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Recognition of Distinct Cross-Reactive Virus-Specific CD8$^+$ T Cells Reveals a Unique TCR Signature in a Clinical Setting

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Human CMV still remains problematic in immunocompromised patients, particularly after solid organ transplantation. CMV primary disease and reactivation greatly increase the risks associated with incidences of chronic allograft rejection and decreased survival in transplant recipients. But whether this is due to direct viral effects, indirect viral effects including cross-reactive antiviral T cell immunopathology, or a combination of both remains undetermined. In this article, we report the novel TCR signature of cross-reactive HLA-A*02:01 (A2) CMV (NLVPMVATV [NLV])–specific CD8$^+$ T cells recognizing a specific array of HLA-B27 alleles using technical advancements that combine both IFN-γ secretion and multiplex nested RT-PCR for determining paired CDR3α/β sequences from a single cell. This study represents the first evidence, to our knowledge, of the same A2-restricted cross-reactive NLV-specific TCR-α/β signature (TRAV3TRAJ31_TRBV12-4TRBJ1-1) in two genetically distinct individuals. Longitudinal posttransplant monitoring of a lung transplant recipient (A2, CMV seropositive) who received a HLA-B27 bilateral lung allograft showed a dynamic expansion of the cross-reactive NLV-specific TCR repertoire before CMV reactivation. After resolution of the active viral infection, the frequency of cross-reactive NLV-specific CD8$^+$ T cells reduced to previremia levels, thereby demonstrating immune modulation of the T cell repertoire due to antigenic pressure. The dynamic changes in TCR repertoire, at a time when CMV reactivation was subclinical, illustrates that prospective monitoring in susceptible patients can reveal nuances in immune profiles that may be clinically relevant. ***The Journal of Immunology*****, 2014, 192: 000–000.**

Human CMV remains an ever-present risk for immunocompromised patients, particularly in solid organ transplant recipients, because of its noneradicable nature and the constant dependence on vigilant immune control. Over the past two decades, prophylactic antiviral strategies in the early months after lung transplantation have greatly reduced the clinical impact of CMV reactivation and disease (1). However, even in the current era, we and others have reported that significant rates of subclinical CMV reactivation can still occur in the lung allograft, and that these are associated with both acute episodes of CMV pneumonitis in the short term and chronic rejection associated bronchiolitis obliterans syndrome and decreased patient survival in the longer term (2, 3). Poorer clinical outcomes in solid organ transplantation have long been associated with ubiquitous DNA viruses such as CMV and EBV, but it was previously thought that this was largely a result of a direct viral effect and/or the amplification of underlying alloreactivity (3–7). However, a more contemporary theory links these two possibilities together by focusing on circulating antiviral memory T cell pools toward latent DNA viruses (8–11) and the cross-reactive capacity of a subset of these memory pools to recognize unrelated HLA molecules (12–19). In this setting, cross-reactive antiviral T cells have the potential to significantly drive immunopathological destruction of the allograft.

Whether cross-reactive antiviral T cells contribute to adverse posttransplant events and reduced survival rates still remains undetermined. Earlier reports examining the contribution of cross-reactive antiviral T cells toward allograft rejection in lung transplant recipients (LTRs) from our group (20) and graft-versus-host disease in hematopoietic stem cell transplant recipients (21) demonstrated that cross-reactive antiviral T cells represented a significant proportion of the alloreactive T cell pool. However, in our previous EBV study, we revealed that baseline frequencies of the cross-reactive T cell pool remained unaltered in the absence of EBV reactivation (thereby potentially negating their ability to become activated, expand, and exert clinically significant damage toward the allograft) (20).

Despite using antiviral prophylaxis for the first 5 mo post-transplantation in all our "at-risk" LTRs, primary disease or reactivation occurs in up to 50% of LTRs who are CMV seropositive (irrespective of donor sero-status), and this rate is even

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Abbreviations used in this article: A2, HLA-A*02:01; B8, HLA-B*08:01; BAL, bronchoalveolar lavage fluid; C1R, class I–reduced; FLR, FLRGRAYGL epitope; HC, healthy control; ICS, intracellular cytokine staining; LTR, lung transplant recipient; MFI, mean fluorescence intensity; NLV, HLA-A2–restricted NLVPMVATV epitope.

higher in the primary CMV mismatch scenario (CMV-seronegative recipient and CMV-seropositive donor) (3). Considering these relatively high rates of CMV reaction in LTR, our present model enabled a greater opportunity to examine the dynamics of cross-reactive antiviral T cells after episodes of viremia requiring medical intervention. We recently reported on the existence of CMV-specific CD8$^+$ T cells dually recognizing the HLA-A*02:01 (A2)–restricted CMV-pp65$_{495–503}$ epitope NLVPMVATV (NLV) and a specific cluster of HLA-B27 alleles in two unrelated individuals, a healthy control (HC5) and LTR5, who received a HLA-B27 bilateral lung allograft (19). Importantly, we demonstrated a significant increase in both the activation and the expansion of these cross-reactive NLV-specific CD8$^+$ T cells in a relevant clinical setting both before (no clinical detection) and during CMV reactivation (clinically relevant requiring pre-emptive treatment), which contracted back to basal levels after resolution of the active infection.

This study identifies the unique TCR signature of NLV-specific CD8$^+$ T cells that cross-react with a subgroup of HLA-B27 alleles (27:05, 27:07, 27:09) in two unrelated individuals. We show that there are significant and dynamic alterations in the TCR profile of an LTR after a clinically significant CMV reactivation episode, propelling the immunodominance of the cross-reactive TCR. Despite the heightened presence of these cross-reactive NLV-specific CD8$^+$ T cells, no associated immunopathology was observed in LTR5. This observation was in keeping with this patient having received a B*27:04 donor lung allograft, which would not be recognized by the cross-reactive T cells.

Materials and Methods

Subjects and PBMC samples

Experimental work was conducted according to Declaration of Helsinki principles. All subjects provided written informed consent with ethics approvals granted by both The Alfred Hospital and Monash University. A2-positive/CMV-seropositive peripheral blood samples from HC5, and LTR3, LTR5, and LTR8 samples were collected in heparinized Vacutainer tubes. The patient demographics of LTR3, LTR5, and LTR8 have been detailed elsewhere (19). PBMCs were isolated by Ficoll-Paque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation and cryopreserved at −180˚C until required.

HLA class I/peptide tetramers and peptide pulsing

A2/NLV tetramer was generated as previously described (22) and HLA-B*08:01/FLRGRAYGL (B8/FLR) tetramer was kindly provided by Dr. Jie Lin (The University of Melbourne). The NLV peptide was synthesized by GenScript (Piscataway, NJ). For peptide pulsing, PBMCs or cell lines were incubated with NLV peptide (10^{-6} M) for 1 h at 37˚C with gentle vortexing every 15 min then washed twice before use.

Generation of T cell lines

Bulk T cell lines were generated by stimulating PBMCs with NLV-pulsed autologous PBMCs (irradiated at 3000 rad) for up to 13 d (37˚C, 5% CO_2) at a 2:1 ratio in complete RF10 media plus 20 U/ml rIL-2 as described previously (23). Cells were then cryopreserved at −180˚C until required.

