TRANSPLANTATION

Comment on "Tracking donor-reactive T cells: Evidence for clonal deletion in tolerant kidney transplant patients"

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In their recent article (*1*), Morris *et al.* apply a strategy that elegantly combines flow sorting of proliferating cells after mixed lymphocyte reaction (MLR) assay and next-generation sequencing (NGS) of the T cell receptors (TCRs), similar to the work from 2003 by Douek and collaborators (*2*), to characterize and track alloreactive T cells in transplant recipients. They use this approach to study a few patients undergoing kidney transplantation and claim that their data provide evidence for deletion of donor-reactive clones as a mechanism of allograft tolerance. However, on the basis of the data presented and a number of questions regarding their methodology, we believe that this conclusion might be premature.

The primary evidence Morris *et al.* use to support their conclusion is that the number of putative donor-reactive clones observed is reduced after transplantation in tolerant patients, but not in a single nontolerant patient. However, a large number of clones considered to be alloreactive remain detectable 14 to 24 months after transplantation in tolerant recipients [subjects 1, 2, and 4 in figs. S3 and S4 in (*1*)]. Thus, even if the number of donor-reactive clones may be reduced in tolerant patients, there still remain enough clones—in similar frequencies to the nontolerant patient—that could maintain an effective anti-donor response.

In addition, it is not clear why only donor-specific clonotypes identified by pretransplant MLR were considered in this analysis. Alloreactive T cell clones identified in posttransplant samples could be different from those detected before transplantation and should be tracked as well, as has been done in other studies (*3*). Although Morris *et al.* performed MLRs at 12 months (M12) after transplantation [fig. S6 in (*1*)], the kinetics of posttransplant donor-reactive clonotypes were not shown. Nevertheless, the authors reported a very low overlap between pre- and posttransplant clonotypes identified by MLR, which suggests drastic changes in the anti-donor specific profile.

We believe that when analyzing the kinetics of specific clonotypes, it is important to do so from different time points of view (tpov). To illustrate this point, we show in a longitudinal NGS-based study of BK virus (BKV)–specific TCRs after transplantation that the numbers of antigen-specific clonotypes functionally characterized in blood at a given time point (tpov M6) indeed decline with time (Fig. 1A). However, these clones are in fact replaced by others, characterized in blood as BKV-specific 3 months later (tpov M9), and tracked back in previous samples (Fig. 1B). We also show how some individual clonotypes remain dominant (Fig. 1C) and others rise in frequency (Fig. 1D), although the number of antigen-specific clonotypes characterized from tpov M6 decline with time (Fig. 1A). Had we analyzed these data only by looking at number of clonotypes from the early tpov (M6), we would have wrongly concluded that anti-BKV immunity fades away in this patient.

Altogether, from the analytical point of view adopted by Morris *et al.*, it is clear that the numbers of putative pretransplant donor-reactive clonotypes decline with time in some of their patients, but it is not clear whether the actual frequencies of donor-reactive cells, including newly, posttransplant expanded clones, do so.

In fact, it is unclear, considering the level of evidence produced, whether all the T cell clonotypes defined by Morris *et al.* as donor-reactive are indeed such. Here, a recipient clone is considered donor-reactive when its frequency increases at least fivefold after in vitro MLR stimulation with donor cells. Therefore, the determination of alloreactivity solely relies on the MLR assay, although it was previously demonstrated that during in vitro MLR, proliferating T cells include bystander responsive T cells that are not alloreactive (*4*). The supernatant of a primary MLR was shown to induce a level of proliferation of naïve cells equivalent to the primary response itself (*4*), indicating how poorly specific in vitro MLR is when it comes to probing alloreactivity. This issue was solved performing in vivo MLRs that do not suffer the same background limitations, but that cannot be easily performed in humans (*5*).

In an attempt to validate the reproducibility and specificity of their alloreactive TCR detection protocol, Morris *et al.* repeat this assay in blood draws from the same healthy individual separated by a 2-week interval. Consistent with the fact that MLR-responsive clones are not necessarily alloreactive, it is striking to observe [Fig. 2 in (*1*)] that less than half of clonotypes considered to be alloreactive at time 0 are detected 2 weeks later. Considering the power of the genetic analysis applied (deep sequencing of TCRs), we find it unlikely that T cell repertoire turnover and/or tissue redistribution might account for such drastic changes within such a short time. We rather argue that a large proportion of clonotypes considered alloreactive are in fact bystander T cell clones randomly activated during the 6-day in vitro culture.

An alloreactive clonotype signature could nevertheless be extracted from in vitro MLR sequence data, but only after subtraction of background noise related to bystander proliferation. Such background noise could be determined using control cultures of lymphocytes stimulated with MLR supernatants, as previously described (*4*), or with T cell growth factors, in the absence of TCR stimulation.

