Heterologous immunity in organ transplantation

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CHAPTER

3

DETECTION OF VIRUS-SPECIFIC CD8$^+$ T CELLS WITH CROSS-REACTIVITY AGAINST ALLOANTIGENS: POTENCY AND FLAWS OF PRESENT EXPERIMENTAL METHODS

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Transplant Direct. 2015 Nov 4;1(10):e40

ABSTRACT

Background

Virus-specific T cells have the intrinsic capacity to cross-react against allogeneic HLA antigens, a phenomenon known as heterologous immunity. In transplantation, these cells may contribute to the alloimmune response and negatively impact graft outcome. This study describes the various techniques that can be used to detect heterologous immune responses of virus-specific $CD8^+$ T cells against allogeneic HLA antigens. The strengths and weaknesses of the different approaches are discussed and illustrated by experimental data.

Methods

Mixed lymphocyte reactions (MLRs) were performed to detect allo-HLA cross-reactivity of virus-specific $CD8^+$ T cells in total peripheral blood mononuclear cells. T-cell lines and clones were generated to confirm allo-HLA cross-reactivity by IFNγ production and cytotoxicity. In addition, the conventional MLR protocol was adjusted by introducing a 3-day resting phase and subsequent short restimulation with alloantigen or viral peptide, whereupon the expression of IFNγ, interleukin-2 (IL-2), CD107a and CD137 was determined.

Results

The accuracy of conventional MLR is challenged by potential bystander activation. T-cell lines and clones can circumvent this issue, yet their generation is laborious and time-consuming. Using the adjusted MLR and restimulation protocol, we found that only truly cross-reactive T cells responded to re-encounter of alloantigen and viral peptide, while bystander-activated cells did not.

Conclusions

The introduction of a restimulation phase improved the accuracy of the MLR as a screening tool for the detection of allo-HLA cross-reactivity by virus-specific $CD8^+$ T cells at bulk level. For detailed characterization of cross-reactive cells, T-cell lines and clones remain the golden standard.

INTRODUCTION

Viral infections are a common complication after transplantation and are associated with rejection and decreased graft survival (103). Viruses may cause transplant injury directly by infecting cells of the graft, or indirectly by activating innate and adaptive immune responses. Local viral infections, for instance initiated by BK virus in kidney transplantation or by airborne viruses in lung transplantation, may harm the graft by lytic viral replication within epithelial cells and immune cell-mediated (bystander) injury (104, 105). In addition, viral infections can alter the cytokine milieu inside the graft or even systemically, affecting the differentiation and function of lymphocytes including alloreactive T cells. For example, cytomegalovirus (CMV) infection induces a systemic immune activation characterized by increased levels of Th1-associated cytokines in both healthy individuals and kidney transplant recipients (106).

The role of viruses in alloimmune responses is illustrated by experimental murine studies. Whereas transplantation tolerance is easily achieved in pathogen-free mice, it is far more difficult to achieve in humans and nonhuman primates. As humans and nonhuman primates are continuously exposed to bacteria and viruses, this suggests that pathogens and acquired immunological memory may affect alloresponses. Indeed, studies using pathogen-free versus pathogen-experienced mice showed that the latter were significantly less susceptible to the induction of tolerance (35). Interestingly, viral infections may affect transplant outcome even if viremia has been resolved long before transplantation, and virus-specific $CD8^+$ T cells may directly contribute to graft rejection (37), suggesting a role for memory T cells induced by viral exposure (35, 107).

A significant part of virus-specific memory $CD8^+$ T cells can recognize allogeneic human leukocyte antigens (allo-HLA) (108). This is due to cross-reactivity of their T-cell receptor (TCR), enabling the recognition of different epitopes by the same TCR. This phenomenon is known as heterologous immunity. Heterologous immunity often occurs in a physiological setting and creates an evolutionary benefit by enhancing the protection against (un)related pathogens. Cross-reactivity is essential for organisms that encompass only a restricted number of T cells and is an intrinsic feature of all TCRs (24). Therefore, it is not surprising that the vast majority of virus-specific $CD8^+$ T cells in healthy individuals can cross-react to 1 or multiple allo-HLA antigens *in vitro* (43).

Compared to naïve T cells, memory T cells tend to be less sensitive to immunosuppressive drugs (109, 110). Therefore, memory T cells that cross-react to donor alloantigens may play a role in T

cell-mediated allograft rejection (111-114). Several studies in heart, kidney, and liver transplant recipients demonstrate a distinct correlation between the frequency of donor-reactive memory T cells before and the incidence and severity of rejection episodes after transplantation (115, 116). Indeed, cross-reactive virus-specific memory T cells have been found in allografts of lung transplant recipients (39, 40, 86).

Clinical studies on cross-reactive virus-specific memory T cells in transplantation are limited, and additional studies are required. A potential obstacle facing these studies is the complex detection of truly cross-reactive responses. Here, we have described the strengths and weaknesses of various approaches that can be used to detect and functionally analyze virus-specific $CD8^+$ T cells with cross-reactivity to allo-HLA antigens. We compared current experimental methods, divided into bulk culture and clonal analyses, for their accuracy, potential applications and limitations. Furthermore, we suggest an altered protocol to more accurately distinguish true cross-reactivity from bystander-activation at bulk level.

MATERIALS AND METHODS

Collection of responder and target cells

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy individuals and anonymous donors (Buffy coats, Sanquin Blood Supply, The Netherlands) after informed consent in accordance with the Declaration of Helsinki. PBMCs were isolated by standard density gradient centrifugation and cryopreserved. Epstein-Barr Virus transformed lymphoblastoid cell lines (EBV-LCLs) were generated by incubating PBMCs with supernatant of the EBV-producing marmoset cell line B95.8 for 1.5 hours at 37°C, and additional culture in RPMI 1640 Medium (Gibco) supplemented with penicillin/streptomycin, glutamine and 10% fetal calf serum (FCS). Single-antigen-expressing cell lines (SALs) were generated as described previously (117). HLA typing was performed by sequence-specific oligonucleotide (SSO) or sequence-specific primer (SSP) genotyping at the Tissue-typing laboratory (Leiden University Medical Center (LUMC), Leiden, The Netherlands).

Generation of virus-specific $CD8^+$ T-cell lines and clones

$CD8^+$ memory T-cell lines and clones were generated from individuals 1 and 2 by fluorescence-activated cell sorting (FACS Aria; BD), as previously described (118). PBMC were stained with phycoerythrin (PE)-labeled viral tetramers CMV pp65(417-426) HLA-B*07:02/TPRVTGGGAM (CMV B7/TPR), EBV EBNA-3A(379-387) HLA-B*07:02/RPPIFIRRL (EBV B7/RPP), and EBV EBNA-3A(458-466) B*35:01/YPLHEQHGM (EBV B35/YPL) (Protein facility, LUMC) and fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies (mAb) against CD4, CD19, CD45-RA,

CD14, CD40, CD16 and CD56. The FITC channel (FL1) served as a dump channel, as concurrent CD8 mAb and major histocompatibility complex (MHC)-tetramer staining may trigger TCR internalization. T-cell receptor (TCR) usage was determined by DNA sequencing using TCR-specific polymerase chain reaction (PCR) primers (119).