Generation of HLA-B27–expressing cell lines

Class I–reduced (C1R) cell lines C1R.B*27:03, C1R.B*27:05, and C1R.B*27:09 were produced as described previously (19) after retrovirus transduction methodologies (24). In brief, cDNA of HLA-B*27:03/05/09 alleles were cloned into pGEM-T Easy Vector (Promega, Madison, WI) before insertion into a murine stem cell virus-based plasmid with an internal ribosome entry site plus sequence encoding enhanced GFP as a reporter, referred to as "pMIG" (kindly provided by Prof. Dario Vignali, St. Jude Children's Research Hospital, Memphis, TN). Similarly for HLA-B*27:02 cDNA, two rounds of site-directed mutagenesis were performed on pGEM.B*27:05 plasmid using sense and antisense primers: first round, 5′-GACCGAGAGAACCTGCGGACCCTG-3′; second round, 5′-AACCTGCGGATCGCGCTCCGCTAC-3′. For HLA-B*27:04 and HLA-B*27:07, cDNA sequences obtained from the ImMunoGeneTics/Human Leukocyte Antigen database (accession numbers HLA00223 and HLA00228, respectively) were synthesized by GenScript via a pUC57 plasmid. Each HLA-B*27:02/04/07 gene was then subcloned into pMIG vectors. Cell-surface expression of HLA class I molecules was achieved by pMIG transfection into 293T packaging cells for subsequent transduction into C1R parental cell lines, as described previously (24, 25). Cell lines expressing high levels of GFP and Bw4 (epitope located on HLA α_1-chain at position 77–83) were bulk sorted, grown, and reanalyzed (>90% GFP$^+$ Bw4$^+$) before use in functional assays. Cell lines were maintained in complete RF10 media (26).

Generation of T cell lines expressing cross-reactive TCR

Full-length human TCRα and TCRβ cDNA were cloned into a self-cleaving 2A peptide-based pMIG vector essentially as described previously (25). LTR5's cross-reactive HLA-A2/NLV–specific CD8$^+$ TCR (referred to as OTN5) composed of TRAV3 and TRAJ31 genes with CDR3α sequence and flanking residues: CAVRNNNARLMFGDGTQLVV; and TRBV12-4 and TRBJ1-1 genes with CDR3β sequence and flanking residues: CASSIV NEAFFGQGTRLTVVED, referred to as pMIG.OTN5. As a nonspecific control, pMIG.LC13 vector (kindly provided by Dr. Zhenjun Chen, The University of Melbourne) containing LC13's full-length TCRαβ constructs (27) was included, in which the TCR dually recognizes HLA-B8/FLR and HLA-B*44:02/EEYLQAFTY. Both pMIG.OTN5 and pMIG.LC13 were then transfected into non–GFP-tagged SKW3.hCD8αβ cells (kindly provided by Dr. Zhenjun Chen) using 293T packaging cells. For enhanced TCR cell-surface expression, pMIG.CD3 (kindly provided by Dr. Richard Berry, Monash University) containing the P2A-linked human CD3 genes (ζγδε) was also added for each retrovirus transfection to generate TCR-expressing cell lines SKW3.hCD8αβ.CD3.OTN5 and SKW3.hCD8αβ.CD3.LC13, respectively. The original SKW3 parental cell line (kindly provided by Dr. Klaus Steube, Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) was negative for endogenous TCRαβ but contained CD3 and its signaling components. SKW3.hCD8αβ cells were derived from transfecting SKW3 parental cells with pMIG.humanCD8αβ (kindly provided by Dr. Zhenjun Chen). All cell lines were maintained in complete RF10 media (26).

Paired TCRαβ analysis of single cross-reactive NLV-specific CD8$^+$ T cell

To single-cell sort functionally viable cross-reactive NLV-specific CD8$^+$ T cells from a mix of polyclonal NLV-specific CD8$^+$ T cells, the IFN-γ Secretion Assay–Detection Kit (APC; Miltenyi Biotec, Auburn, CA) was used after incubation with or without HLA-B27 target cells and costaining with anti-CD8 and A2/NLV tetramer. Cryopreserved T cell lines were thawed and rested overnight in RPMI 1640 containing 10% HSA plus supplements, not FCS, to avoid nonspecific binding as per manufacturer's instructions. T cell lines (up to 10×10^6 cells) were incubated with either C1R.B*27:03, C1R.B*27:04, C1R.B*27:05, C1R.B*27:07, or C1R.B*27:09 cell lines (2:1 ratio) or remained unstimulated for 2 h (37˚C/5% CO_2) before detection with anti–IFN-γ Ab according to manufacturer's instructions. Single cells were sorted (FACSAria I; BD Biosciences, San Jose, CA) directly into chilled 96-well PCR plates (Eppendorf, Hamburg, Germany) based on the following: 1) CD8$^+$tetramer$^+$ T cells for the unstimulated conventional group, and 2) CD8$^+$tetramer$^+$IFN-γ$^+$ T cells for the stimulated groups. Sorted plates were immediately stored at −80˚C until required. Analysis of paired CDR3α and β regions was carried out by RT-PCR, multiplex nested PCR, and then sequencing of α and β products essentially as described previously (28). Both external and internal rounds of PCR included 40 TRAV and 27 TRBV forward primers, and a TRAC and TRBC reverse primer. All primers have been detailed elsewhere (28) and have been individually validated by our group (Supplemental Fig. 1) and previously by Wang and colleagues (28). Sequences were analyzed according to the ImMunoGeneTics/V-QUEry and STandardization web-based tool (29). All TCR nomenclature was according to Folch et al. (30). CDR3 amino acid sequences described within the text start from CDR3-position 3, which is equivalent to amino acid position 107 of the TRAV and TRBV segments, and end at TRAJ-position 10 or TRBJ-position 6.

Functional T cell assays

NLV-specific CD8$^+$ T cells were assessed for cross-reactivity via IFN-γ production using intracellular cytokine assay in combination with CD8 and A2/NLV-tetramer staining as essentially described previously (19). Responder cells (2×10^5 cells) were cultured with HLA-B27–expressing C1R cells for 6 h at a 2:1 ratio. Activation of SKW3.hCD8αβ.CD3.OTN5 cells was assessed using cell-surface CD69 upregulation after 16–20 h incubation (31) with HLA-B27–expressing C1R cells (5×10^4 responder

cells, 1:1 ratio). The CD69 expression profiles were measured as mean fluorescence intensity (MFI) to provide more meaningful evaluation of changes in the relative amounts of expressed protein per cell. These cells were then costained with A2/NLV-tetramer and mouse anti-human CD3 and CD8 mAbs. SKW3.hCD8αβ.CD3.LC13 cells and B8/FLR-tetramer staining were included as controls. For all experiments, stimulation with NLV-pulsed C1R.A*02:01 was included as a positive control, and non-pulsed C1R.A*02:01 cells or autologous T cells alone were included for negative controls. Flow cytometry data were all analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis

Samples of the TCR repertoire as shown in Fig. 4B on different days posttransplant were compared with the TCR repertoire pretransplant using Fisher's exact test, as implemented by fisher.test in R (32, 33).

Results

Hierarchical order of HLA-B27 cross-reactivity is analogous between HC5 and LTR5

Previously, we investigated A2-NLV/B27 cross-reactivity based on the commonly represented HLA-B27 alleles within our population (B*27:05, B*27:03, and HLA-B*27:09) (19, 34). HLA-B*27:09 showed a significant increase in functional cross-reactivity in both HC5 (up to 5-fold) and LTR5 (up to 10-fold) compared with HLA-B*27:05, with no cross-reactivity observed for HLA-B*27:03. In this study, we have extended our stimulator panel to include a broader set of C1R target cell lines expressing the HLA-B27 alleles: HLA-B*27:02, HLA-B*27:03, HLA-B*27:04, HLA-B*27:05, HLA-B*27:07, and HLA-B*27:09 (Table I). NLV-specific $CD8^+$ T cells from HC5 and LTR5 (30 mo posttransplant time interval) were autologously stimulated with NLV peptide and in vitro expanded for 13 d before performing a 6-h intracellular cytokine staining (ICS) assay. Cross-reactivity was determined by the percentage of A2/NLV-$tetramer^+$ $CD8^+$ T cells producing IFN-γ in response to a C1R cell line transfected with a specific HLA-B27 allele. Of the six HLA-B27 alleles, the hierarchy of immune reactivity was identical for both HC5 and LTR5: B*27:07 > B*27:09 > B*27:05, with no reactivity toward B*27:02, B*27:03, and B*27:04 (Fig. 1).

