

Research paper

Cell permeabilization for the assessment of T lymphocyte polyfunctional capacity

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Abstract

The rapid progress in flow cytometry means that an increasing number of parameters can be looked at simultaneously, which is highly relevant for the assessment of lymphocyte characteristics, in particular their multifunctional profile. However, procedures using less complex technology need now optimization in order to take into account the new reagents and applications related to polychromatic flow cytometry. Through optimization of an immunomonitoring protocol to assess the functional profile of antigen specific T-cells using 9–10 colour flow cytometry, we tested the efficacy of three distinct standardized permeabilization buffers for the staining of relevant intracellular molecules. We show significant discrepancies in staining sensitivity for cytokine and cytotoxic factor expression from one permeabilization kit to another, which can lead to different data and interpretation. It is important to be aware of this potential bias and to design specific application/experimental procedures in order to obtain optimal results.

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1. Introduction

Antigen specific CD8⁺ T-cells are key players in the immune response against viruses. They achieve control of viral replication through a variety of effector mechanisms, which include the secretion of soluble anti-viral factors such as cytokines and chemokines and the direct killing of infected cells, involving degranulation of cytotoxic molecules. The analysis of their characteristics

has become central in the immunomonitoring of patients enrolled in clinical trials aimed at boosting T-cell mediated immunity. New technological development in flow cytometry, in particular related to the number of fluorescent parameters that can be assessed at once, renders possible a systematic assessment of multiple T-cell functions from the donors' peripheral blood (Perfetto et al., 2004). Recent data suggest that their ability to fight an infection may be related to their capacity to present several of these functions simultaneously (Darrach et al., 2007). This technology and the increasing need to assess multiple parameters has led to the development of a new range of reagents (e.g. monoclonal antibody/fluorochrome pairs, buffers). However, revision or optimization of protocols that were commonly used with previous applications is required.

Abbreviations: PBMC, peripheral blood mononuclear cell; SEB, staphylococcal enterotoxin B; IFN γ , interferon gamma; TNF α , tumour necrosis factor alpha; IL-2, interleukin 2; MIP-1 β , macrophage inflammatory protein 1 beta; MHC, major histocompatibility complex.

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In the context of setting reproducible and reliable immunomonitoring procedures, we have established a protocol to assess the expression of up to six function related analytes simultaneously on T-cells, including antigen specific CD8⁺ T-cells identified using tetramers. The choice of antibody/fluorochrome pairs, lasers and filters, as well as fine tuning of the flow cytometers are central to the process of polychromatic FACS staining. These issues have been highlighted and discussed in detail by Perfetto et al. (2004) and Chattopadhyay et al. (2006). We do not consider these issues further here; the combinations of antibody/fluorochrome pairs and filters selected in our procedures are described in Table 1. The assessment of single T-cell functionality is usually based on the analysis of molecules with active biological properties, that are located intracellularly (e.g. cytokines or cytotoxic factors). It is therefore necessary to permeabilize cell membranes to allow fluorochrome conjugated antibodies to access the different analytes, which represents a critical parameter in the methodology. By setting up a procedure to assess polyfunctionality, we have compared three different permeabilization buffers, obtained from three suppliers (BD, Caltag and eBioscience). We decided to use standardized commercial buffers or kits, instead of self made buffers, because of the necessity for assay standardization/reproducibility in immunomonitoring. Our results show discrepancies between the buffers in terms of staining sensitivity for a range of molecules of interest, and thus caution is advised when using such buffers or kits.

2. Methods

2.1. Samples

Heparinized blood samples were obtained from healthy volunteers and peripheral blood mononuclear cells (PBMC) were separated from the blood using a Ficoll gradient according to the manufacturer's recom-

mendations to be used directly or cryopreserved. Thawed PBMC were rested overnight at 37 °C in complete RPMI medium (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, L-glutamine and antibiotics) before use. For tetramer positive cell analysis, sample viability after thawing was >90% when examined by trypan blue exclusion. For cytokine staining after SEB stimulation or cytotoxic factor staining, both frozen and freshly isolated PBMC were used.

