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Comment on “Diversification of the antigen-specific T cell receptor repertoire after varicella zoster vaccination”

Christophe Parizot,1,2,3* Hélène Brisson,1,2,3* Jehane Fadlallah,1,2 Delphine Sterlin,1,2,3 Guy Gorochov1,2,3†

Analysis of antigen-specific T cells can be confounded by T cell activation in vitro.

The Qi et al. study (1) analyzed the repertoire of T cells from individuals receiving varicella zoster virus (VZV) immunization. Although the biostatistical approach undertaken is impressive, we would like to argue that the definition of vaccine-specific T cells, at the center of the study, may affect the conclusions.

The first issue we would like to raise is related to the fact that the determination of VZV specificity relies on in vitro T cell proliferation and cytokine secretion after stimulation with a lysate of cells infected with VZV. It is well-established that antigen-specific stimulation is accompanied 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 appropriate control for bystander stimulation would have been stimulation of peripheral blood mononuclear cells (PBMCs) with antigen-presenting cells expressing recall antigens other than VZV, in order to induce not only an irrelevant proliferative response but also concomitant bystander stimulation.

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

A technical issue in the Qi et al. (1) study should also be discussed. In an effort to study the repertoire diversity of VZV-reactive T cells, the authors labeled naïve T cells with the fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE) and mixed them with mononuclear cells from the same individual, labeled with a different dye, CellTrace Violet (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 of CFSE- or CTV-related fluorescence. In principle, this approach permits the study, within the same experimental setting, of T cell responses derived from either memory or naïve T cell pools by sorting CFSEdim and CTVdim cells and comparing their respective T cell receptor sequences.

We have used the same flow cytometric approach to determine alloreactive T cell repertoire in transplant patients but were so far unable to exploit the data because of difficulties with interpretation of the results. As expected, we observed, in a mixed lymphocyte reaction (MLR) in which CFSE-labeled donor cells were stimulated with CTV-labeled recipient cells, the appearance of CFSEdim and CTVdim cells, presumably corresponding to proliferating donor- and recipient-derived cells, respectively (Fig. 1). However, we consistently observed the concomitant appearance of a CFSEhighCTVhigh double-labeled population that did not correspond to cells doublets (Fig. 1, middle panel). Surprisingly, such double-labeled cells are absent from the data shown by Qi et al. [Fig. 4B in (1)]. In our own experiments, these double-labeled cells have dye-related fluorescence that is as high as that of cells analyzed immediately.

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1Sorbonne Universités, Université Pierre et Marie Curie (Université Paris 06), Unité Mixte de Recherche de Santé CR7, Centre d’Immunologie et des Maladies Infectieuses (CIMI), F-75013 Paris, France. 2INSERM, Unité 1135, CIMI, F-75013 Paris, France. 3Assistance Publique-Hôpitaux de Paris (AP-HP), Département d’Immunologie, Hôpital Pitié-Salpêtrière, F-75013 Paris, France. *These authors contributed equally to this work. †Corresponding author. Email: guy.gorochov@upmc.fr

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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<sup>−</sup>CTV<sup>−</sup>CD3<sup>+</sup> cells. CFSE-labeled PBMCs from a healthy donor (donor A) were mixed in equal proportions with CTV-labeled 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). An unexpectedly high proportion of CTV/CFSE double-labeled cells among CD3<sup>+</sup> cells (dotted line gate) is recorded in the MLR setting only (central panel). One representative result out of five independent experiments.
after dye exposure. In our view, it is 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 expect to observe only low-fluorescence background events.

In order to understand the nature of these CFSE\textsuperscript{high}CTV\textsuperscript{high} double-labeled 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 (Fig. 1, right panel). Under these experimental conditions, no CFSE\textsuperscript{high}CTV\textsuperscript{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\textsuperscript{high}CTV\textsuperscript{high} double-stained cells remains unclear. It might therefore have been important for Qi et al. (1) to elucidate the phenomenon by including these double-labeled cells in their repertoire analysis. We would like to argue that the presentation of the full array of results generated by co-culture of dye-labeled cells is important to researchers that will use the same technical approach and also will be important to permit an unbiased, meaningful interpretation of the data.

REFERENCES
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