A novel strategy to identify the TCRαβ repertoire of cross-reactive T cells. Our next focus was then to determine the TCR repertoire usage of NLV-specific $CD8^+$ T cells and to identify the cross-reactive TCR. Identification of individual CDR3α and/or CDR3β gene segments has been previously limited to either bulk T cell cultures or extensive cloning methodologies (35–40). However, a recent report by Wang et al. (28) has outlined new technology for concurrent characterization of both human CDR3α and CDR3β repertoires in epitope-specific $CD8^+$ T cells by combining RT-PCR and multiplex nested PCR for simultaneous amplification of CDR3αβ transcripts from a single cell. We have modified this single-cell flow cytometric strategy, which had previously used common T cell phenotypic markers and MHC class I/peptide tetramers, by adding an IFN-γ secretion assay step to enable identification of functionally active, cross-reactive NLV-specific $CD8^+$ T cells responding toward the specific HLA-B27 molecules.

Day 13 NLV-specific $CD8^+$ T cell lines generated from HC5 and LTR5 (at various time points after lung transplantation) were thawed and rested overnight before immunophenotyping with CD8 and A2/NLV-tetramer. T cell cross-reactivity of the A2/NLV-$tetramer^+$ population was determined by an IFN-γ secretion assay after incubation with C1R.B27 target cells that elicited the strongest responses from Fig. 1 (C1R.B*27:07, C1R.B*27:09, and C1R.B*27:05). We also included C1R.B*27:04 target cells as representative of the donor lung allograft received by LTR5. TCR signatures of single-cell sorted, unstimulated, NLV-specific $CD8^+$ T cell repertoire (conventional; $CD8^+$A2/NLV-$tetramer^+$) were compared with the cross-reactive NLV-specific $CD8^+$ T cell repertoire (stimulated; $CD8^+$A2/NLV-$tetramer^+$IFN-γ^+) following the two-step strategy displayed in Fig. 2A and 2B.

A unique TCRαβ signature is revealed for cross-reactive NLV-specific $CD8^+$ T cells. Conventional HLA-A2–restricted, NLV-specific $CD8^+$ T cells display TCR repertoires that are highly oligoclonal between individuals, with each individual generally having a limited number of dominant and subdominant TCRs (41–43). Given its oligoclonal nature, many public TCRα and TCRβ motifs, as well as a few paired TCRαβ motifs, as defined by the common sharing of CDR3α and/or CDR3β amino acid sequences, have been described in a number of unrelated individuals (28, 35, 37–40). The most featured NLV-specific public TCR is RA14, which consists of TRAV24-TRAJ49 and TRBV6-5-TRBJ1-2 genes, and has recently been crystallized (44). Yet, no data exist for NLV-specific TCR repertoires that are cross-reactive to HLA alloantigens (12, 45). To explore this, we examined both the conventional (unstimulated) and the C1R.B27-stimulated (cross-reactive) NLV-specific TCR repertoires in HC5 (HLA-A2, -A11; HLA-B51, -B61) and LTR5 (HLA-A2, -A3; HLA-B7, -B18), who received a B27 bilateral lung allograft (LTR5 donor: HLA-A11, -A33; HLA-B*27:04, -B58), to determine whether similar or identical TCR signatures existed. For comparison, we also analyzed conventional NLV-specific TCR repertoires from LTR3 and LTR8, who have previously shown no functional cross-reactivity toward HLA-B27 by way of IFN-γ production (19).

The conventional NLV-specific TCR repertoire for HC5 was mainly dominated by a single TCR clonotype bearing TRAV35TRAJ42_TRBV12-4TRBJ1-2 (81% frequency) (Table II). Although the CDR3β sequence SIQALL was not a known public motif, clonotypes bearing TRBV12-4TRBJ1-2 have been frequently observed in other NLV-specific TCR studies solely looking at β-chain usage (39, 40, 42). Three less frequent TCRs with different TRVαβ and TRJαβ usage were also observed, one having public α and β motifs ($_n$TSYDKV and SSAYYGY, respectively; $_n$ = variable number of amino acids) and one having a public α motif ($_n$GNQF; Table II, underlined). However, after stimulation with C1R.B27 allelic target cells, there was a shift in immunodominance toward clonotypes that had

Table I. Amino acid residue polymorphisms within the HLA-B27 H chain (exons 2 and 3)

Residue	59	77	80	81	97	113	114	116	131	152	211
B*27:05[a]	Y	D	T	L	N	Y	H	D	S	V	A
B*27:02		N	I	A							
B*27:03	H										
B*27:04		S								E	G
B*27:07					S	H	N	Y	R		
B*27:09								H			

[a]HLA-B*27:05 is the most frequent allele (4% Caucasians, North America) (32), shown as the reference sequence.

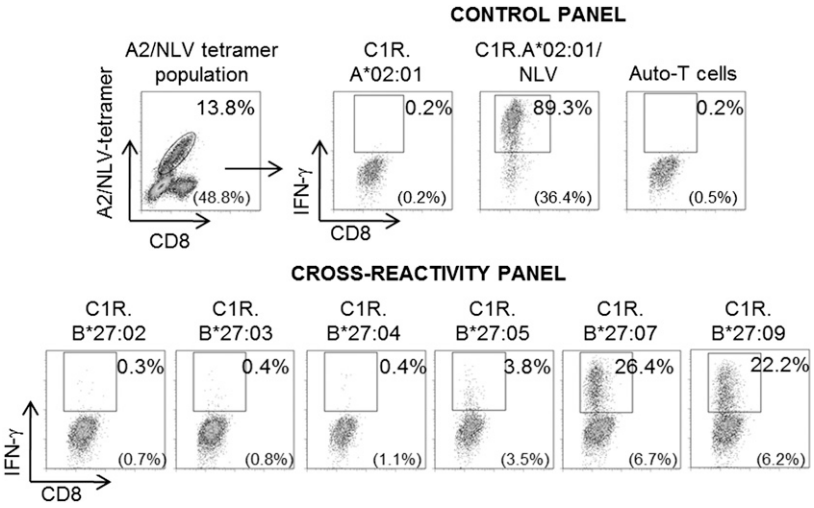


FIGURE 1. Cross-reactive NLV-specific CD8+ T cells display a hierarchical response toward HLA-B27 subtypes. NLV-specific CD8+ T cells from LTR5 (30 mo posttransplant) and HC5 were autologously expanded in the presence of NLV peptide for up to 13 d before performing a 6-h ICS assay using a panel of C1R transfectants expressing a single HLA-B27 allele. Cross-reactivity was measured by IFN-γ–producing CD8+tetramer+ cells after an initial forward scatter and side scatter lymphocyte gate. FACS plots are depicted for LTR5, whereas HC5 percentages are shown in parentheses.

a paired TRAV3TRAJ31 and TRBV12-4TRBJ1-1 gene usage. This pairing was initially not detected in the conventional pool, but was widely observed after C1R.B*27:07 (80% frequency) and C1R.B*27:09 (70% frequency) stimulations with identical CDR3β sequences (SSVNEA) and similar CDR3α regions (RXXNARL, "X" denotes any amino acid). However, stimulations with the less or non–cross-reactive cell lines (C1R.B*27:03, C1R.B*27:04, and C1R.B*27:05) revealed fewer TCR sequences closely resembling the cross-reactive signature TRAV3TRAJ31_TRBV12-4TRBJ1-1 (20, 7, and 0%, respectively). In these experiments, CD8+tetramer+ cells were sorted on very low to background levels of IFN-γ because of poorer functional responses observed with these subtypes. TRBV12-4 was the only common feature between the conventional and the cross-reactive TCR signatures (Table II).