2.2. Reagents

Standardized permeabilization buffers were purchased from three different suppliers: the Cytotfix/Cytoperm buffer set from Becton Dickinson (San Jose, CA), the Fix&Perm buffer set from Caltag (Carlsbad, CA) and the Foxp3 Staining buffer set from eBioscience (San Diego, CA). Directly conjugated monoclonal antibodies were obtained from the following vendors: BD Biosciences: CD3 (Pacific Blue), CD8 (AmCyan), CD4 (allophycocyanin-cyanine 7, APC-Cy7), CCR7 (phycoerythrin-cyanine 7, PE-Cy7), CD40L (PE), granzyme A (fluorescein isothiocyanate, FITC), perforin (FITC or PE), granzyme B (Alexa 647), CD107a (PE-Cy5), MIP-1 β (PE), IL2 (APC), IFN γ (Alexa 700), and TNF α (PE-Cy7); Beckman Coulter: CD4 (PE-Texas Red), CD45RA (PE-Texas Red or PE); Caltag: granzyme B (PE-Texas Red); R&D Systems (Abingdon, UK): MIP-1 β (FITC). HLA-A2 CMV pp65-NLVPMTATV and HLA-B8 EBV BZLF1-RAKFKQLL tetrameric complexes ("tetramers") were produced as previously described (Altman et al., 1996) and conjugated with PE.

2.3. Flow cytometry analysis

For stimulation, at least 10⁶ cryopreserved or freshly isolated PBMC were incubated in the presence of specific peptide (5 μ M) (but pre-stained with titrated tetramer), or

Table 1
Combinations of antibody/fluorochrome used

Lasers	Violet laser 405 nm		Blue laser 488 nm			Red laser 633–635 nm				
<i>Machine settings</i>										
Actual filters	450/50	525/50	530/30	575/26	610/20	660/20	780/60	660/20	730/45	780/60
Long pass		505	505	550	600	655	755		710	755
Fluorochrome	Pacific Blue	AmCyan	FITC	PE	PE-Texas Red	PE-Cy5	PE-Cy7	APC	Alexa 700	APC-Cy7
<i>Combination</i>										
1. Tetramer/function	CD3	CD8	MIP1 β	Tetramer	Granzyme B	CD107a	TNF α	IL-2	IFN γ	
2. Immunomonitoring/ function	CD3	CD8	MIP1 β	CD40L	CD4	CD107a	TNF α	IL-2	IFN γ	
3. Cytotoxic/ differentiation	CD3	CD8	Granzyme A	Perforin	CD45RA		CCR7	Granzyme B		CD4

SEB (2 µg/ml), and anti-CD107a antibodies (10 µl) for 1 h at 37 °C in a 5% CO₂ incubator, followed by an additional 5 h in the presence of the secretion inhibitors monensin (2.5 µg/ml; Sigma-Aldrich) and Brefeldin A (5 µg/ml; Sigma-Aldrich). Negative controls were obtained in the absence of peptide and pre-staining with tetramer. BD Cytotfix/Cytoperm™, Caltag or eBioscience

buffers were used according to the manufacturers' recommendations to permeabilize the cells prior to staining for intracellular markers with titrated monoclonal antibodies (anti-MIP-1β, anti-granzyme B and perforin antibodies were also titrated with the three different buffers). In order to minimize any potentially detrimental effects of the fixating/permeabilizing reagents on cell

