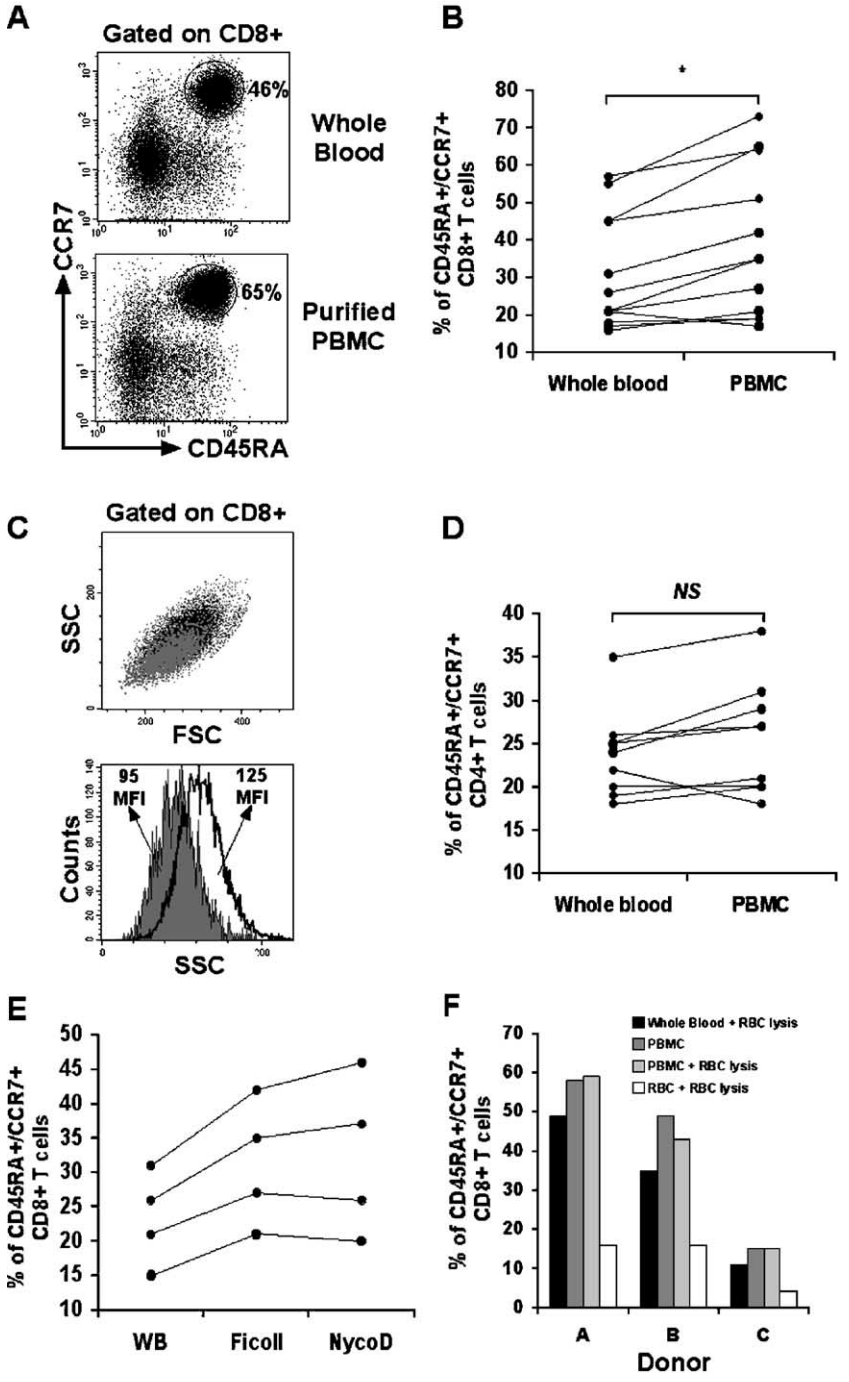

Fig. 1. Proportions of CD8+ T cells with a naïve phenotype (CD45RA+/CCR7+) in whole blood or fresh purified PBMC. (A) Representative example of CD45RA/CCR7 staining on CD8+ gated T cells, from whole blood or purified PBMC from the same sample. Percentages of naïve T cells within the CD8+ compartment are indicated. (B) Proportion of naïve T cells (CD45RA+/CCR7+) within the CD8+ compartment ($n=12$ samples) comparing whole blood and PBMC. *$P=0.0024$ with a paired t-test. (C) Granularity of naïve CD8+ T cells (in grey) measured on the SSC channel, compared with antigen experienced cells (black or bold line). (D) Proportions of naïve T cells (CD45RA+/CCR7+) within the CD4+ compartment ($n=9$ samples) comparing whole blood and PBMC. NS, non-significant. (E) Comparison between whole blood and PBMC purified, using Ficoll–Hypaque™ or NycoPrep™, as regards the percentage of naïve CD8+ T cells in four donors. (F) Percentage of CD45RA+/CCR7+ T cells within the CD8+ population, measured in whole blood, purified PBMC, or the red blood cell fraction, all after treatment with red blood cell lysis buffer, or in non-treated purified PBMC, from three different donor samples.