Having identified a cross-reactive NLV-specific TCR signature in HC5, we applied our TCR repertoire analysis to LTR5 at various time intervals (pretransplant and 0.5, 3, 7, 30 mo posttransplant) against the cross-reactive stimulation panel: C1R.B*27:05, C1R.B*27:07, and C1R.B*27:09 cells, wherever possible (Table III). In the conventional NLV-specific CD8+ TCR populations, a strikingly similar TCR clonotype (CDR3α: RNNNARL; CDR3β: SIVNEA, referred to as OTN5 hereafter) bearing the same TRAV3TRAJ31_TRBV12-4TRBJ1-1 gene usage as that of HC5's was largely apparent at the pretransplant time interval (65%) and during the posttransplant period (70–100%). More importantly, the OTN5 TCR remained present after stimulation with C1R.B*27:07 (84–100%), C1R.B*27:09 (43–100%), and C1R.B*27:05 (59–85%) throughout the first 3 y posttransplant and represented the cross-reactive NLV-specific TCR signature (Table III). As a negative control, stimulation with the non–cross-reactive HLA-B*27:04 cell line at 30 mo posttransplant did not detect any paired TCR sequences resembling the OTN5 TCR signature. To our knowledge, this is the first evidence of the cross-reactive NLV-specific TCRαβ signature of TRAV3TRAJ31_TRBV12-4TRBJ1-1 in two genetically distinct individuals.

To validate that the cross-reactive NLV-specific TCR signature is only attributed to certain individuals who are capable of mounting a dual immune response toward HLA-A2/NLV epitope and HLA-B27 alloantigens, we screened LTR3 (HLA-A2; HLA-B27, -B44), who received an HLA-B27 bilateral lung allograft (LTR3 donor: HLA-A2, -A32; HLA-B13, -B27), and LTR8 (HLA-A1, -A2; HLA-B8, -B44), who received a non–HLA-B27 bilateral lung allograft (LTR8 donor: HLA-A2, -A30; HLA-B7, -B62). Previously, both LTR3 and LTR8 have not shown T cell cross-reactivity using this model (19). Not surprisingly, their NLV-specific TCRαβ repertoires did not possess the same cross-reactive NLV-specific TCR signature as identified in HC5 and LTR5. Instead, LTR3 and LTR8 possessed a relatively high proportion of previously described public CDR3α and/or CDR3β motifs, with 48–95% observed in LTR3 (pretransplant, 12 mo posttransplant) and 79–82% in LTR8 (3 and 7 mo posttransplant; Table IV). For example, the pairing of TRAV24TRAJ49 and TRBV6-5TRBJ1-2 genes was commonly observed in LTR3's NLV-specific TCR repertoire (38–69%), which has been frequently reported in immunosuppressed patients (44).

Because of relatively high frequencies of an ex vivo population of A2/NLV-tetramer+ cells present in LTR3 (2.5–17.6% of CD8+ T cells at different time intervals), we were able to compare ex vivo–derived and in vitro–generated NLV-specific TCR repertoires and observed analogous TCR repertoires both pretransplant (52 versus 48% containing public motifs, respectively) and at 12 mo posttransplant (87 versus 95%, respectively; Table IV), indicating that our in vitro data impartially reflected the ex vivo NLV-specific TCR profile. There was no evidence of the cross-reactive TCR signature present in LTR3 or LTR8, or in previously published literature (reviewed in Ref. 28).

Functional confirmation of the cross-reactive NLV-specific TCR signature OTN5. To confirm that LTR5's cross-reactive OTN5 TCR was indeed responsible for mounting functional cross-reactivity between HLA-A2/NLV and HLA-B27 allelic subtypes, we generated a pMIG vector containing full-length TCRαβ constructs from OTN5 for retroviral transduction into SKW3.hCD8αβ T cell lines (kindly provided by Dr. Zhenjun Chen, The University of Melbourne) along with pMIG.CD3 (kindly provided by Dr. Richard Berry, Monash University) using 293T packaging cell lines for functional studies. As a negative control, SKW3.hCD8αβ T cells expressing the B8/FLR-specific LC13 TCR were also generated with pMIG.LC13 (kindly provided by Dr. Zhenjun Chen) plus pMIG.CD3. The functional outcome was measured by the CD69 activation assay and measured by flow cytometry (31, 46). The advantages of the SKW3.hCD8αβ T cell line were that it was clonally stable and did not possess endogenous TCRαβ, providing a rich source of reagent for TCR recognition studies. In addition, SKW3.hCD8αβ T cells were chosen for TCR cell-surface expression because previous attempts of TCR transduction with the parental SKW3 cell line (without CD8 costimulatory molecule) failed to bind tetramer and displayed weaker CD69 upregulation upon stimulation with our cross-reactive panel (data not shown).