Mixed lymphocyte reaction

Responder PBMC ($5x10^5$ cells) were labeled with carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes, 5mM), and co-cultured for 6 days with irradiated stimulator PBMC (3000 Rad, responder:stimulator ratio 1:1) in a 24-well flat bottom plate at a slant. Culture medium consisted of either RPMI 1640 Medium (Gibco) supplemented with penicillin/streptomycin, glutamine and 15% human serum (HS) or Iscove's Modified Dulbecco's Medium (IMDM, Lonza) supplemented with 10% HS, penicillin/streptomycin and 0.00036(v/v)% β-mercaptoethanol. For culture beyond 6 days, medium was supplemented with IL-2 (10 U/mL) to ensure T-cell survival. Cells were stained with fluorescence-labeled CD8 and CD3 antibodies, a viability dye (fixable viability dye eFluor 506, eBioscience) and viral tetramer conjugated with PE or allophycocyanin (APC) (Protein facility, LUMC, or Sanquin, Amsterdam, The Netherlands). Flow cytometric analyses were performed on FACS Calibur and FACS CANTO (BD Biosciences).

Proliferation assay with correction for bystander activation: Mixed lymphocyte reaction followed by restimulation

After 6 days of MLR with unmanipulated responders and allogeneic stimulators (see above), medium was replaced with culture medium containing 10 U/ml IL-2, and cells were cultured for additional 3 days to allow downregulation of activation markers. Importantly, addition of viral peptide during the first MLR is discouraged because this will lead to preferential expansion of T-cell clones with a high affinity for the viral peptide. Next, the cells were taken up in stimulation medium (IMDM + 10% HS + βME + P/S + a-CD28 (2 µg/ml) + a-CD29 mAb (1 µg/ml)) and restimulated with: PMA (10 ng/ml, Sigma-Aldrich) and ionomycin (1 µg/ml, Sigma-Aldrich) (TCR-independent positive control), the original allogeneic stimulators ($2x10^6$), autologous cells ($5x10^5$) loaded with 10-100 ng viral peptide (TCR-dependent positive control), or co-stimulation alone (negative control) in a non-tissue-culture-treated round-bottom 96-wells plate. Stimulator cells were labeled with Celltracker Violet BMQC (Invitrogen) to allow discrimination between responders and stimulators. Costimulation through anti-CD28 and anti-CD29 antibody binding was provided to ensure optimal responses (120). The kinetics of the functional markers were previously analyzed: cytokine production and CD107a exposure peaked after 6 hours of restimulation, while the induction of CD137 and other activation markers was most prominent after 24 hours (data not shown). a-CD107a-PE antibody (BD Pharmingen) was added during

the 6-hour restimulation and after 1 hour monensin (0.7 µg/ml; GolgiStop, BD Pharmingen) and brefeldin A (10 µg/ml; Invitrogen) were administered to inhibit protein secretion. Next, the cells were harvested and stained intracellularly for IL-2 (IL-2-PE-Cy7, BioLegend) and IFNγ (IFNγ-allophycocyanin (APC)-eFluor 780, eBioscience). CD137 was measured by cell-surface staining (CD137-PE, BD Pharmingen) at 24 hours after restimulation (without addition of monensin and brefeldin A). All parameters were analyzed by flow cytometry (FACS CANTO; BD Biosciences).

Cytokine production assay

IFNγ levels were measured in a standard enzyme-linked immunosorbent assay (ELISA), performed according to the manufacturer's protocol (U-CyTech ELISA kit; U-CyTech, the Netherlands). $CD8^+$ T-cell lines and clones were stimulated by a panel of 11 EBV-LCLs or 6 SALs. $5x10^3$ $CD8^+$ T cells were incubated with $5x10^4$ EBV-LCLs or SALs in triplicate wells for 24 hours at 37°C in IMDM supplemented with penicillin/streptomycin, glutamine, 5% FCS, 5% HS, and IL-2 (10 U/mL), after which supernatants were collected.

Cytotoxicity assay

For optimal culture conditions, $CD8^+$ T-cell lines and clones were cultured with irradiated PBMCs (4000 Rad) from anonymous buffy coats 8 days prior to cytotoxicity testing. Cytotoxic capacity was assessed by ^{51}Chromium-release (^{51}Cr) assay (121). Serial dilutions (responder/stimulator ratio 30:1; 10:1; 1:1; 0.1:1) of responder $CD8^+$ T-cell lines and clones were stimulated with ^{51}Cr-labeled EBV-LCLs and/or phytohaemagglutinin (PHA) blasts in round-bottom 96-wells plates for 4 hours at 37°C, in IMDM, penicillin/streptomycin, glutamine, 5% FCS, 5% HS, and IL-2 (10 U/mL). PHA blasts were generated by incubation of $1x10^6$ cells PBMCs with PHA (0.8mg/mL; Murex Biotec Limited). Supernatants were collected for analysis on a γ-counter (PerkinElmer 2470 Wizard^2), and specific lysis was determined by the following calculation: *(Experimental ^{51}Cr release -Spontaneous ^{51}Cr release)/(Maximum ^{51}Cr release - Spontaneous ^{51}Cr release) x100*. Maximum ^{51}Cr release of the target cells was determined in PBS 1% Triton X-100, and spontaneous ^{51}Cr release in medium. Values for specific ^{51}Cr lysis represent the mean ± standard deviation of triplicate wells.

RESULTS

Techniques to assess virus-specific T cells with cross-reactivity to alloantigen in bulk cultures

Cross-reactivity of virus-specific T cells can be assessed in bulk cultures using PBMCs. PBMCs are easily obtained from blood samples, do not need pre-culturing, and are considered to be a fair representation of the immune repertoire.

Mixed lymphocyte reaction: a tool to screen for cross-reactivity and determine precursor frequencies of cross-reactive T cells

A widely used method to determine alloreactive lymphocytes in vitro at bulk level is the mixed lymphocyte reaction (MLR). Responder PBMCs are cultured with irradiated allogeneic stimulator PBMCs, whereupon proliferation and expression of activation markers can be assessed. Figure 1 shows how MLR can be used to determine proliferation of cross-reactive $CD8^+$ T cells that recognize both viral and alloantigen epitopes.

