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T-cell receptor (TCR) repertoire analysis has clearly gained the status of a major tool to profile the immune response in health and disease. Although the rapid, technological advances in the field of high-throughput sequencing have widened the landscape of immunological repertoire studies, their application also results in the generation of complex, large volume, datasets that poses a challenge as to their analysis and interpretation. In particular, barriers still remain with respect to the definition of TCR specificities and the integration of functional data with such, large scale, quantitative analyses. To put it plainly, whereas it is feasible to generate extensive quantitative genetic datasets on given T cell subsets, it still remains problematic to assign a clear in vivo function to these cells.

The Qin *et al.* study (*1*). illustrates some of these issues in their attempt to use repertoire analysis to predict the success of vaccination. Although the bio-mathematical approach undertaken is impressive, we would like to argue that basic issues in the definition of vaccine-specific T cells, at the center of the study, undermine the conclusions of the authors.

The first issue raised by the Qin *et al.* study is related to the fact that the determination of varicella zoster virus (VZV)-specificity relies on in vitro T cell proliferation and cytokine secretion after stimulation with a lysate of cells infected with VZV. It is a well-known observation that antigen-specific stimulation is accompagnied by so-called bystander activation (*2*). The authors attempt to control for in vitro bystander stimulation by using uninfected cell lysates as negative controls and report that mock stimulation induces only minimal cellular activation (Fig.1A in (*1*). However, we would like to propose that a more proper control for bystander stimulation would have been stimulation of PBMCs with antigen presenting cells expressing recall antigens other than VZV, in order to induce not only an irrelevant proliferative response, but also a concomitant

bystander stimulation.
In the absence of this appropriate control we argue that a large proportion of clonotypes considered to be VZV- specific are in fact derived from bystander T cell clones randomly activated during the 8-day in vitro culture and, as a consequence, will hamper efforts to quantify the antigen-specific response. This notion is corroborated by results demonstrating that booster vaccination induces high magnitude bystander immune responses in vivo (*2*). It is nevertheless important to stress that, as previously suggested (*3*), a specific clonotype signature could nevertheless be extracted from in vitro sequence data, but only after subtraction of background noise related to bystander proliferation.

There are also basic technical issues that are not discussed in the Qin *et al.* report. In an effort to study the repertoire diversity of VZV-reactive T cells, the authors labeled naïve T cells with the fluorescent dye CFSE, and mixed them with mononuclear cells from the same individual, labeled with a different dye, referred to as CTV. The cell mixture was stimulated with a VZV lysate and analyzed by flow cytometry for the presence of dividing cells, as demonstrated by decreased expression levels of CFSE- or CTV-related fluorescence. In principle, this approach permits to elegantly study, within the same experimental setting, T cell responses derived from either memory or naïve T cell pools by sorting $CFSE^{dim}$ and CTV^{dim} cells and comparing their respective TCR sequences.

We have previously used the same flow cytometric approach to determine alloreactive T cell repertoires in transplant patients, but were so far unable to exploit the data because of difficulties to interpret the results. As expected, we observed, in a mixed lymphocyte reaction (MLR) in which CFSE-labeled donor cells were stimulated with separately CTV-labeled recipient cells, the appearence of $CFSE^{dim}$ and CTV^{dim}cells, presumably corresponding to proliferating donor- and recipient-derived cells, respectively. However, we very consistently observed the concomitant appearance of a $CFSE^{high}CTV^{high}$double labelled population that did not correspond to cells doublets (Fig. 1). Surprisingly, such double labeled cells are absent from the data shown by Qin *et al.* [Fig.4B in (*1*)], although it cannot be excluded that the corresponding dots might have been erased in the plot shown in this figure. In our own experiments, these double labeled cells have dye-related fluorescent levels that are just as high as those of cells analyzed immediately after dye exposure. It is therefore highly unlikely that the cells expressing both fluorescent dyes are the result of dye leakage occurring during in vitro culture, because if this were the case, we would rather expect to observe only low

fluorescence background events. We also excluded the possibility that the presence of these double labeled cells was the result of the formation of cell syncytia, by monitoring HLA-mismatched MLRs using HLA-$B27^+$ HLA-$A2^-$ and HLA-$B27^-$ HLA-$A2^+$ PBMCs, and confirming that no HLA-$B27^+$ $A2^+$ cells were detected after in vitro culture (not shown).