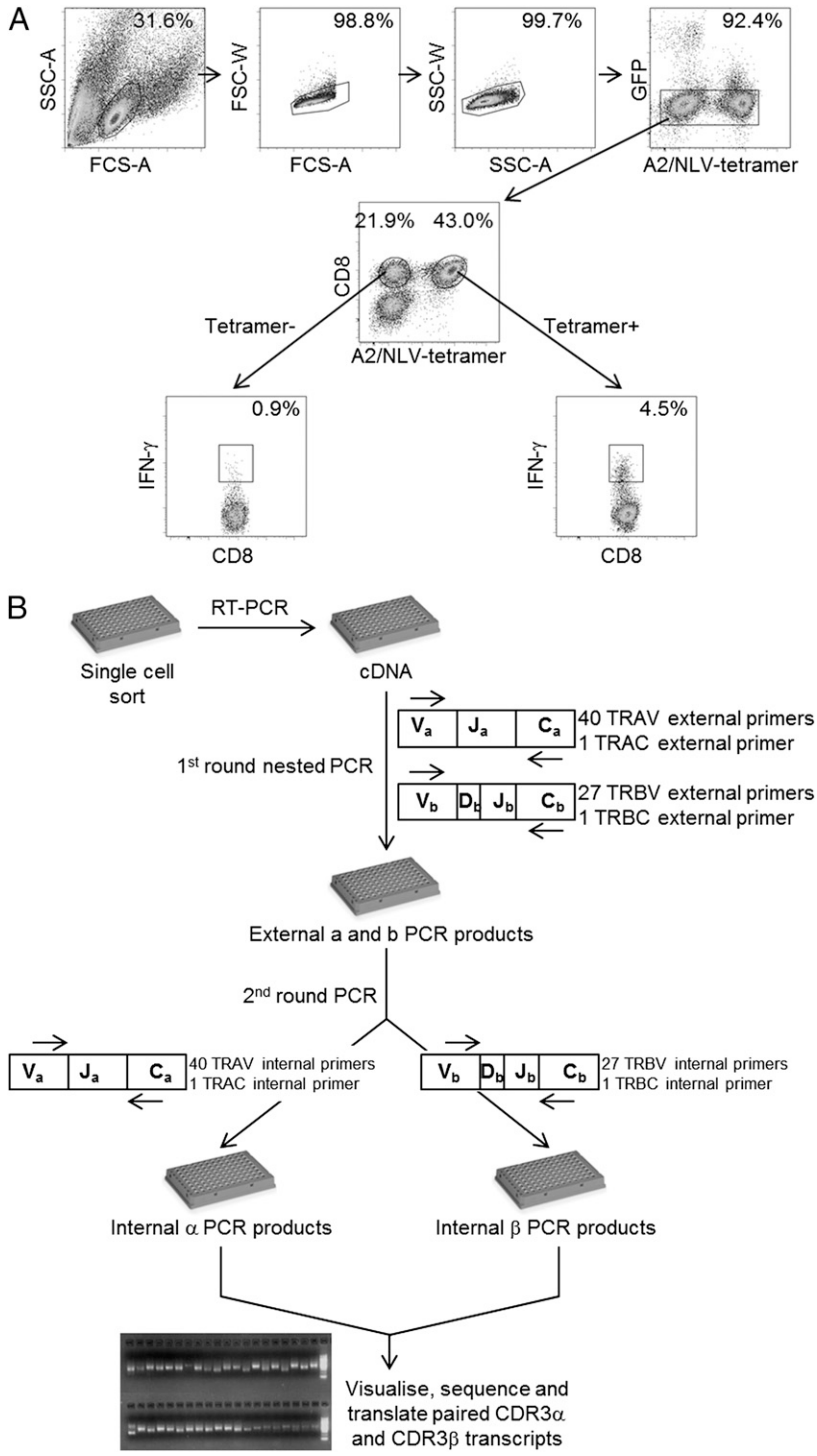


FIGURE 2. Single-cell TCRαβ analysis of NLV-specific $CD8^+$ T cells. (**A**) NLV-specific $CD8^+$ T cell lines underwent single-cell sorting after several gating of forward scatter (FSC) and side scatter (SSC) for lymphocytes, selection of GFP^- cells to exclude stimulator cells, and dual staining with CD8 and A2/NLV tetramer for detection of NLV-specific $CD8^+$ T cells. The $CD8^+$A2/NLV-$tetramer^+$ double-positive cells were then gated on IFN-γ^+ cells after stimulation with GFP^+ C1R.B27 allele cell lines and represent the cross-reactive T cell pool. The unstimulated $CD8^+$ $tetramer^-$ population shows minimal/nonspecific IFN-γ production. Percentages are based on parent populations. (**B**) Schematic diagram of multiplex nested RT-PCR for simultaneous analysis of CDR3α and CDR3β regions. RT-PCR was performed on single cells to generate cDNA before undergoing first-round nested PCR using a full panel of external sense TRAV- and TRBV- and antisense TRAC- and TRBC-specific primers. External PCR products underwent second-round nested PCR for CDR3α and CDR3β regions, using a corresponding panel of internal sense TRAV- and antisense TRAC-specific primers and internal sense TRBV- and antisense TRBC-specific primers, respectively. PCR products were then visualized, sequenced, and translated for paired CDR3αβ data. Primer details are described elsewhere (28).

After retroviral transduction of SKW3.hCD8αβ cell lines with pMIG.OTN5/pMIG.CD3 and pMIG.LC13/pMIG.CD3, high GFP/CD3-expressing cells were sorted and used for functional analysis (Fig. 3A). The specificity of SKW3.hCD8αβ.CD3.OTN5 cells was verified after complete binding to A2/NLV-tetramer, but not to the nonspecific B8/FLR-tetramer (Fig. 3B). As expected, the B8/FLR-tetramer stained positively with SKW3.hCD8αβ.CD3.LC13 cells, but not with SKW3.hCD8αβ.CD3.OTN5. In fact, the MFI increased 10-fold when either T cell line was complexed with their corresponding tetramer in comparison with the nonspecific tetramer (Fig. 3B). After incubation with C1R transfectants, SKW3.hCD8αβ.CD3.OTN5 cells showed high upregulation of CD69 expression toward the positive control (NLV-pulsed C1R.A*02:01), which was 17-fold higher in MFI compared with the nonpulsed C1R.A*02:01-negative control (Fig. 3C). Most importantly, there was a distinct hierarchy in CD69 activation levels in response to the different HLA-B27 allelic subtypes, corresponding with our earlier ICS data (Fig. 1). The order of recognition was as follows: B*27:07 (MFI increase; 17-fold) > B*27:09 (13-fold) > B*27:05 (5-fold) and negligible reactivity to B*27:02, B*27:03, and B*27:04 (between 1- and 2-fold). Although the control SKW3.hCD8αβ.CD3.LC13 cell line was capable of activation using anti-CD3/anti-CD28 beads or PMA/Ionomycin stimulation (data not shown), they failed to respond

Table II. HC5: conventional NLV-specific TCRαβ profiles alter in response to alloantigenic stimulation

TRBV	CDR3β	TRBJ	TRAV	CDR3α	TRAJ	Conv.	B*27:03	B*27:04	B*27:05	B*27:07	B*27:09
12-4	SIQALL	1-2	35	QASQGNL	42	81%	69%		76%		20%
12-4	SIQALL	1-2		ND							5%
	ND		35	QASQGNL	42		8%	7%			
12-4	SSVNEA	1-1	3	RGTNARL	31		12%			47%	45%
	ND		3	RGTNARL	31					7%	
12-4	SSVNEA	1-1	3	RDTNARL	31		4%				
12-4	SSVNEA	1-1	3	RDVNARL	31						25%
12-4	SSVNEA	1-1	3	RGVNARL	31			7%		33%	
12-4	SSVNEA	1-1		ND						7%	
12-4	SIVNEQ	2-1	3	RDTNARL	31		4%		10%		
6-5	SRDPYNEQ	2-1	3	RGTNARL	31					7%	
	ND		3	RDTNTPL	29			7%			
4	SQEEGPGNQPQ	1-5	5	TRSRDYKL	20	5%					
4	SQEEGPGNQPQ	1-5		ND		5%			10%		
11-2	SLGGLGGGIQETQ	2-5	22	TPGTSYDKV[a]	50		4%	7%			
12-4	AITSTQ	2-3	22	ADTGNQF[b]	49	5%					
12-4	SSAYYGY[c]	1-2	8-6	TSYGKL	52			13%	5%		
			8-6	TSYGKL	52			40%			
12-4	SSAYYGY[c]	1-2	35	PRETSYDKV[a]	50	5%					
12-4	SSAYYGY	1-2		ND							5%
13	SQEXGGSYEQ	2-7	38-1	MKLTYSGGGADGL	45			7%			
	ND		26-2	DNNNDM[a]	43			13%			
					No. of sequences	21	26	15	21	15	20

In vitro–cultured NLV-specific CD8+ T cells from HC5 were left unstimulated for conventional NLV-specific TCR analysis or restimulated with C1R.B27 allelic transfectants for 2 h in an IFN-γ secretion assay before single-cell sorting and multiplex nested RT-PCR. Each TCR repertoire was measured in one experiment per stimulation condition.

[a]Known public motifs are underlined (28, 35).

[b]Known public motif is underlined (41).

[c]Known public motif is underlined (28, 35, 37).

Conv., conventional; X, undetermined amino acid.

to the C1R.A2 and C1R.B27 APC panel, thereby validating the TCR specificity observed with our SKW3.hCD8αβ.CD3.OTN5 cell line (Fig. 3C).