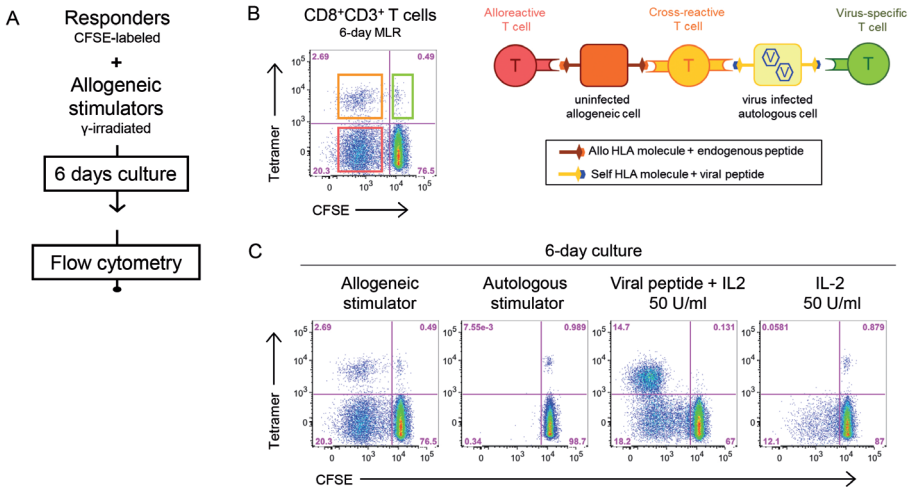
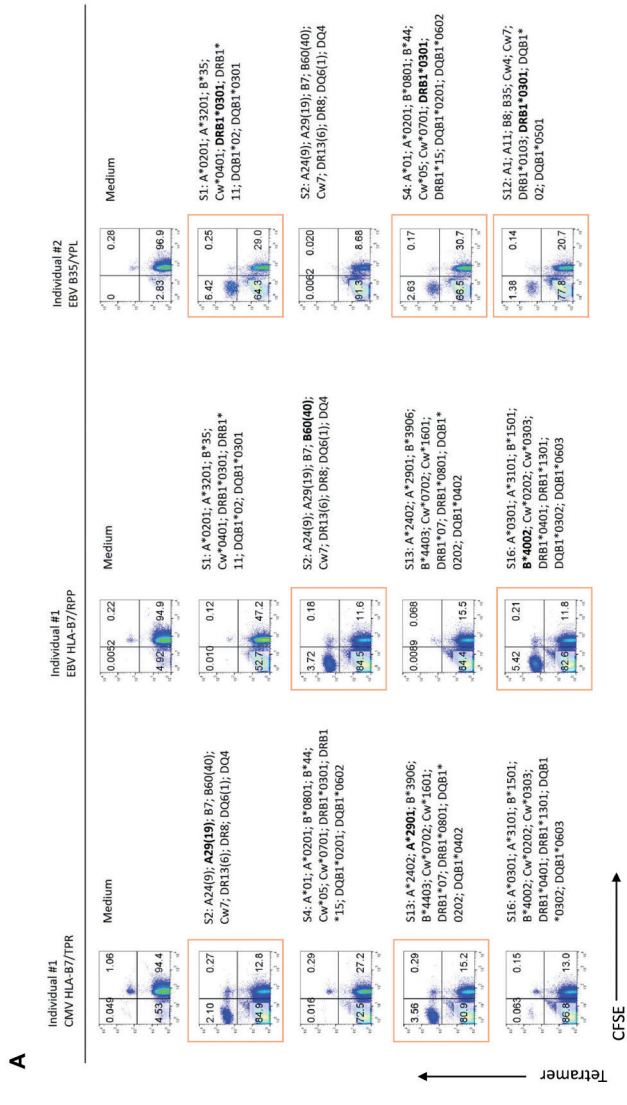


Figure 1. Identification of virus- and alloantigen cross-reactive $CD8^+$ T cells by combining MLR with viral tetramer staining. A) Flow chart of the experimental setup for a standard MLR. B) Flow-cytometric analysis of $CD8^+$ T cells after a 6-day MLR. Plotting viral tetramer against CFSE can distinguish between virus-specific cells ($CFSE^+tetramer^+CD8^+CD3^+$ T cells, green), cross-reactive cells ($CFSE^{dim}tetramer^+CD8^+CD3^+$ T cells, orange), and alloreactive cells ($CFSE^{dim}tetramer^-CD8^+CD3^+$ T cells, red). Schematic overview of allo-, virus- and cross-reactive T cells (right panel) C) Overview of different FACS plots after a 6-day MLR showing from left to right: a proliferative response of tetramer-positive and tetramer-negative cells upon allogeneic stimulation; no proliferative response of tetramer-positive and tetramer-negative cells upon autologous stimulation; a proliferative response of tetramer-positive and tetramer-negative cells upon viral peptide + IL-2 stimulation; a proliferative response of tetramer-negative cells but no proliferative response of tetramer-positive cells upon IL-2 stimulation alone.

We composed a panel of 16 different HLA-typed stimulator PBMCs, which covered the most common HLA class I molecules in the Western European population (>5%) (Table S1, SDC, http://links.lww.com/TXD/A15). The PBMCs of two HLA-typed healthy individuals were screened against this panel in MLR. In both individuals, $CD8^+$ T cells directed against different viral epitopes proliferated upon encounter with 1 or more stimulator targets (Table S2, SDC, http://

links.lww.com/TXD/A15). CMV B7/TPR and EBV B7/RPP T-cell responses of individual 1, as well as EBV B35/YPL T-cell responses of individual 2, revealed potential cross-reactivities to allo-HLA antigen (Figure 2A). Additional MLRs were performed, which confirmed allo-HLA cross-reactivity (Figure 2B). CMV B7/TPR T cells proliferated strongly in response to stimulators expressing HLA-A29, whereas EBV B7/RPP T cells responded toward HLA-B40-expressing stimulators. EBV B35/YPL T cells appeared to recognize the HLA class II molecule HLA-DRB1*03:01.



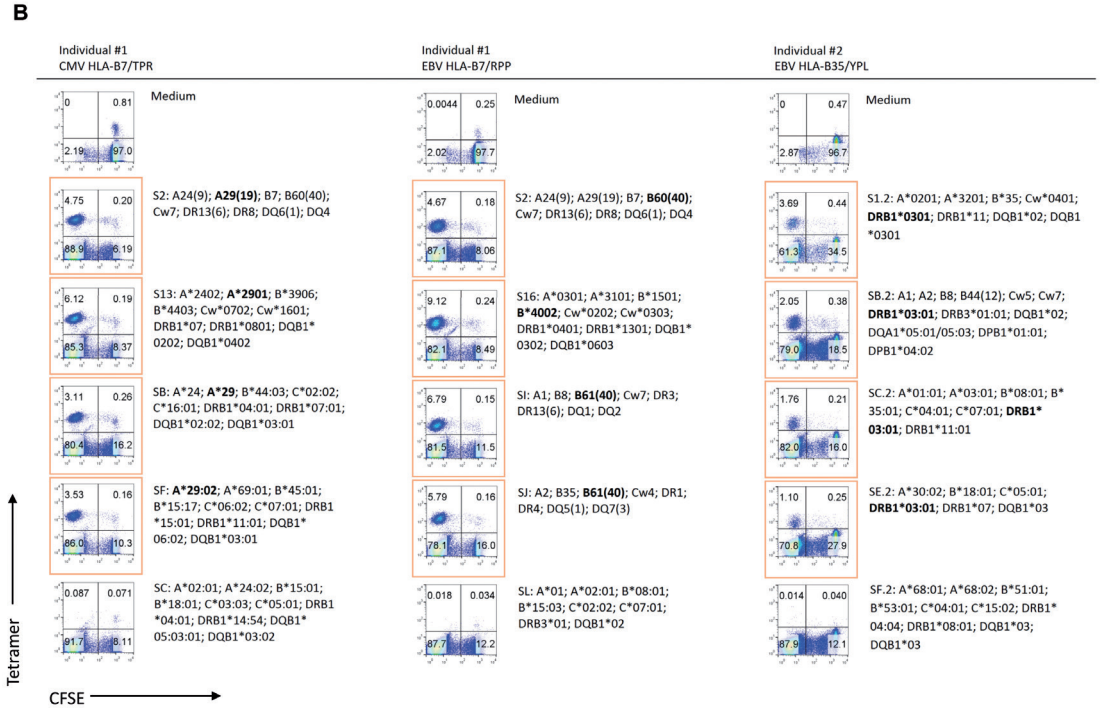


Figure 2. Cross-reactivity of CMV- and EBV-specific T cells to multiple allogeneic stimulators. A) FACS plots showing proliferation of CMV B7/TPR-, EBV B7/RPP- and EBV B35/YPL-specific CD8⁺ T cells following MLRs against the HLA-typed stimulator PBMC panel (n = 16). Illustrated are proliferative responses against stimulators S1, S2, S4, S12, S13 and S16, and presumed cross-reactive HLA antigens are indicated in bold. B) FACS plots showing proliferation of CMV B7/TPR-, EBV B7/RPP- and EBV B35/YPL-specific CD8⁺ T cells after MLRs against stimulator PBMCs derived from healthy donors expressing the presumed cross-reactive HLA antigens (indicated in bold). Plots are gated on CD8⁺ lymphocytes. Left column: responder R1 CMV B7/TPR. Middle column: responder R1 EBV B7/RPP. Right column: responder R2 EBV B35/YPL. X-axis: CFSE; Y-axis: tetramer-PE.