Nonetheless, there was an overall trend towards a smaller percentage of tetramer detected in purified PBMC, and significant discrepancies were sometimes observed between the two procedures. The analysis of circulating lymphocytes from an HLA-A2 healthy donor, in which virus (e.g. EBV) specific (antigen experienced) cells as well as Melan-A specific cells with naïve phenotype can be identified (Zippelius et al., 2002), provides an interesting example, as shown in Fig. 2B. Striking differences were observed, with an increased percentage of a Melan-A tetramer positive naïve population and a decreased percentage of an EBV tetramer positive antigen experienced population in purified PBMC. Analysis of Melan-A tetramer positive cells from a Melan-A peptide vaccinated individual, which therefore included mostly antigen experienced cells (Speiser et al., 2005), shows a decreased percentage of tetramer positive cells with a bias towards CD45RA+/CCR7+ cells in PBMC (Fig. 2C). Although such discrepancies may be relatively infrequent and it is difficult to provide a definitive PBMC/whole blood ratio as regards tetramer positive cell frequency (in particular considering the potential intra-assay variations inherent in the analysis of tetramer positive cells in low frequencies), it is important to be aware that tetramer analysis on purified PBMC can sometimes be incorrect and therefore potentially misleading. Overall, CD8+ T cell analysis directly from whole blood yields more accurate results, both in terms of frequency and phenotype.