Narrowing of virus-specific TCR diversity before a clinically relevant CMV reactivation episode. The diversity of virus-specific memory cytotoxic T cells or TCR clonal focusing is a function of the overall dynamics of the viral immune response. "Adaptive" TCR narrowing may therefore be caused by successive and repetitive stimulation upon chronic CMV infection or during periods of immunodepression, resulting in fewer dominant clones bearing high-affinity TCRs (39). We investigated the diversity of the conventional NLV-specific TCR repertoire in LTR5 before and after a clinically relevant CMV reactivation episode that required prophylactic treatment (Fig. 4A), bearing in mind that LTR5's OTN5 TCR should not be cross-reactive to the B*27:04 lung allograft based on our in vitro experimental evidence. Fig. 4B represents the TCR diversity of the conventional unstimulated NLV-specific CD8+ T cell population both pretransplant and posttransplant. Unpaired CDR3α or CDR3β data represented 6.4% of single cells analyzed (7/109 cells) and were grouped together in the analysis. Within the first 3 mo posttransplant (days 13 and 88), OTN5 constituted between 77 and 88% of the total TCR repertoire similar to that of pretransplant levels (day 0, 65%). Statistical evaluation of the TCR repertoires between pretransplant and posttransplant time intervals showed no significant difference between days 0 and 13 ($p = 0.08$) and days 0 and 88 ($p = 0.1$). However, the TCR repertoire underwent significant dynamic modulation by day 193, being dominated by OTN5 (100%) even in the absence of CMV viremia (Fig. 4B, $p = 0.008$ for comparison of day 0 and day 193 TCR repertoire). CMV reactivation was detected in the bronchoalveolar lavage fluid (BAL) on day 270 with a viral titer measurement of 18,600 copies/ml, and CMV pneumonitis was observed in the transbronchial biopsy after routine clinical follow-up. Initiation of antiviral treatment with valganciclovir was warranted, with establishment of immune control by day 375 based on further sampling where CMV viral load was undetected in the BAL and no other CMV-related events were reported up to day 1252 when CMV viral load was undetected in the blood. Interestingly, we observed a reduction in OTN5 to 70% of the conventional TCR repertoire on day 909, which was again more similar to the pretransplant level (Fig. 4A, $p = 0.04$ for comparison of day 0 and day 909 TCR repertoire).

Our observations support our previous functional data describing the gradual increase in cytokine production and expansion of cross-reactive NLV-specific CD8+ T cells recognizing B*27:09, which preceded clinical detection of viremia in BAL samples but returned to baseline levels after CMV treatment and viral clearance (19). Similarly, in this study, we observed a highly focused narrowing of the cross-reactive NLV-specific TCR repertoire before CMV reactivation, which then reduced in immunodominance long after cessation of viral infection. Therefore, our observation supports previous reports (39, 47) that antigenic pressure offers to shape the T cell repertoire.

Discussion

Human CMV-specific memory CD8+ T cells are a heterogeneous population with high-affinity TCRs representative of continual Ag exposure via the initial infection and subsequent outbreaks of viral proliferation. The HLA-A2–restricted NLV-specific CD8+ T cell response ($pp65_{495–503}$) is oligoclonal, despite its epitope being within a highly conserved region (48). Previous studies have described both public and private specificities of the conventional NLV-specific TCR repertoire with individuals generally having one or a few dominant clonotypes and some subdominant clonotypes (28, 35, 38–40, 42). However, TCR signatures of cross-reactive CMV-specific T cells have not been previously characterized. Besides studies of the well-defined EBV LC13 TCR investigations of T cell cross-reactivity, limited data exist

Table III. LTR5: dynamics of NLV-specific TCRαβ repertoires before and after lung transplantation

						Pretx	0.5 mo			3 mo				7 mo				30 mo				
TRBV	CDR3β	TRBJ	TRAV	CDR3α	TRAJ	Conv.	Conv.	B*27:05	B*27:07	Conv.	B*27:05	B*27:07	B*27:09	Conv.	B*27:05	B*27:07	B*27:09	Conv.	B*27:04	B*27:05	B*27:07	B*27:09
12-4	SIVNEA	1-1	3	RNNNARL	31	65%	88%	85%	100%	77%	64%	100%	50%	100%	63%	88%	100%	70%		59%	87%	43%
12-4	SIVNEA	1-1		ND							5%		10%								7%	
	ND		3	RNNNARL	31		4%								21%				13%		7%	
25-1	SEFGGTERTQETQ	2-5	3	RNNNARL	31											13%						
12-4	SSVTEA[a]	1-1	3	RGTNARL	31													4%				
2	SEGELAGATDGEQ	2-7	26-2	RDRYPDKL	34	5%																
2	SEGELAGATDGEQ	2-7		ND		5%																
6-2/3	SYEAGGAKNIQ	2-4	35	QMGPTGANSKL	56																	5%
6-4	SDSSRGXTDTQ[b]	2-3	1-2	GDSNYQL	33															5%		
6-5	SYQTGTIYGY[a]	1-2		ND																		29%
6-5	HPTSGGLYTGEL	2-2		ND																5%		
7-6/7	SLAPGTTNEKL	1-4	26-2	DNNNDM[c]	43		4%												6%			
	ND		26-2	DNNNDM[c]	43														13%			
12-4	SLPPGRGYTEA[a]	1-1	10	SENYGGSQGNL	42	5%																
12-4	SFIGGVDGY	1-2	5	IPNYGGSQGNL	42					14%	32%		35%					22%		14%		5%
	ND		5	IPNYGGSQGNL	42										5%				6%			
14	SQGKYQPQ	1-5	4	AQGDNYGQNF[d]	26			4%														
15	SSDSTFGTEA[a]	1-1	5	IPASGGSYIP	6														25%	14%		5%
15	SSDSTFGTEA[a]	1-1		ND														4%				
16	SQGELAGGLEQ	2-7	26-2	TGANNL[e]	36		4%			5%												
	ND		26-2	TGANNL[e]	36								5%									
20-1	SRGATNEKL	1-4		ND																		5%
24-1	SDSGQGRTDTQ	2-3	3	RAPGGFNKF	21	5%																
25-1	SETPQRPGGYEQ	2-7		ND		5%																
27	SLSNVAGVTDTQ[b]	2-3		ND				11%		5%					11%					5%		10%
	ND		3	RDHSGQNF[d]	26	5%																
	ND		3	RDYNAGKS	27	5%																
	ND		24	NTGNQF[e]	49														38%			
					No. of sequences	20	24	27	14	22	22	14	20	20	19	16	19	23	16	22	15	21

Single-cell multiplex nested RT-PCR was performed on in vitro–cultured NLV-specific $CD8^+$ T cells at various time points after an IFN-γ secretion assay in the presence or absence of C1R.B27 transfectants to determine cross-reactive and conventional NLV-specific TCR repertoires, respectively. Each TCR repertoire was measured in one experiment for each time interval per stimulation condition.

[a]Known public motifs are underlined (28, 35).

[b]Known public motif is underlined (28, 35, 37).

[c]Known public motif is underlined (28, 34–36).

[d]Known public motif is underlined (28, 33–37, 41).

[e]Known public motifs are underlined (28, 33–37).

Conv., conventional; Pretx, before transplant; X, undetermined amino acid.

Table IV. LTR3 and LTR8: TCRαβ repertoires of non–cross-reactive NLV-specific CD8$^+$ T cells

TRBV	CDR3β	TRBJ	TRAV	CDR3α	TRAJ	Pretx Ex Vivo	Pretx In Vitro	3 mo In Vitro	7 mo In Vitro	12 mo Ex Vivo	12 mo In Vitro
LTR3											
2	SEGLFGTTKGHEQ	2-7	26-2	RDEYGNKL	47		3%				5%
4	SQEQGPGNQPQ	1-5	5	VPSNDYKL	20	10%	10%				
6-1	SETLIAPYEQ	2-7	26-2	REAHYGGSQGNL	42	5%					
6-5	SAQTGASYGY[a]	1-2	24	NTGNQF[b]	49	33%	29%			43%	9%
6-5	SYQTGAGGYGY[a]	1-2	24	NTGNQF[b]	49	5%	6%			4%	
6-5	SPITGTGAYGY[a]	1-2	24	NTGNQF[b]	49		3%			13%	36%
6-5	SYVTGTGAYGY[a]	1-2	24	NTGNQF[b]	49					9%	18%
6-5	RAVTGSIYGY	1-2	24	NTGNQF[b]	49		6%			4%	
12-4	SSVSGY	1-2	5	VPSNDYKL	20	5%					
12-4	SSVSGY	1-2	22	TPPPSYDKV	50	14%	3%				
12-4	SSVNEQ	2-7	3	RDTDARL	31		6%				
27	QLQGHTEA	1-1	24	PYNNNDM[c]	43	5%	3%			9%	18%
29	EKGGVWGY	1-2	24	NTGNQF[b]	49						5%
30	SVILGVGGRRTGEL	2-2	19	SEQGSGTYKY	40		3%				
12-4	SSVSGY	1-2		ND		14%	26%			4%	
20	HFGTPNYGY	1-2		ND						4%	
	ND		24	NTGNQF[b]	49	10%				4%	9%
	ND		26-2	RDEYGNKL	47					4%	
					No. of sequences	21	31			23	22
LTR8											
2	SYFDEKL	1-4	1-1	RDNSITGGFKT	9		8%		5%		
12-4	ASANYGY	1-2	35	PRETSYDKV[d]	50			18%			
28	NGGSNYGY	1-2	5	LGGSSNTGKL	37			9%			
30	SVSDVANTEA[c,e]	1-1	26-2	SNNNDM[c,e]	43			64%	16%		
27	SLSAGTPLGEL	2-2		ND			92%	9%	11%		
	ND		1-1	RDNSITGGFKT	9				5%		
	ND		26-2	SNNNDM[c]	43				58%		
	ND		35	PRETSYDKV[d]	50				5%		
					No. of sequences		25	22	19		
					No data				3		