Responder HLA typing:
R1: A*02; A*03; B*07; B*35; C*04; C*07; DRB1*01; DRB1*08; DQB1*04:02; DQB1*05:01;
R2: A*02; A*03; B*07; B*35.

Proliferating alloreactive T cells produce IL-2 and additional cytokines that can promote T-cell activation and proliferation in an antigen-independent manner. Consequently, it is difficult to determine which responses are truly cross-reactive. IL-2-mediated bystander proliferation is illustrated by CMV B7/TPR T cells of individual 3 in Figure 3B. To get an impression of potential bystander activation, the extent to which virus-specific T cells proliferate in response to IL-2 can be assessed (Figure 1). However, unresponsiveness to IL-2 alone does not exclude bystander activation and proliferation in response to IL-2 alone does not exclude true cross-reactivity toward alloantigen. The probability of bystander activation can be assessed by performing additional MLRs with various HLA-typed stimulators. Overall, these findings demonstrate that performing MLRs against a broad panel of HLA-typed targets can aid in identifying HLA class I and II antigens recognized by cross-reactive virus-specific CD8⁺ T cells, yet one should be aware of bystander activation.

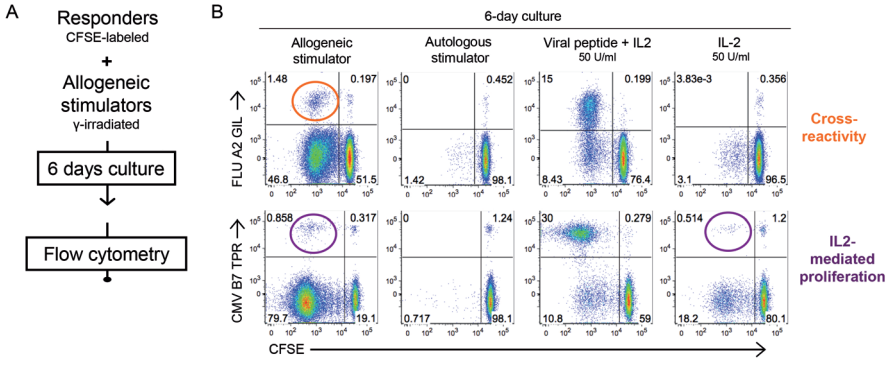


Figure 3. Potential cross-reactivity of virus-specific CD8⁺ T cells can be misinterpreted due to bystander activation in a mixed lymphocyte reaction. A) Flow chart of a 6-day MLR. B) FACS plots depicting the extent of proliferation and tetramer-reactivity of CD8⁺ T cells after 6-day culture with (from left to right): allogeneic stimulators, autologous stimulators (negative control), viral peptide and IL-2 (positive control) or IL-2 alone (cytokine-mediated bystander activation).

Responder HLA typing:
R3 (FLU A2/GIL): A1, A2, B8, B44(12), Cw5, Cw7, DR1, DR4
R4 (CMV B7/TPR): A2, B7, remainder unknown
Allogeneic stimulator HLA typing:
FLU A2/GIL: A2, A19, B7, B16, DR2, DR6
CMV B7/TPR: A1, A31(19), B8, B51(5), DR13(6), DR3

MLR with restimulation: identification of cross-reactive T cells in total PBMC, an optimized protocol to detect true cross-reactivity at bulk level

Performing multiple MLRs is time-consuming, and it would thus be beneficial to rule out bystander activation in a single experiment. This could be achieved by combining a primary MLR with a subsequent short restimulation with the same allogeneic responder (122). This approach ensures a more accurate and sensitive detection of alloreactivity due to clonal expansion and diminished activation requirements of prestimulated alloreactive cells. Cross-reactive cells responding during the primary MLR will respond quickly against the original stimulator cells, yet only modestly to other allogeneic stimulator cells (123).

We introduced a 3-day resting period and restimulation phase following the conventional MLR to identify truly cross-reactive T cells and simultaneously elucidate their function by assessment of cytokine production, exposure of the degranulation marker CD107a, and expression of the activation marker CD137 (124, 125). The FLU A2/GIL and CMV B7/TPR responses of the responder-stimulator combinations that were previously investigated in conventional MLR (Figure 3B) were now investigated in MLR with restimulation. Proliferating FLU A2/GIL T cells expressed comparable levels of interferon γ (IFNγ), IL-2, CD107a and CD137 upon restimulation with either TCR-independent stimulus (PMA-ionomycin), autologous cells pulsed with viral peptide, or allogeneic stimulator cells. This indicated true cross-reactivity (Figure 4B). In contrast, CMV B7/TPR cells showed no IFNγ, IL-2, CD107a and negligible CD137 expression levels upon allogeneic restimulation compared to TCR-independent stimulus and autologous cells with viral peptide, suggesting that the CMV B7/TPR T cells indeed proliferated in an alloantigen-independent manner (as indicated by IL-2-mediated proliferation; Figure 3B) and thus were not truly cross-reactive. When cross-reactive or bystander-activated cells were not restimulated, they expressed no or very little functional and activation markers. These findings were reproduced in independent experiments with the same responder-stimulator pairs. The examples depicted in Figure 4 are representative for n = 15 responses of T cells specific for epitopes of CMV, EBV and FLU. The altered MLR with restimulation protocol is thus a suitable tool to identify true cross-reactivity at bulk level.