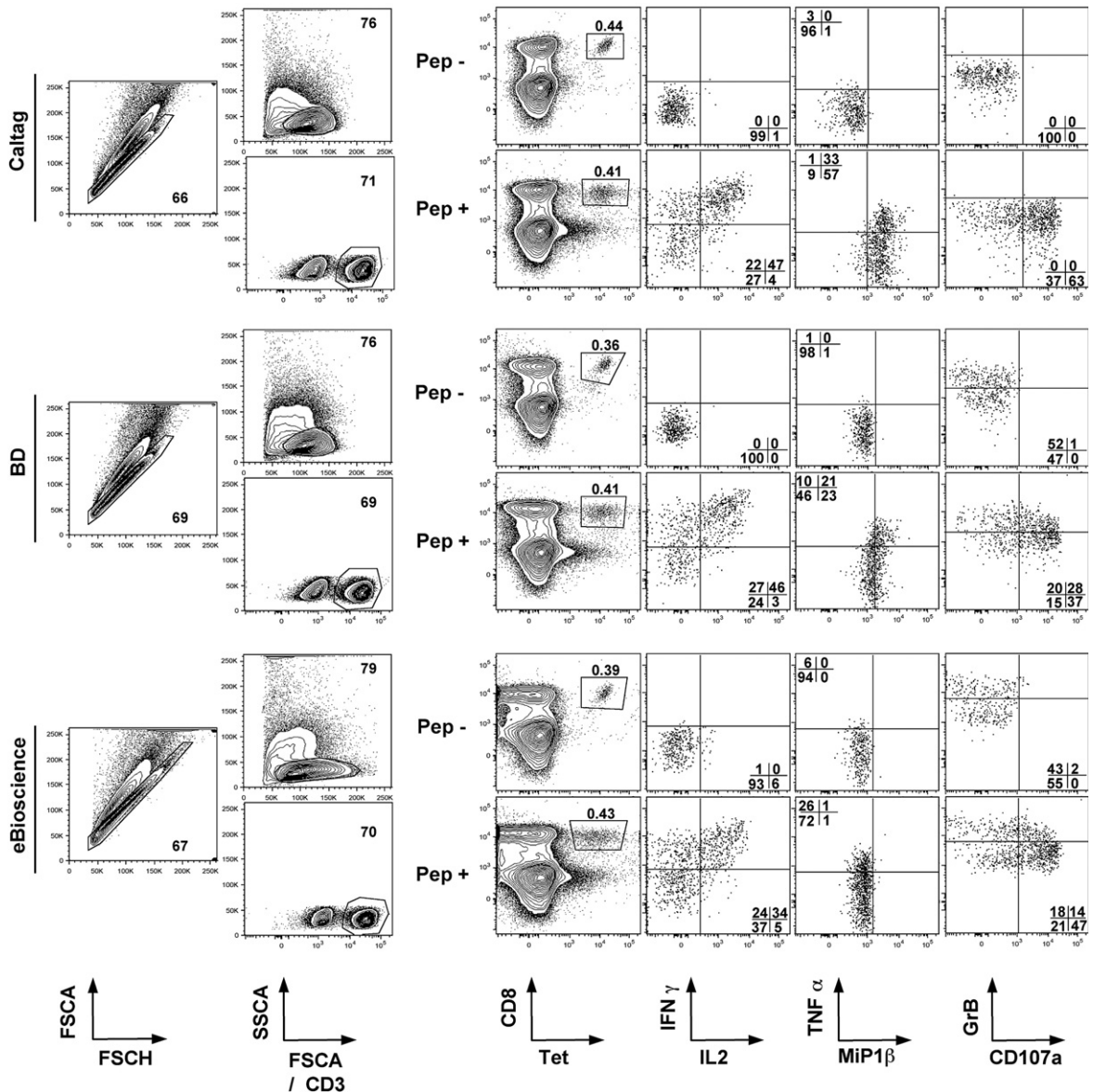


Fig. 1. Polyfunctional assessment of antigen specific CD8+ T-cells. EBV specific CD8+ T-cells, identified using tetramers (HLA-B8 RAK) were left unstimulated (pep-) or stimulated with the cognate peptide (pep+) for 6 h before permeabilization using three different buffers and staining for functional molecules simultaneously (cytokine production: IFNγ, TNFα, MIP-1β, IL-2 and cytotoxic potential: CD107a and granzyme B). Functional markers are shown on tetramer gated cells. There was no background in the whole CD8+ T-cell population under all conditions. Similar results with CMV specific CD8+ T-cells (HLA-A2 NVP). On the left panels, percentages of gated cells are shown. On the right panels, percentages of tetramer positive T-cells within the CD8+ compartment as well as percentages in the different quadrants are indicated.

surface receptors and/or their respective staining, staining for extracellular markers (i.e. CCR7, CD45RA as well as CD3, CD8 or CD4) was performed before the fixation/permeabilization steps. Three staining combinations were used: (1) tetramer/function, (2) immunomonitoring/function and (3) cytotoxic/differentiation (Table 1). Cells were analyzed shortly after the last wash to minimize degradation of the fluorochromes. A BD LSRII (see Table 1 for settings) was used to acquire the samples and the data were analyzed using FlowJo v8.2 (Tree Star, Inc) and DIVA software. FMO controls were performed once for all cytokines on CD8+ T-cells following stimulation with SEB to check for positive staining.