Ex vivo– and in vitro–cultured NLV-specific CD8$^+$ T cells were single-cell sorted based on CD8 and A2/NLV-tetramer costaining before performing multiplex nested RT-PCR. Each TCR repertoire was measured in one experiment per donor and time interval.

[a]Known public motifs are underlined (28, 35, 37).

[b]Known public motifs are underlined (28, 33–37).

[c]Known public motifs are underlined (28, 35).

[d]Known public motifs are underlined (28, 33–37, 41).

[e]Known public motifs including paired motifs are underlined (28, 35).

Pretx, before transplant.

in other viral models. CMV has greater clinical significance in terms of primary disease and reactivation, as well as the increased risk associated with incidences of chronic allograft rejection and decreased survival in transplant recipients. Thus, we focused our study on determining the TCR signature of cross-reactive NLV-specific CD8$^+$ T cells in a CMV-reactivated lung transplant patient (CMV$^+$HLA-A2$^+$ recipient, HLA-B27$^+$ donor) and an unrelated healthy individual.

The tetramer and cytokine secretion capture assay in combination with the multiplex nested RT-PCR methodology provides an effective framework for investigating TCRαβ signatures of distinct cross-reactive virus-specific T cells. We identified a cross-reactive TCR clonotype in two unrelated individuals consisting of TRAV3TRAJ31 and TRBV12-4TRBJ1-1 genes and comprising the same number of CDR3αβ amino acid residues (CDR3α: RXXNARL, CDR3β: SXVNEA). We postulated that the slight differences (denoted by "X") observed between HC5 and LTR5 at CDR3α positions 4–5 (Gly-Thr to Asn-Asn, respectively) and CDR3β position 4 (Ser to Ile, respectively) may not be so critical for peptide/MHC binding. To the best of our knowledge, this particular TCRαβ paired clonotype has not been previously described in the literature, which may be explained by either: 1) limited TCRαβ paired data available, 2) limited coverage in our population, or 3) private or less public specificity of this clonotype in our population. Based on our and others' data (42), TCRβ chains comprising TRBV12-4TRBJ1-2 genes were more frequently observed for conventional NLV-specific CD8$^+$ T cells, whereas TRBV12-4TRBJ1-1 TCRβ chains, being a key feature of our cross-reactive OTN5 clonotype, have been previously reported at very low frequencies (38, 40, 47, 49). Nevertheless, this is the first study, to our knowledge, to demonstrate a TRBV12-4TRBJ1-1 TCRβ chain pairing with TRAV3TRAJ31 in NLV-specific CD8$^+$ T cells.

Key polymorphic amino acid positions within the HLA-B27 molecule have been associated with differential tapasin-associated Ag processing (50), modulation of peptide–MHC binding (51), and TCR–peptide–MHC interaction that directly influence the downstream effects of T cell activation and immune reactivity (52–54). Although the exact mechanisms for pathogenesis is still unclear, disease susceptibility to ankylosing spondylitis has been well associated with the HLA-B*27:05 subtype, but not HLA-B*27:09 (55), which differs only by one amino acid at position 116 (Asp116His). Furthermore, investigations of immunological responses among HLA-B44 alleles that differ by a single amino acid difference at either position 156 (Asp to Leu) between B*44:02 and B*44:03 or position 116 (Asp to Tyr) between B*44:02 and B*44:05 promote alterations associated with the diversity of the peptide repertoires (26), Ag presentation kinetics (56), and T cell alloreactivity dynamics (23). Therefore,

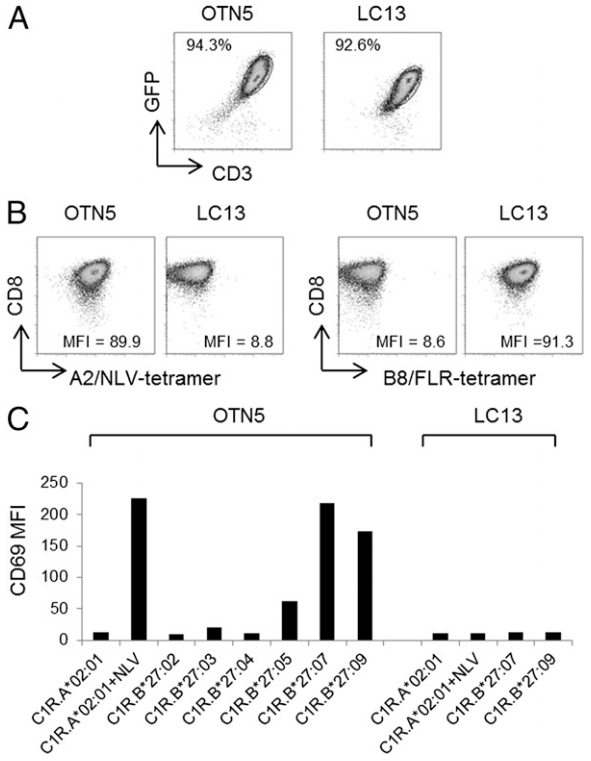
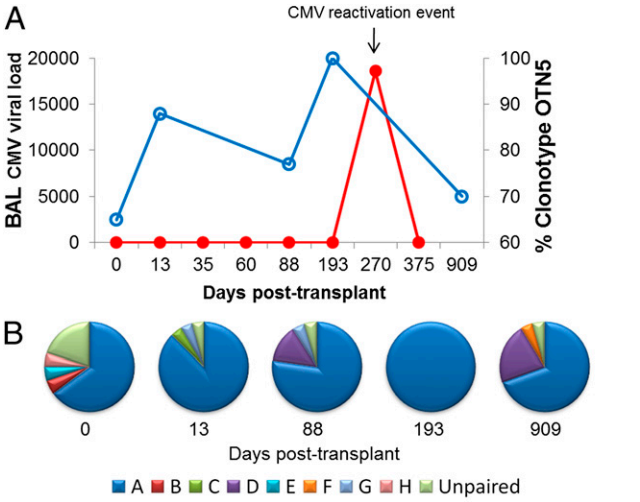


FIGURE 3. Activation of SKW3.hCD8αβ.CD3.OTN5 cells by HLA-B27–expressing C1R cells. (**A**) Newly generated SKW3.hCD8αβ.CD3.OTN5 and SKW3.hCD8αβ.CD3.LC13 cells were sorted for reculture based on their high levels of GFP and CD3 expression. (**B**) Specificity of LTR5's cross-reactive OTN5 TCR was confirmed using A2/NLV-tetramer and anti-CD8 staining. Cells were originally gated on live cells (FSC versus SSC), then GFP^+CD3^+ cells. SKW3.hCD8αβ.CD3.LC13 and B8/FLR-tetramer were included as irrelevant controls (MFI). (**C**) Activation was measured by cell-surface CD69 upregulation and flow cytometry after 16–20 h incubation with C1R transfectants. CD69 MFI values were taken after gating on live cells (FSC versus SSC), $CD8^+$ cells, then GFP^+CD3^+ cells. Data shown in (B) and (C) are from one experiment and are representative of two separate experiments in duplicate.