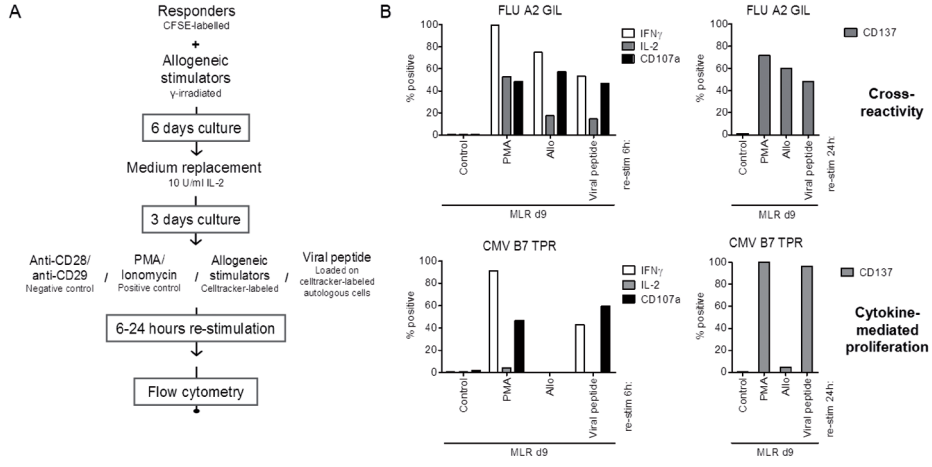


Figure 4. Restimulation after an MLR can help to discriminate true cross-reactivity from bystander activation. A) Flow chart of the 9-day MLR followed by 6-24 hr restimulation. B) Expression of IFNγ, IL-2, CD107a and CD137 in FLU A2/GIL$^+$ T cells that cross-react to alloantigen, and CMV B7/TPR$^+$ T cells that have proliferated independent of alloantigen. Responder and stimulator cells correspond to the ones used in Figure 3B. The examples of true cross-reactivity and bystander activation are representative for n = 15 responses for epitopes of CMV, EBV and FLU.

Responder HLA typing:
R3 (FLU A2/GIL): A1, A2, B8, B44(12), Cw5, Cw7, DR1, DR4
R4 (CMV B7/TPR): A2, B7, remainder unknown
Stimulator HLA typing:
FLU A2/GIL: A2, A19, B7, B16, DR2, DR6
CMV B7/TPR: A1, A31(19), B8, B51(5), DR13(6), DR3

Techniques to assess virus-specific T-cells with cross-reactivity toward alloantigen using T-cell lines and clones

To determine in-depth characteristics and cytotoxicity of cross-reactive CD8$^+$ T cells, CD8$^+$ T-cell lines and clones are recommended. In addition, they can be used to support MLR findings.

T-cell lines and clones: accurate detection and in-depth characterization of TCR cross-reactivity at clonal level

To confirm allo-HLA cross-reactivity of CMV- and EBV-specific T cells, we generated CD8$^+$ T-cell lines and clones of the following viral specificities: CMV B7/TPR, EBV B7/RPP, and EBV B35/YPL. Homogeneity of the lines and clones was confirmed by TCR usage (Table S3, SDC, http://links.lww.com/TXD/A15). The T-cell lines and clones were first stimulated with a panel of HLA-typed immortalized EBV-LCLs, whereupon IFNγ production was determined by ELISA (Figure 5A, 5B).

Significant amounts of IFNγ were produced by EBV B35/YPL T-cell line 2A6 upon recognition of HLA-DRB1*03:01+ EBV-LCLs and by CMV B7/TPR T-cell line 5A1 upon recognition of HLA-A29+ EBV-LCLs. The EBV B7/RPP T-cell clones 9G6 and 10D8 produced moderate levels of IFNγ upon recognition of HLA-B40+ EBV-LCLs. T-cell lines and clones with similar viral specificity but different TCR usage did not produce IFNγ in response to the same allo-HLA molecules, demonstrating that cross-reactivity is mediated by a subpopulation of virus-specific T cells with defined TCR usage. In contrast to EBV-LCLs, SALs did not induce significant IFNγ production, suggesting that the recognized endogenous peptide might not be expressed by these cells (Figure 5C). This highlights the importance of testing cross-reactivity with different cell types.

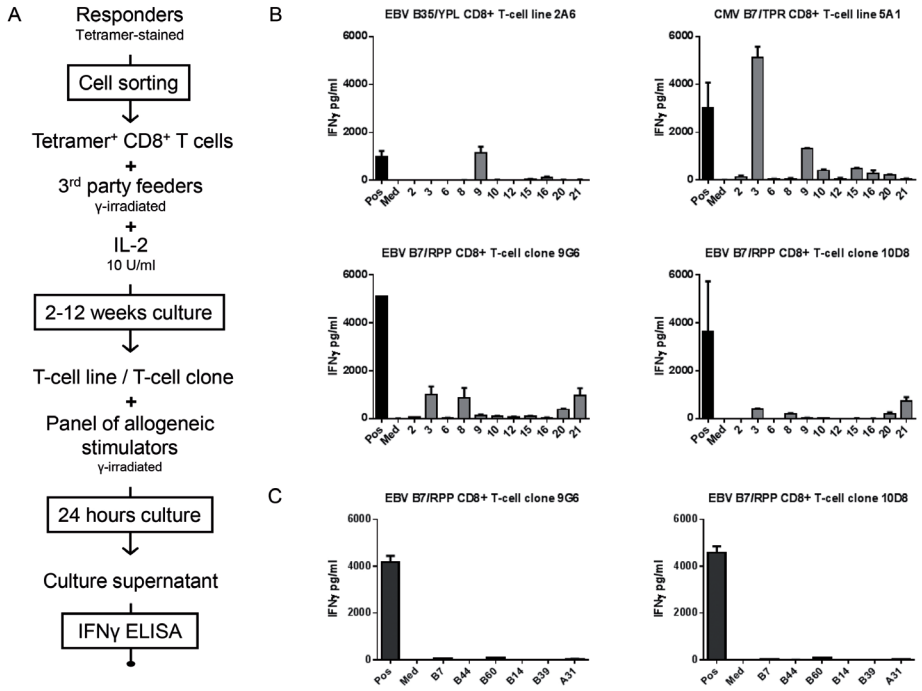


Figure 5. Generation of virus-specific T-cell lines and clones followed by analysis of cross-reactivity based on IFNγ secretion. A) Flow chart of the procedure to generate CD8+ T-cell clones and subsequent measurement of IFNγ production upon stimulation with allogeneic cells by ELISA. B) IFNγ production by CD8+ T-cell lines 2A6 (EBV B35/YPL; R2) and 5A1 (CMV B7/TPR; R1) and CD8+ T-cell clones 9G6 and 10D8 (both EBV B7/RPP; R1) upon stimulation with HLA-typed EBV-LCLs. EBV B35/YPL T-cell line 2A6 responded to EBV-LCL 9 (HLA-DR3), CMV B7/TPR T-cell line 5A1 to EBV-LCL 3 and 9 (both HLA-A*29:02), and EBV B7/RPP T-cell clones 9G6 and 10D8 responded to EBV-LCL 3 (HLA-B60), 8 (HLA-B*40:01), 21 (HLA-B61). Positive control: EBV-LCL sharing the autologous HLA antigen (B35/B7) loaded with viral peptide. C) IFNγ production of EBV B7/RPP T-cell clones 9G6 and 10D8 upon stimulation with SALs expressing depicted HLA molecules. All bars represent the mean of duplicates.