3. Results and discussion

We aimed to establish a procedure to assess the capacity to produce four soluble factors with distinct properties (three cytokines: IFN γ , TNF α , IL-2; and one chemokine: MIP-1 β), together with cytotoxic potential (granzyme B measurements indicate the capacity for cytotoxicity, and CD107a upregulation indicates degranulation suggesting recent cytotoxic activity) by antigen specific CD8+ T-cells identified using MHC/peptide complexes. Cell membrane permeabilization for these types of assays is usually achieved using a saponin-based solution, however, the use or addition of other permeabilizing agents (e.g. Triton, digitonin or methanol) can influence the eventual result, as shown in other applications (Lanza et al., 1997; Verdier et al., 2000; Krutzik and Nolan, 2003; Chow et al., 2005). In order to set up a standardized procedure applicable for immunomonitoring, we tested three different permeabilization buffers, held to be optimized permeabilizing reagents for intracellular cytokine FACS staining by their respective suppliers (the exact formulation is kept undisclosed).

Background staining (with no peptide stimulation) on tetramer positive specific CD8+ T-cells was equivalent and satisfactory with all three buffers (Fig. 1). However, upon stimulation with cognate peptides, we found staining discrepancies between buffers. The use of the Caltag buffer yielded strong staining for IFN γ , TNF α , IL-2, MIP-1 β and CD107a, but a weak shift in granzyme B staining after stimulation. In contrast, the eBioscience buffer resulted in a clearer shift of granzyme B staining after stimulation, concomitant with CD107a upregulation; cytokine staining was satisfactory with this buffer, apart from MIP-1 β , for which the signal intensity was low and discrimination between positive and negative cells was poor. The BD buffer yielded an intermediate picture: with good staining for granzyme B, as well as IFN γ , TNF α and IL-2, and

detectable although weak staining for MIP-1 β . The discrepancies did not seem to be due to the loss of certain subpopulations of antigen specific CD8+ T-cells, since the percent frequency of tetramer+ cells was equivalent for each set of conditions.

In order to confirm these observations beyond the specific study of tetramer positive CD8+ T-cells, we performed a comparative analysis on whole CD8+ or CD4+ T-cell populations upon stimulation with SEB to focus on cytokine staining. SEB is a specially adapted stimulus for setting up protocols; it stimulates, in an antigen-like manner (in contrast to mitogens like PHA), a large number of both CD4+ and CD8+ T-cells (i.e. all cells expressing TCR-BV 3, 12, 14, 15, 17 or 20). For CD4+ T-cells, the marker of degranulation, CD107a, was replaced by CD40L (CD154), a receptor involved in the interaction with and activation of B-cells, whose expression is assessed intracellularly upon activation (Chattopadhyay et al., 2005; Frentsch et al., 2005). While staining for IFN γ , TNF α , CD107a and CD40L expression was equivalent with all buffers, significant discrepancies between permeabilizing solutions were observed for IL-2 and in particular MIP-1 β staining (Fig. 2a,b). In line with our observations on tetramer positive CD8+ T-cells, the strongest staining sensitivity was obtained with the Caltag buffer, and the weakest with the eBioscience buffer. We also tested pools of HIV overlapping peptides to stimulate PBMC from HIV-infected patients. Although these stimuli are not particularly adapted to the comparative study of permeabilizing buffers since CD4+ T-cell responses are very low and CD8+ T-cells produce very little IL-2 under these conditions, similar observations were made as regards IFN γ , TNF α , CD107a and MIP-1 β (data not shown).

Next we examined whether the inconsistency observed with granzyme B staining intensity was analyte specific or could extend to other cytotoxic factors. We looked at the expression of two additional cytotoxic factors (granzyme A and perforin) within CD8+ T-cells, directly *ex vivo* (without stimulation). Permeabilization with the Caltag buffer resulted in weak staining for both granzymes A and B, and perforin, hence gave inconsistent results with regard to expression of these molecules, in clear contrast with the eBioscience and BD buffers (Fig. 2c). In general, the eBioscience buffer led to the brightest staining of cytotoxic factors (Fig. 2d). Similar observations (i.e. differences in signal intensity according to permeabilization buffer) were observed when looking at highly differentiated cytotoxic CD4+ T-cells (data not shown).