Altogether, the work of Morris *et al.* raises interesting issues, but a better definition of alloreactive clonotypes and more information on the kinetics

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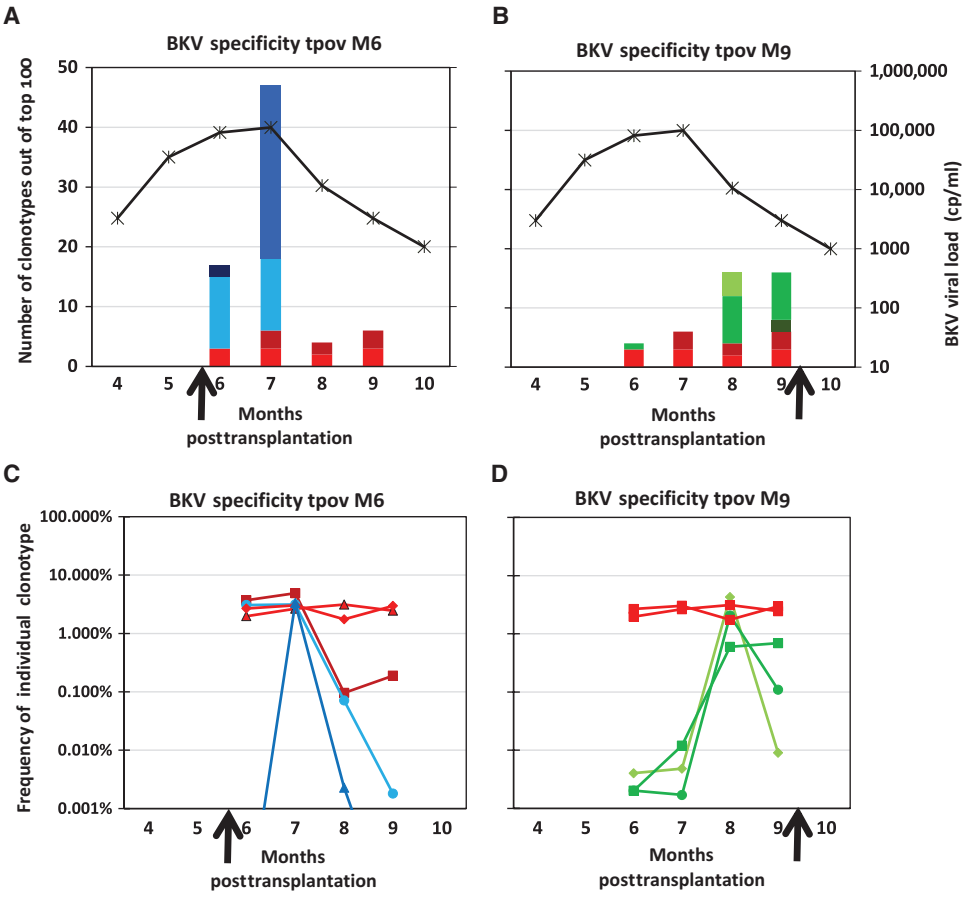

Fig. 1. Kinetic analysis that allows tracking both the number (A and B) of antigen-specific clonotypes and their frequencies (C and D) from different time points of view (tpov). A 70-year-old male underwent kidney transplantation and had BKV reactivation, resulting in an elevation of BKV viral load (black curve). BKV-specific cells were sorted from peripheral blood mononuclear cells (PBMCs) drawn at months 6 (M6) and 9 (M9) after transplantation (as indicated by arrows), on the basis of interferon-γ secretion after BKV antigen stimulation in vitro, and their TCR repertoire was analyzed using deep sequencing. BKV-specific clonotypes were then tracked by deep sequencing in unstimulated PBMC samples at indicated time points. Each color represents a group of clonotypes that is found at several time points. A reduction in the number of BKV-specific clonotypes among the top 100 dominant CD8 clonotypes is observed (A) when using an M6 tpov. Nevertheless, at least two BKV-specific clonotypes remain in high frequencies, as shown by the kinetics of a number of representative clonotypes (C). Furthermore, from an M9 tpov, the number of BKV-specific clonotypes (B) and their frequencies (D) show different kinetics, which together indicates a repertoire shift rather than a decline in BKV-specific immunity.

of such clonotypes posttransplant are needed to determine whether tracking of bona fide alloreactive clones might indeed unravel the mechanisms of spontaneous tolerance. Finally, it is an occasion to emphasize that high-throughput deep sequencing of TCR and antibody repertoires remains a new area, where the correct experimental and statistical approaches are still being developed.

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