Responder HLA typing:

R1: A*02; A*03; B*07; B*35; C*04; C*07; DRB1*01; DRB1*08; DQB1*04:02; DQB1*05:01

R2: A*02; A*03; B*07; B*35

Stimulator HLA typing:

EBV-LCL 2: A*24:02; A*33:01:01; B*14:02:01; C*02:02/02:32; C*08:02/08:29; DR1; DQ*05:01; DP1; DP4

EBV-LCL 3: A*03:01/03:22; **A*29:02/29:09**; B*07:02/07:61/07:114; B*44:03/44:105; C*07:02:01; C*16:01:01; DR2

EBV-LCL 6: A*01:01:01:01; A*26:01:01; B*08:01:01; B*49:01:01; C*07:01:01; DR01; **DR*03:04**; DQ*03:04; DQ*05:04

EBV-LCL 8: A*02:03:01; A*24:02; B*38:02:01; **B*40:01:02**; C*03:04:01; C*07:02:01; DR2; DR5

EBV-LCL 9: **A*29:02:01**; A*31:01:02; B*18:01/18:17N; B*58:01:01; C*05:01; C*07:18/07:01; **DR3**; DR8; DQ2

EBV-LCL 10: A*24:03:01; B*51:01:01; C*15:02:01; DR*11:04; DQ*03:01; DP*04:02

EBV-LCL 12: A*24:02:01; A*30:01:01; B*51:01:01; B*58:01:01; C*01:02:01; C*03:02:02; DR1; DR7; DQ1; DQ2

EBV-LCL 15: A*24:02; A*31:01:02; B*39:01; B*55:01:01; C*03:03:01; C*12:03:01; DR13; DQ1; DP2; DP4

EBV-LCL 16: A*30:01:01; A*68:02:01; B*42:01:01; C*17:01:01; **DR*03:02**; DQ*04:02; DP*01:01; DP*04:02

EBV-LCL 20: A31; A*24:02; B7; Cw4; Cw7; DR12; DR15

EBV-LCL 21: A*02:10; A30; B13; **B61**; Cw6; DR7; DR9

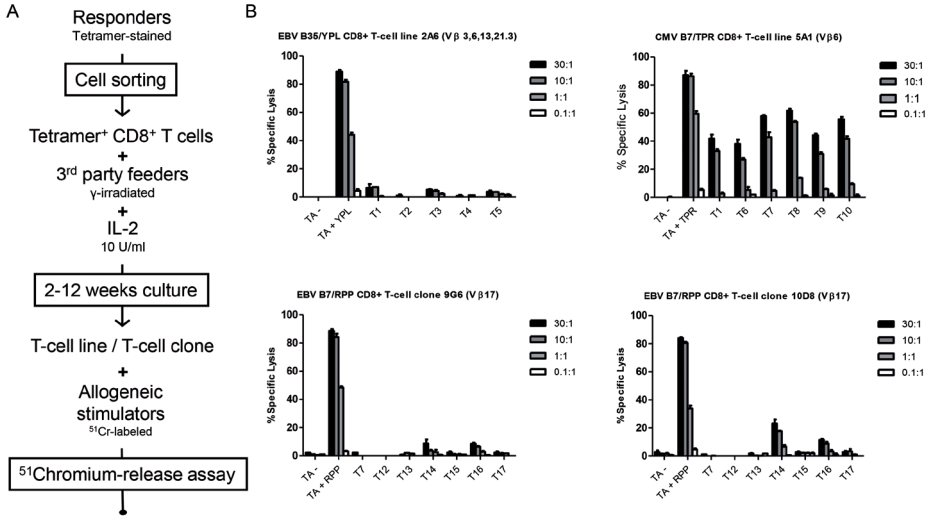


Figure 6. Cytotoxic potential of cross-reactive T-cell lines and clones. A) Flow chart of the generation of CD8+ T-cell clones and subsequent measurement of cytotoxicity toward allogeneic cells in a 51Chromium-release assay. B) Percentage specific lysis of 51Chromium-labeled target cells by CD8+ T-cell lines 2A6 (EBV B35/YPL; R2) and 5A1 (CMV B7/TPR; R1) and CD8+ T-cell clones 9G6 and 10D8 (both EBV B7/RPP; R1). Negative control: autologous PHA blasts without peptide (TA-). Positive control: autologous PHA blasts loaded with viral peptide (TA + YPL, TA + TPR, TA + RPP). All bars represent triplicate wells with standard deviation.

Responder HLA typing:
R1: A*02; A*03; B*07; B*35; C*04; C*07; DRB1*01; DRB1*08; DQB1*04:02; DQB1*05:01
R2: A*02; A*03; B*07; B*35
Stimulator HLA typing:
T1: EBV-LCL 9: **A*29:02:01**; A*31:01:02; B*18:01/18:17N; B*58:01:01; C*05:01; C*07:18/07:01; **DR3**; DR8; DQ2
T2: PHA-blast S1.2 Figure 2B: A*02:01; A*32:01; B*35; Cw*04:01; **DRB1*03:01**; DRB1*11; DQB1*02; DQB1*03:01
T3: PHA blast SB.2 Figure 2B: A1; A2; B8; B44(12); Cw5; Cw7; DRB1*03:01; **DRB3*01:01**; DQB1*02; DQA1*05:01/05:03; DPB1*01:01; DPB1*04:02
T4: PHA blast SC.2 Figure 2B: A*01:01; A*03:01; B*08:01; **B*35:01**; C*04:01; C*07:01; **DRB1*03:01**; DRB1*11:01
T5: PHA blast SE.2 Figure 2B: A*30:02; B*18:01; C*05:01; **DRB1*03:01**; DRB1*07; DQB1*03
T6: EBV-LCL 3: A*03:01/03:22; **A*29:02/29:09**; B*07:02/07:61/07:114; B*44:03/44:105; C*07:02:01; C*16:01:01; DR2
T7: PHA blast S2 Figure 2B: A24(9); **A29(19)**; B7; **B60(40)**; Cw7; DR13(6); DR8; DQ6(1); DQ4
T8: PHA blast S13 Figure 2B: A*24:02; **A*2901**; B*39:06; B*44:03; Cw*07:02; Cw*16:01; DRB1*07; DRB1*08:01; DQB1*02:02; DQB1*04:02
T9: PHA blast SE: A2; **A29(19)**; B57(17); B55(22); Cw3; Cw6; DR14(6); DR7; DQ5(1); DQ9(3)
T10: PHA blast SF Figure 2B: **A*29:02**; A*69:01; B*45:01; B*15:17; C*06:02; C*07:01; DRB1*15:01; DRB1*11:01; DQB1*06:02; DQB1*03:01
T12: EBV-LCL 8: A*02:03:01; A*24:02; B*38:02:01; **B*40:01:02**; C*03:04:01; C*07:02:01; DR2; DR5
T13: PHA blast SG: A*01:01; A*02:01; B*08:01; **B*40:01**; C*03:04; C*07:01; DRB1*03:01; DRB1*13:02; DQB1*06:04; DQB1*02:01
T14: EBV-LCL 21: A*02:10; A30; B13; **B61**; Cw6; DR7; DR9
T15: PHA blast S16 Figure 2B: A*03:01; A*31:01; B*15:01; **B*40:02**; Cw*02:02; Cw*03:03; DRB1*04:01; DRB1*13:01; DQB1*03:02; DQB1*06:03
T16: PHA blast SI Figure 2B: A1; B8; **B61(40)**; Cw7; DR3; DR13(6); DQ1; DQ2
T17: PHA blast SJ Figure 2B: A2; B35; **B61(40)**; Cw4; DR1; DR4; DQ5(1); DQ7(3)

Furthermore, the cytotoxic capacity of the T-cell lines and clones was determined in a ^{51}Chromium-release assay, which is the golden standard for measuring cytotoxicity of cross-reactive T cells (126). CMV B7/TPR T-cell line 5A1 efficiently lysed HLA-A*29:01^{+} EBV-LCLs, whereas EBV B35/YPL T-cell line 2A6 and EBV B7/RPP T-cell clones 9G6 and 10D8 were not cytotoxic toward the EBV-LCLs that induced IFNγ production (Figure 6). This is in concordance with previous data, demonstrating a similar discrepancy of cross-reactive CD8^{+} T cells that produce IFNγ, but lack cytotoxic capacity in response to alloantigen (127).