In order to understand the nature of these $CFSE^{high}CTV^{high}$ double labelled cells, we analyzed the fluorescence of PBMCs from the same individual, either labeled with CFSE or CTV, that had been pooled and stimulated with anti-CD3 and anti-CD28 antibodies to induce T cell proliferation. Under these experimental conditions, no $CFSE^{high}CTV^{high}$ cells were detected, which suggests that the generation of such double labeled events might require contact between cells, mediated by interactions between their cognate surface receptors and resulting in the intercellular transfer of fluorescent dye-labeled material. In this respect, it has been reported that lymphocytes may actively take up plasma membrane fragments released by antigen presenting cells (*4*). The physiological relevance of these $CFSE^{high}CTV^{high}$ double stained cells remains however unclear, but cannot a priori be excluded. It might therefore have been important to Qin *et al.* to elucidate the phenomenon by including these double-labeled cells in their repertoire analysis. In any case, we would like to argue that the presentation of the full array of results generated by co-culture of dye-labelled cells is important to researchers that will use the same technical approach, and also important to permit an unbiased, meaningful interpretation of the data.

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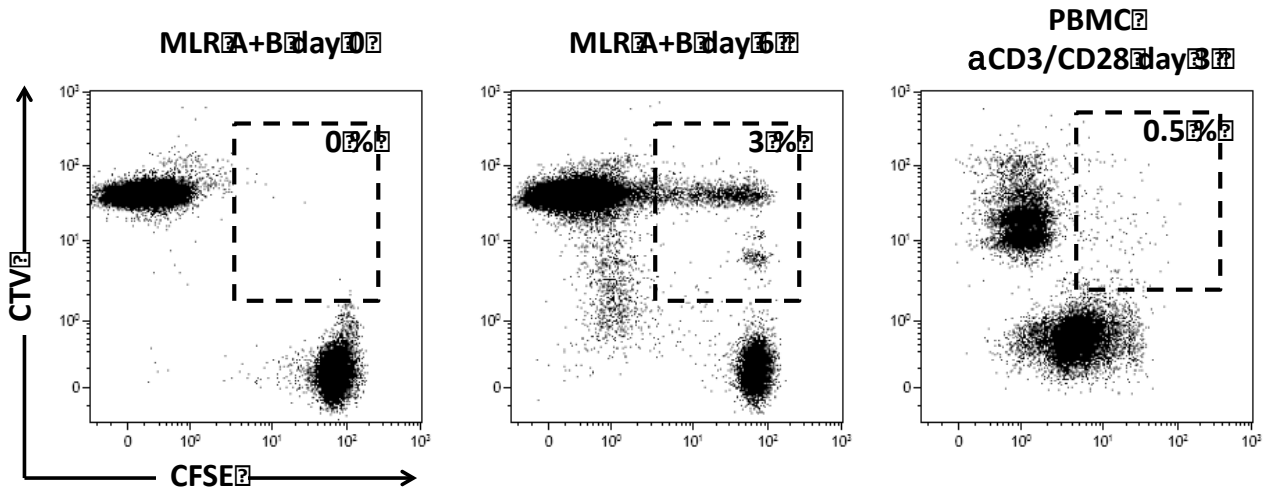

Fig. 1. Flow cytometric analysis of cell division by co-dilution of CFSE and CTV reveals the unexpected presence of CFSE+ CTV+ CD3+ cells. CFSE-labelled PBMCs from an healthy donor (donor A) were mixed in equal proportions with CTV-labelled PBMCs from an unrelated healthy individual (donor B). The cell mixture was analyzed by flow cytometry before (left panel) and after 6 days of in vitro co-culture (central panel). PBMCs from donor A were also separately labeled with either CFSE or CTV, then mixed and analyzed after 3 days of anti-CD3/anti-CD28 stimulation (right panel). As shown, an unexpectedly high proportion of CTV/CFSE double labeled cells among CD3+ cells (doted line gate) is recorded in the MLR setting only (central panel). One representative experiment out of 5.