Clonotype	TRBV	CDR3β	TRBJ	TRAV	CDR3α	TRAJ
A (OTN5)	**12-4**	**SIVNEA**	**1-1**	**3**	**RNNNARL**	**31**
B	2	SEGELAGATDGEQ	2-7	26-2	RDRYPDKL	34
C	7-6/7	SLAPGTTNEKL	1-4	26-2	DNNNDM	43
D	12-3/4	SFIGGVDGY	1-2	5	IPNYGGSQGNL	42
E	12-3/4	SLPPGRGYTEA	1-1	10	SENYGGSQGNL	42
F	12-3/4	SSVTEA	1-1	3	RGTNARL	31
G	16	SQGELAGGLEQ	2-7	26-2	TGANNL	36
H	24-1	SDSGQGRTDTQ	2-3	3	RAPGGFNKF	21

FIGURE 4. Narrowing of TCR repertoire after viral reactivation episode. (**A**) LTR5's CMV viral load (*left* y-axis, red line) was measured in BAL up to day 375 posttransplant. OTN5 represents the cross-reactive TCR that recognizes the HLA-B27 alloantigen (*right* y-axis, blue line). (**B**) Pie graphs of paired NLV-specific $CD8^+$ TCRαβ clonotypes pretransplant and posttransplant from the unstimulated conventional sort gate. Unpaired CDR3α and CDR3β sequences have been grouped together in the analysis but are listed individually in Table III under the conventional columns.

we predict that an amino acid substitution at position 116 (Asp, Tyr, His) of B*27:05, B*27:07, and B*27:09, respectively, would be pivotal in regulating the tiered interactions between the peptide–MHC and the TCR–peptide–MHC complex. HLA-B*27:07 comprises four additional amino acid substitutions (positions 97, 113, 114, 131) compared with B*27:09 (Table I), which could also impact on strengthening the resulting TCR–peptide–MHC interactions leading to the higher production of IFN-γ observed. In contrast, amino acid substitutions at other positions within the subtypes, B*27:02, B*27:03, and B*27:04, could have a weakening or null effect on the TCR–peptide–MHC complex, as evidenced by the lack of immune reactivity generated.

Our previous work has shown the ability of cross-reactive EBV-specific T cells to induce immune reactivity toward HLA molecules expressed on both PBMCs and BAL mononuclear cells (20, 23, 26). Of particular importance, a seminal study confirmed that recognition of allogeneic HLA molecules by virus-specific memory T cells is dependent on self-peptide presentation by the allogeneic target cell (57). The more readily testable EBV model yielded key observations, with FLR-specific $CD8^+$ T cells showing very weak recognition of HLA-B*44:02 target cells because of lack of EEYLQAFTY peptide presentation in specific tissues (proximal tubular epithelial cells and HUVECs). Moreover, differential allorecognition of virus-specific T cells against normal cell subsets (including B cells, T cells, monocytes, DCs, and fibroblasts) has also been shown (12). In our CMV model of T cell cross-reactivity, identification of the allopeptide must be determined before we can embark on tissue-specific experiments across a range of cellular and tissue (i.e., lung, kidney) subsets. Allopeptide discovery would also facilitate structural investigations to identify TCR–peptide–MHC binding interactions, and a direct comparison with the binding configurations of the NLV-specific public RA14 TCR (44) versus our OTN5 TCR. In addition, an analysis between the cross-reactive NLV-specific $CD8^+$ TCR and de novo–generated HLA-B27–specific $CD8^+$ TCR profiles warrants further investigation.

One of the major complications in lung transplantation in the first postoperative year relates to either primary CMV disease or reactivation after cessation of antiviral prophylaxis at 5 mo posttransplant, which has been significantly associated with the onset of bronchiolitis obliterans syndrome/chronic rejection in LTR (1, 58). Previously, we have reported that cross-reactive antiviral T cells constitute a significant proportion of the alloreactive T cell pool, but there was no association with allograft rejection in the absence of active viral infection; thus, there was no potential amplification of the cross-reactive T cell pool (20). Building on this knowledge, we hypothesized that an active viral infection may be a major amplifier of the destructive potential of cross-reactive T cells (19). In this study, we were able to investigate the TCR dynamics of LTR5's NLV-specific $CD8^+$ T cell repertoire both before and after a clinically relevant viral reactivation episode. We observed an increase in dominance of the cross-reactive OTN5 clonotype from 65% pretransplant to 100% by day 193 in the absence of detectable CMV reactivation, but before viral reactivation on day 270. Following medical intervention and viral clearance with no other

CMV-related event, OTN5 clonotype had reduced to basal levels on day 909 (70%) similar to what was observed before transplant.

Because LTR5's donor lung allograft had the non–cross-reactive HLA-B*27:04 subtype, it was not surprising that LTR5 experienced no allograft damage as measured by physiological lung function, acute cellular rejection (cellular infiltrate, tissue architecture), and survival dynamics (>3 y posttransplant). If LTR5 had received a donor lung allograft that was HLA-B*27:07, HLA-B*27:09, or HLA-B*27:05 to a lesser extent, we hypothesize that these cross-reactive T cells could potentially mediate allograft damage in the presence of active CMV infection, especially given the immunodominant nature of the cross-reactive OTN5 TCR and dynamic skewing of OTN5 TCR associated with CMV viremia. We further speculate that a single viral reactivation event, which was successfully treated in LTR5, did not have a sustainable antigenic load to intensify the cross-reactive T cell pool, and that multiple hits of viral reactivation may be needed in combination with the right cross-reactive T cell to cause significant immunopathological effects in the lung allograft.

We have previously shown that differential immunogenicity of HLA molecules exists across different HLA class I alleles (23, 26). This study demonstrated that of the six most commonly expressed Caucasian HLA-B27 molecules (B*27:02, 27:03, 27:04, 27:05, 27:07, 27:09), a hierarchy of immunogenicity also exists for T cell cross-reactivity (B*27:07 > 27:09 > 27:05 and no reactivity toward B*27:02, 27:03, 27:04). The fact that LTR5 was "lucky" not to receive one of the immunogenic HLA-B27 alleles is a chance event, and their uneventful clinical course posttransplant supports this observation. Interestingly, studies of the autoimmune disease ankylosing spondylitis have shown B*27:04 to be more immunogenic as a risk factor compared with B*27:02 and B*27:05, with other B27 alleles being identified as either protective or as having no association with susceptibility (59, 60).

Although we have deciphered the TCR signature of the cross-reactive T cell, there are some limitations in this study. We acknowledge that our clinical evaluation in lung transplantation was limited by the number of suitable patients who were accessible. Ideally, another LTR ($A2^+B27^-$) receiving a $B27^+$ allograft would have been valuable to corroborate the findings of LTR5. In addition, access to samples obtained before, during, and after lung reactivation signals (i.e., active CMV viremia) are dependent on clinical decision-making relating to bronchoscopic sampling.

In conclusion, to our knowledge, this study is the first report to provide a detailed insight into the unique TCRαβ signature of cross-reactive NLV-specific T cells and the potential benefits that may follow from prospective immune monitoring of the TCR repertoire in transplant recipients.

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Disclosures

The authors have no financial conflicts of interest.

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