DISCUSSION

This article summarizes the advantages, limitations and applications of commonly used experimental methods for the detection of virus-specific $CD8^+$ T cells with cross-reactivity to allogeneic HLA antigen (see overview in Table S4, SDC, http://links.lww.com/TXD/A15).

We conclude that MLR can be a useful tool to screen for cross-reactivity of virus-specific T cells against alloantigen at bulk level. By using determined responder-stimulator combinations, donor-specific cross-reactivity can be identified and characterized. Furthermore, precursor frequencies of cross-reactive T cells can be calculated and unknown allo-HLA cross-reactivity can be identified by using a broad array of HLA-typed targets. Accordingly, we were able to identify allo-HLA specificity in conventional MLR: CMV B7/TPR $CD8^+$ T cells proliferated in response to HLA-$A29^+$, EBV B7/RPP T cells to HLA-$B40^+$ and EBV B35/YPL $CD8^+$ T cells proliferated in response to HLA-DRB1*03:01 stimulators. Interestingly, the latter alloresponse was mediated by $CD8^+$ T cells cross-reacting toward HLA class II alloantigen. Recognition of an HLA-DRB1*03:01-derived peptide within an HLA class I molecule was unlikely as not all stimulators shared HLA class I molecules. Although cross-reactivity of virus-specific $CD8^+$ T cells toward HLA class II molecules is rare, it has been reported previously for CMV-reactive T cells (43, 128). Therefore, when evaluating MLR results, one ought to keep in mind that TCR cross-reactivity is not restricted by the rules of cognate pMHC recognition.

Although MLR provides a suitable tool for cross-reactivity screening, it has limitations. First, a vast amount of both responder and stimulator cells is needed. Second, due to the usage of bulk PBMC cultures, high-affinity alloresponses may dominantly overgrow low-affinity alloresponses, leading to an underestimation of the latter. Third, cross-reactive cells with a low precursor frequency may not, or only incidentally, be detectable at bulk level depending on the number of analyzed responder cells. Fourth, the composition of cell types within the PBMC compartment may differ between stimulators, which could lead to further overestimation or underestimation of alloantigen recognition. MLRs are thus insufficient in detecting the full spectrum of cross-reactivity, resulting in only a moderate sensitivity of the assay.

In addition, one should keep in mind that allo-HLA cross-reactivity is directed against the combination of allogeneic HLA and endogenous peptide (129). Because cells derived from different individuals may differ in HLA expression levels and/or the ability to present cross-reactive peptides, the strength of an alloresponse could vary between individuals (Figure 2A). Moreover, tissue-specific expression of endogenous peptides could influence alloreactivity

(130, 131), and thereby affect transplantation outcome of different organs. Indeed, tissue-specific cross-reactivity of virus-specific T cells has been described: cross-reactive EBV-induced $CD8^+$ T cells showed decreased cytotoxic capacity toward epithelial and endothelial target cells compared to PBMCs due to poor presentation of the cross-reactive peptide in these cell types (87). Because MLR uses PBMCs as targets, potential recognition of tissue-specific peptides remains unnoticed. The latter could be overcome by modifying the protocol using different stimulator cell types such as epithelial and endothelial cells.

Finally, one of the major issues affecting the accuracy of MLR is bystander activation. In our experience, a vigorous proliferative response against allogeneic stimulator cells by virus-specific T cells in MLR gives a fair indication for cross-reactivity. On the contrary, less pronounced responses are more difficult to interpret. These responses could represent truly cross-reactive responses with low TCR affinity and/or low precursor frequencies, but they may also be the result of cytokine-mediated bystander activation. Hereto, a resting period and short restimulation phase was introduced in the conventional MLR protocol. We have shown that this experimental approach accurately identifies truly cross-reactive T cells based on proliferative capacity, cytokine production, degranulation and activation state upon encounter of alloantigen or viral peptide.

Cross-reactive T cells may respond differently to restimulation with alloantigen, depending on the TCR affinity and the levels of allo-HLA and peptide presented on the stimulator cells. The extent of the response may be less reproducible when studying cross-reactive responses with a very low precursor frequency. The number of cross-reactive T cells at the start of each MLR could differ by chance, and this difference is enlarged during the 9-day culture period. This protocol should therefore not be used for determining the strength of an alloresponse, but rather as a quick tool to discriminate cross-reactivity from bystander activation.

Compared to bulk level protocols, T-cell lines and clones can provide more detailed characterization and do not suffer from bystander activation and dominant overgrowth of TCR cross-reactivity. T-cell lines and clones can be screened against large panels of different cell types that express a wide array of HLA antigens (87, 117, 121, 132), and because they constitute a homogeneous population, the cross-reactive HLA antigen and peptide can be identified (44). Also, the mechanism underlying TCR cross-reactivity can be investigated. Molecular mimicry and alternate TCR docking modes have been identified as mechanisms for TCR cross-reactivity (44, 133-135). Moreover, T-cell lines and clones can be used to determine cytotoxicity of cross-reactive T cells, which is important because differences in effector function can influence the

impact on transplantation outcome. Our data show that cross-reactive T cells producing IFNγ upon alloantigen encounter are not always cytotoxic toward the same targets and highlight the importance of performing multiple functional assays for proper characterization of the cross-reactive response.

Despite these advantages, T-cell lines and clones have limitations as well. First, their generation is labor-intensive and time-consuming and they only represent part of the total T-cell repertoire generated against a specific viral epitope. Moreover, the precursor frequency, which has prognostic value for the impact of alloreactive T-cell responses on transplantation outcome (136), cannot be determined.

A major limitation of all techniques is the availability of viral peptide-HLA tetramer complexes. Although available tetramers are believed to cover the most dominant epitopes in individuals with the corresponding HLA type, it is not possible to address the total impact of all virus-specific $CD8^+$ T cells. Unfortunately, it is thereby impossible to uncover all cross-reactivities of virus-specific T cells. Moreover, limited availability of HLA class II tetramers impairs the analysis of the cross-reactive potential of virus-specific $CD4^+$ T cells, leaving their role in heterologous immune responses underexposed. Finally, current methods studying cross-reactive responses are labor-intensive and costly, which might hamper large scale screening of transplant recipients required to address the impact of cross-reactive T-cell responses on transplant function and outcome.

In conclusion, the cross-reactive potential of virus-specific T cells against allogeneic HLA antigen can be studied by using the techniques discussed, provided that one should be aware of their limitations. Depending on the research question and the availability of cells and resources, one can apply bulk MLR cultures for fast broad-spectrum screening, or T-cell lines and clones for in-depth characterization of heterologous immune responses.

ACKNOWLEDGMENTS

Financial support by the Dutch Kidney Foundation (Project CP09.04) is gratefully acknowledged. Furthermore, we would like to thank Prof. R.A.W. van Lier (Sanquin, Amsterdam, The Netherlands) for valuable advices and constructive criticisms.

SUPPLEMENTARY MATERIAL

Table S1. HLA class I antigens represented in the HLA-typed stimulator panel composed of 16 different stimulators.