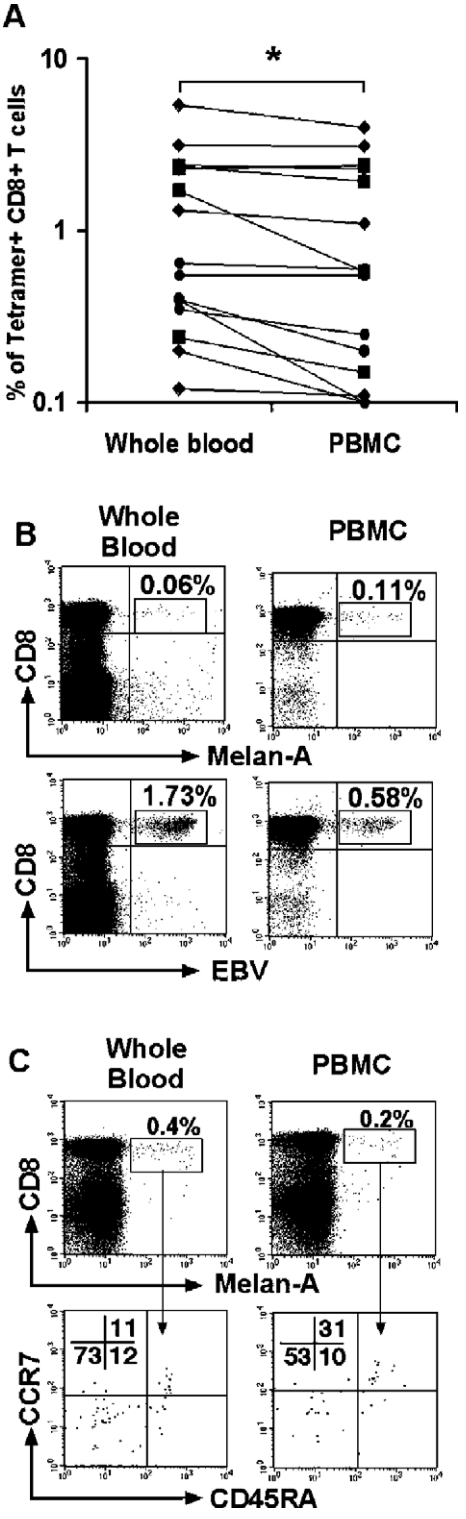

Fig. 2. Percentage of tetramer positive CD8+ T cells in whole blood or fresh purified PBMC. (A) Proportion of tetramer positive T cells (including memory Melan-A, EBV or CMV specific T cells) within the CD8+ compartment comparing whole blood and PBMC ($n=16$ tetramer positive populations analysed). *$P=0.035$ with a paired t-test. (B) Tetramer staining (Melan-A "naïve" and EBV "memory") in whole blood and PBMC from one HLA-A2 healthy donor. Percentages of tetramer positive T cells within the CD8+ compartment are indicated. (C) Tetramer staining (Melan-A memory) and CD45RA/CCR7 staining on tetramer+ gated T cells in whole blood and PBMC from one HLA-A2 melanoma patient vaccinated with Melan-A peptide.

3.3. Longitudinal monitoring of CD8+ T cells using whole blood

To perform longitudinal studies of natural or therapy induced T cell responses, it is common to analyse series of cryopreserved PBMC samples. This has two major practical advantages compared to whole blood analysis: purified PBMC can be stored frozen until analysis to avoid on the spot analysis (i.e. at the time point of blood withdrawal), and several samples (or time points) can be analysed simultaneously, which favour the analysis (e.g. subpopulation gating and comparison between time points) as staining conditions and flow cytometry

parameters are identical for all samples analysed at the same time. Nonetheless, based on the quality of data obtained from whole blood, we have performed longitudinal immuno-monitoring directly from whole blood. This may have the disadvantage that staining settings (e.g. due to decline of reagent quality with time or use of different batches), as well as parameter settings (even though modern flow cytometers are rather stable) may vary slightly over the time it takes to perform longitudinal immuno-monitoring.

Fine sample analysis is required each time in order to overcome these problems. This implies that gating and/or setting of limits may vary slightly from one sample to another (i.e. previous gating or limit setting may not apply exactly). Gating and/or setting of limits, for instance for marker expression on tetramer positive cells, may also be challenging, as no comparison is provided with other samples (Fig. 3A). Internal controls such as the whole CD8+ T cell population and CD8− T cells are used as reference and for standardisation. For