In contrast to the intracellular analytes, staining for a cell surface marker (e.g. CD45RA using PE conjugated

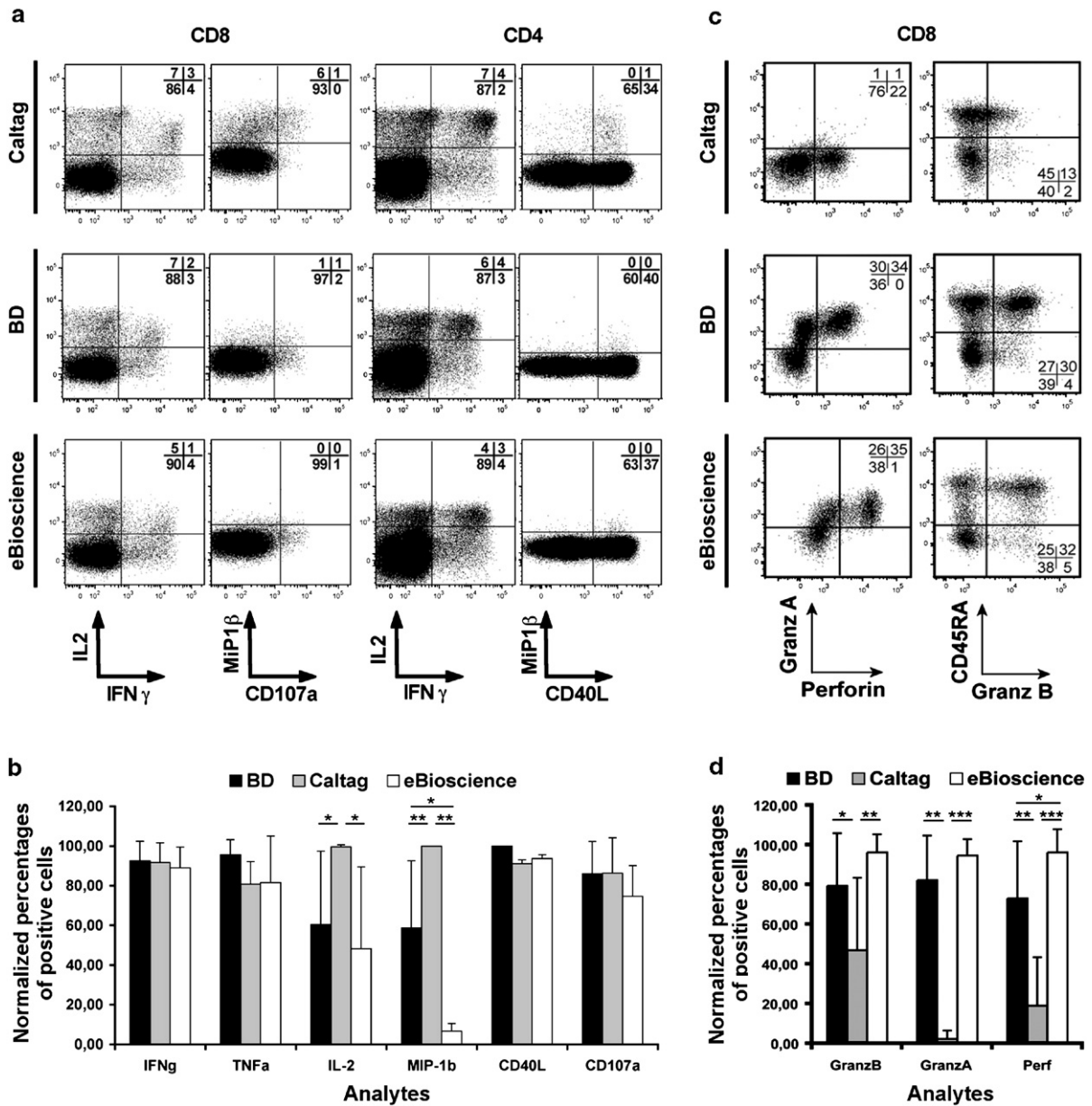


Fig. 2. Assessment of cytokine secretion and cytotoxic factor expression in CD8+ or CD4+ T-cells. (a) Representative staining of PBMC stimulated with SEB for 6 h prior to permeabilization using three different buffers and staining for the functional molecules IFN γ , MIP-1 β , IL-2, CD107a and CD40 L. Cells were gated on either CD8+ or CD4+ populations; percentages in the different quadrants are indicated. (b) Bar graph showing mean values \pm SD of the percentage of positive cells normalized to the highest value obtained between the different permeabilizing solutions tested on seven donors (data for IFN γ , TNF α , MIP-1 β and CD107a staining, or for IL-2 and CD40L staining were obtained on CD8+ or CD4+ gated cells, respectively). MIP-1 β and IL-2 staining was always strongest with the Caltag buffer, which explains their minimal SD for the normalized value. (c) Representative staining of granzymes A and B, and perforin expression within the CD8+ T-cell population after permeabilization using three different buffers. Percentages in the different quadrants are indicated. (d) Bar graph showing mean values \pm SD of the percentage of positive cells normalized to the highest value obtained between the different permeabilizing solutions tested on nine donors. * P <0.01, ** P <0.001 and *** P <0.0001 with the Mann–Whitney test.

antibodies, stained before fixation/permeabilization) led to equivalent separation of the populations with all three buffers (Fig. 2c). However, we noticed that staining with

tandem conjugated antibodies (e.g. anti-CD45RA PE-Texas Red) could be affected (i.e. less effective separation of the populations) in particular when using

the eBioscience procedure. This appeared to affect only the staining for extracellular receptors, probably since fluorescent tandems, which are more fragile than single fluorochromes, stay in contact with the fixation/permeabilization solutions for a significantly longer time (a step that is also longer in the eBioscience procedure), compared to antibodies specific for intracellular molecules (added at the end of the procedure). To ensure that our observations of differences in intracellular staining according to the permeabilization method depended on the analyte rather than on the fluorochrome used, we tested anti-MIP-1 β antibodies coupled with FITC or PE, anti-granzyme B antibodies coupled with Alexa 647 or PE-Texas Red as well as anti perforin antibodies coupled with FITC or PE. Inconsistencies in staining were observed independently of the antibody/fluorochrome tested (data not shown). In addition, the results were similar regardless of whether the freshly isolated PBMC or frozen samples were used.