HLA-A	HLA-B	HLA-C
A1	B7	Cw1
A2	B8	Cw2
A3	B13	Cw3
A11	B14	Cw4
A24	B18	Cw5
A25	B35	Cw6
A26	B38	Cw7
A29	B37	Cw8
A30	B39	Cw12
A31	B41	Cw14
A32	B44	Cw15
A33	B51	Cw16
A*68:01	B55	Cw17
A*68:02	B57	
A66	B58	
	B60	
	B61	
	B62	

Table S2. Virus-specific $CD8^+$ T cells in two healthy individuals show proliferation upon encounter with one or more allogeneic stimulators[1,2]

	CMV	EBV	FLU
R1	B7/TPR B35/IPS	B7/RPP	A2/GIL
R2		A2/GLC B35/EPL B35/YPL	A2/GIL

[1] Two healthy individuals (R1 and R2) selected on the presence of $CD8^+$ T cells directed against CMV, EBV and/or FLU were tested in mixed lymphocyte reactions against a panel of HLA-typed stimulators and analysed by flow cytometry.

[2] The viral epitopes that showed cross-reactivity to 1 or more allogeneic stimulators, defined as cells that bound indicated viral tetramers and proliferated in the MLR, are depicted.

Table S3. TCR usage of the generated CD8+ T-cell lines and clones[1]

	Name	TRAV	TRAJ	CDR3α	TRBV	TRBJ	CDR3β
B35/YPL	1C1	TRAV21*01/02	TRAJ13*01/02	C A V L F W G Y Q K V T L	TRBV7-8*01	TRBJ2-1*01	C A S S L A G G G G N E Q F F
	2A6	TRAV8-2	TRAJ23	C V V S D G W D R G G K L I F	TRBV7-8*01	TRBJ2-1*01	C A S S L A G G G G N E Q F F
		TRAV12-1	TRAJ7	C V V N P Y Y G N N R L A F	TRBV6-1*01	TRBJ2-7*01	C A S S L V L A G E E T Q Y F
					TRBV11-2*01	TRBJ2-5*01	C A S S L V L A G E E T Q Y F
	3A6	TRAV8-4	TRAJ42	C A V S D Y G G S Q G N L I F	TRBV7-8*01	TRBJ2-1*01	C A S S L A G G G G N E Q F F
		TRAV21*01/02	TRAJ13*01	C A V L F W G Y Q K V T F	TRBV6-2*01/3*01	TRBJ2-1*01	C A S S S R T S G V P L G D E Q F F
B7/TPR	**5A1**	TRAV2*01/02	TRAJ12*01	C A V E P R M D S S Y K L S S	TRBV7-9	TRBJ2-7*01	C A S S L P D G A N Y E Q Y F
	5A3	TRAV2*01	TRAJ12*01	C A V E P R M D S S Y K L I F	TRBV7-9	TRBJ2-7*01	C A S S L R D G A N Y E Q Y F
	5A4	TRAV2*01	TRAJ12*01	C A V E P R M D S S Y K F S S	TRBV7-9	TRBJ2-7*01	C A S S L R D G A N Y E Q Y F
	5B2	TRAV17*01	TRAJ12*01	C A T E R R M D S S Y K L I F	TRBV7-9	TRBJ2-7*01	C A S S S R D V G A Y E Q Y F
	5B8	TRAV14/DV4*02	TRAJ12*01	C A M R E G G D S S Y K L I F	TRBV7-9	TRBJ2-7*01	C A S S S H D S T G F N S P L H F
	5D3	TRAV2*01	TRAJ12*01	C A V E P R M D S S Y K L I F	TRBV7-9	TRBJ2-7*01	C A S S L R D G A N Y E Q Y F
B7/RPP	9A12	TRAV38-2DV8	TRAJ54	C A Y T A G A Q K L V F	TRBV4-1*01	TRBJ2-7*01	C A S S Q E A Y N Y E Q Y F
		TRAV24*01	TRAJ24*01	C A F F T T D S W G K F Q L	TRBV25-1*01	TRBJ2-1*01	C A S S D M A G V G N E Q F F
					TRBV6-6*01/03	TRBJ2-5*01	C A S S L W V G Q Q E T Q Y F
					TRBV2*01/02	TRBJ1-3*01	C A S S D H S G G D G N T I Y F
	9G6	TRAV14/DV14*01	TRAJ9*01	C A M R D D T G G F K T I F	TRBV19*01/02/03	TRBJ2-7*01	C A S S I S S G V A Y E Q Y F
	10D8	TRAV14/DV14*01	TRAJ9*01	C A M R D D T G G F K T I F	TRBV19*01/02/03	TRBJ2-7*01	C A S S I S S G V A Y E Q Y F

[1]Cross-reactive T-cell lines and clones are indicated in bold

Table S4. Overview of strengths and limitations of experimental protocols to detect cross-reactivity

Detection method	Advantages	Disadvantages	Applications
Mixed Lymphocyte Reaction (bulk PBMC level)	+ Information on precursor frequency and clonality + Functional characterization + Fast results + Simultaneous analysis of multiple viral epitopes + Analysis of multiple allogeneic stimulators	- Vast amount of cells needed - Potential selective outgrowth of high-affinity responses - Moderate sensitivity - No cytotoxicity assay possible - Variation in target cells: composition of PBMC compartment and HLA expression - Bystander activation bias	> Screening for presence of cross-reactivity to a specific donor > Screening against HLA-typed targets > Analysis of frequency of cross-reactivity > Analysis of multiple viral epitopes > Analysis of clonality of cross-reactive responses > Identification of public cross-reactivities
Mixed Lymphocyte Reaction followed by restimulation (bulk PBMC level)	+ Information on precursor frequency and clonality + Functional characterization + Fast results + Simultaneous analysis of multiple viral epitopes + Analysis of multiple allogeneic stimulators + Discrimination of bystander activation versus cross-reactive responses	- Vast amount of cells needed - Potential selective outgrowth of high-affinity responses - Moderate sensitivity - No cytotoxicity assay possible - Variation in target cells: composition of PBMC compartment and HLA expression	> More accurate screening for true cross-reactive responses compared to MLR > Functional characterization of cross-reactivity > Screening against HLA-typed targets > Analysis of frequency of cross-reactivity > Analysis of multiple viral epitopes > Analysis of clonality of cross-reactive responses > Identification of public cross-reactivities

Table S4. Continued

Detection method	Advantages	Disadvantages	Applications
T-cell lines	+ Cytokine production by ELISA + Cytotoxicity assays + Screening against target cells of various origins + Minimal bystander activation	- Labor-intensive - Time-consuming - No information about precursor frequency - Selection bias - Potential selective outgrowth - Restricted to a single viral epitope	> Screening against HLA-typed targets > Analysis of cross-reactivity within a single viral epitope > Characterization of the effector function of cross-reactive T cells
T-cell clones	+ (Allo-HLA) specificity of a single TCR can be determined + Cytokine production by ELISA + Cytotoxicity assays + Screening against target cells of various origins + No bystander activation + Functional read-out specific for single clone + Inclusion of low affinity, low proliferating cells	- Labor-intensive - Time-consuming - No information about precursor frequency - Selection bias - Restricted to a single viral clone	> Screening against HLA-typed targets > Characterization of the effector function of cross-reactive T cells > Identification of the recognized allo-antigen > Affinity/avidity analyses