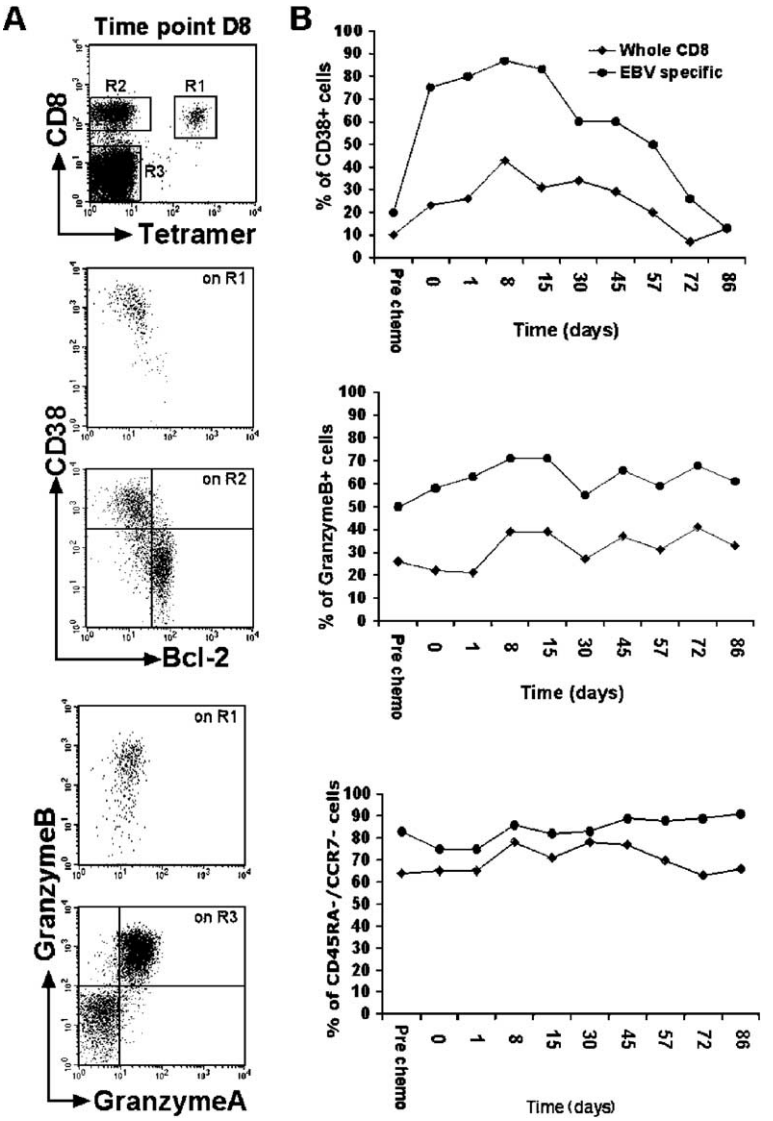

Fig. 3. Monitoring of tetramer positive CD8+ T cell frequencies and characteristics in whole blood. (A) Example of gating for extracellular and intracellular markers to identify and characterise T cell subpopulations. (B) Example of longitudinal follow up of the activation marker CD38, cytotoxic factor granzyme B, and differentiation marker CD45RA/CCR7 on EBV specific CD8+ T cells in one patient who was subjected to a lympho-depleting chemotherapy (day 0 is just after treatment with busulfan (2 mg/kg p.o.) for 2 days followed by fludarabine (30 mg/m^2 i.v.) for 3 days, agents that are usually used in the context of non-myeloablative immuno-suppressive conditioning regimens, to favour the graft versus malignancy effect prior to allogeneic transplantation (Slavin et al., 1998)).

even further precision, a standard (e.g. a cryopreserved sample reference) may be used each time to check the integrity of gating.

Using such fine analysis, it becomes possible to perform trustworthy longitudinal analysis on whole blood samples, to follow either stability or variation of cellular features. For instance, we have monitored the activation status (CD38 expression) and expression of Granyzme B by EBV specific CD8+ T cells compared to the whole CD8+ T cell population in the whole blood of a cancer patient who was subjected to a lympho-depleting chemotherapy, resulting in transient immuno-suppression. Following chemotherapy, we observed a strong and transient activation of EBV specific CD8+ T cells in contrast to the whole CD8+ T cell population, which may be the consequence of EBV reactivation associated with immuno-suppression (Fig. 3B). In contrast, Granzyme B levels in these cells remained more stable and higher than those of the whole CD8+ T cell population, in line with the expression of CD45RA and CCR7 (defining memory subsets) by these cells over time (Fig. 3B).

The need to store cryopreserved PBMC and the importance of performing subsequent analysis of these samples are obvious and unquestionable. Nonetheless, it is important to keep in mind the potential bias associated with such a procedure, in addition to the risk that cryopreservation of PBMC may also result in depletion of some fragile cells, as recently exemplified (Dong et al., 2004). While longitudinal analysis on whole blood is certainly a more demanding procedure than cryopreserved PBMC analysis, in terms of commitment to process the samples (i.e. staining and data acquisition) and to perform careful detailed analysis of the data, it is feasible and can yield more accurate data. Nonetheless, before adopting such a procedure, it is also important to take into consideration the potential downside associated with the use of whole blood, that may prevent correct staining of markers of interest. For instance, soluble cell surface proteins in whole blood may interact with marking antibodies, soluble ligands for cell surface proteins may prevent antibody binding to the same protein, and plasma proteins may interact with the peptides used for identification of antigen specific cells (after short stimulation and intracellular cytokine staining). The levels of these proteins may also vary between individuals, which can cause unpredictable variations. Overall, the use of whole blood for immuno-monitoring may be considered the method of choice, whenever possible and after verification that no pitfall precludes accurate assessment of markers of interest.

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