Overall, all three permeabilizing buffers appear to have led to the permeabilization of the cells: staining for cytokines like IFN γ and TNF α or for CD107a yielded practically equivalent results in each case. However, the staining intensity for other intracellular analytes (MIP-1 β , IL-2 and cytotoxic molecules) appeared to differ depending on the conditions, suggesting different performance of the buffers. Our data do not permit us to conclude the exact reason(s) for these discrepancies. Cytokines, chemokines and cytotoxic factors are thought to be expressed and stored in distinct intracellular compartments (Wagner et al., 1998; Catalfamo et al., 2004). One could speculate that a particular buffer might be more or less efficient at permeabilizing distinct intracellular compartments. While they may all give access to classical secretory vesicles (where IFN γ and TNF α can be found upon stimulation), other cellular compartments (e.g. cytolytic granules or those containing CC chemokines) might be permeabilized with different efficacy thus giving variable access to their content and leading to variable staining intensity. This can lead to a disparity of the results according to the permeabilizing buffer used and therefore represents a potential concern in the context of multiparametric functional analysis. One may need to consider systematic testing of reagents such as permeabilization buffers using a comparative approach for every new analyte to be assessed, and design application-specific procedures to obtain optimal results. In our setting, the Caltag buffer seems most appropriate to monitor T-cell responses based on their cytokine/chemokine function (since it yielded the brightest IL-2 and MIP-1 β staining); the eBioscience buffer seems particularly useful to assess cytotoxic potential in T-cells (since it yielded the most

consistent granzyme and perforin staining); while the BD buffer represents a good compromise to analyze both functions at the same time (i.e. convenient for the analysis of tetramer positive cells; Fig. 1). It is important to be aware of such potential inconsistency for the analysis and interpretation of T-cell functional potential, in particular when comparing results from different immunomonitoring studies. A particular buffer could lead to the conclusion that some cells would not have the possibility to secrete a chemokine like MIP-1 β , or that cytotoxic CD4+ or CD8+ T-cells are not present within populations of antigen specific cells. For instance, a study published in 2003 concluded that CMV specific CD8+ T-cells expressed low levels of perforin, and HIV specific CD8+ T-cells showed inconsistent expression of granzyme A (Zhang et al., 2003), in disagreement with other reports (Appay et al., 2002; Shacklett et al., 2004; Miles et al., 2007). Interestingly, staining in this study was performed using the Fix&Perm Caltag kit (as mentioned in Section 2 of that report), which can explain these observations since this buffer yields weak staining sensitivity of these molecules as shown here.

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