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β-cell-specific CD8 T cell phenotype in type 1 diabetes reflects chronic autoantigen exposure

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Abstract

Autoreactive CD8 T cells play a central role in the destruction of pancreatic islet β-cells that leads to type 1 diabetes, yet the key features of this immune-mediated process remain poorly defined. In this study, we combined high definition polychromatic flow cytometry with ultrasensitive peptide-human leukocyte antigen class I (pHLAI) tetramer staining to quantify and characterize β-cell-specific CD8 T cell populations in patients with recent onset type 1 diabetes and healthy controls. Remarkably, we found that β-cell-specific CD8 T cell frequencies in peripheral blood were similar between subject groups. In contrast to healthy controls, however, patients with newly diagnosed
Type 1 diabetes displayed hallmarks of antigen-driven expansion uniquely within the β-cell-specific CD8 T cell compartment. Molecular analysis of selected β-cell-specific CD8 T cell populations further revealed highly skewed oligoclonal T cell receptor (TCR) repertoires comprising exclusively private clonotypes. Collectively, these data identify novel and distinctive features of disease-relevant CD8 T cells that inform the immunopathogenesis of type 1 diabetes.

Type 1 diabetes is an autoimmune disease characterized by T cell-mediated destruction of insulin-producing β-cells in the islets of Langerhans (1; 2). Several lines of evidence implicate the CD8 T cell lineage in this process: (i) CD8 T cells predominate in islet-centred leukocytic infiltrates close to diagnosis (3; 4); (ii) autoreactive CD8 T cells, with β-cell epitope specificities have been detected in such early infiltrates (3); (iii) CD8 T cell clones specific for preproinsulin-derived peptides can kill β-cells in vitro (5; 6); and (iv) large genetic association studies link disease susceptibility to the inheritance of specific human leukocyte antigen class I (HLA-I) alleles (7). This mounting functional and epidemiological evidence, combined with the expanding array of reported HLAI-restricted β-cell epitopes (8; 9), provides a strong rationale for detailed studies of autoreactive CD8 T cells in type 1 diabetes.

Technological advances have facilitated the design of CD8-centric studies, enabling enhanced data retrieval from cell-limited samples to illuminate fundamental immunobiological processes. In particular, antigen-specific CD8 T cells can now be enumerated routinely by flow cytometry irrespective of functional outputs due to the advent of recombinant peptide-HLAI (pHLAI) proteins in various fluorochrome-tagged multimeric formats (10-13). Moreover, developments in instrumentation and fluorochrome technology continue to expand the horizons of polychromatic flow cytometry (14; 15), facilitating the identification of functionally distinct T cell subsets across a spectrum of phenotypic heterogeneity (16). The collective application of such innovations has transformed our understanding of T cell ontogeny in response to infectious “foreign” antigens. However, it is unclear whether the emerging conceptual frameworks extend similarly to autoimmune processes.

Although β-cell epitope-specific CD8 T cell expansions have been identified in the peripheral blood of patients with type 1 diabetes (6; 17), the functional and phenotypic properties of these cells in the context of disease relevance remain largely uncharacterized. This is a significant knowledge gap for two important reasons. First, it cannot be assumed that autoreactive CD8 T cells will follow the rules of antigen engagement established in previous studies. Indeed, autoreactive T cell receptors (TCRs) characteristically display low affinity interactions with their cognate pHLA antigens (18; 19), presumably reflecting the effects of thymic culling to eliminate potentially dangerous self-specific clonotypes from the peripheral repertoire. Moreover, autoantigens are expressed continuously and guarded by peripheral tolerance mechanisms designed to limit cognate T cell expansion (1). Second, immune intervention strategies designed specifically to target either effector T cells or innate inflammatory pathways that could impact adaptive immune responses are currently being trialed in type 1 diabetes (20-23). The identification of T cell-related biomarkers could facilitate immune monitoring in this setting and delineate correlates of therapeutic efficacy.
Accordingly, we undertook a multiparametric analysis of β-cell-specific CD8 T cell populations in patients with type 1 diabetes and healthy controls to identify the key cellular features associated with disease.

RESEARCH DESIGN AND METHODS

Study subjects

The study cohort comprised 14 HLA-A*0201+ patients (mean age, 30 years +/- SD = 6.4) with newly diagnosed type 1 diabetes (mean disease duration, 4 months) and 14 HLA-A*0201+ healthy controls (mean age, 30 years +/- SD = 5.0). Autoantibodies against GAD65 and IA-2 were detected in 64% (9/14) and 71% (10/14) of patients in the type 1 diabetes group, respectively. Local research ethics committee approval (National Research Ethics Committee, Bromley NRES Committee, Ref 08/H0805/14) was granted at each participating center and written informed consent was obtained in all cases.

Blood samples

Fresh venous blood was collected into heparinized tubes and transported for processing within 3 h of collection. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Nycomed), washed twice in RPMI-1640 supplemented with 1% penicillin/streptomycin and 2% human AB serum (all Life Technologies), then resuspended in freezing medium comprising 90% heat-inactivated, filter-sterilized fetal bovine serum and 10% dimethyl sulfoxide (Sigma-Aldrich). Aliquots of 10-20 × 10^6 cells/mL per vial were cooled overnight at a controlled rate of −1°C/min to −80°C prior to storage in liquid nitrogen. All samples were analyzed within two years of cryopreservation.

Tetrameric pHLAI complexes

Soluble, fluorochrome-conjugated pHLA-A*0201 tetramers were produced as described previously (24). Incorporated peptides were synthesized at >95% purity (BioSynthesis). The corresponding epitopes are summarized in Table 1.

Polychromatic flow cytometry

Thawed PBMCs were pre-treated with 50 nM dasatinib before tetramer staining to enhance the detection of low avidity T cells as described previously (25). The following monoclonal antibodies (mAbs) were used for phenotypic analysis: (i) αCD3-H7APC, αCD4-V500, αCD45RO-PECy7, αCD57-FITC, αCD95-PE and αCCR7-PerCPCy5.5 (BD Biosciences); (ii) αCD8-QD705 (Life Technologies); and (iii) αCD27-PECy5 (Beckman Coulter). Dead cells were excluded from the analysis using the amine-reactive dye ViViD (Life Technologies); monocytes and B cells were eliminated in the same dump channel after staining with αCD14-PacBlue and αCD19-PacBlue, respectively (Life Technologies). Stained samples were acquired using an LSRFortessa™ flow cytometer (BD Biosciences) and analyzed with FlowJo version 9.4 (TreeStar Inc.). The gating strategy is illustrated in Supplementary Figure 1. For quality control purposes, tetramers were batch-tested prior to experimentation using specific CD8 T cell clones where available (5). Assay variability was monitored throughout the study using aliquots of cryopreserved PBMCs drawn from a single healthy donor at a single time point (Supplementary Figure 2).
**T cell receptor clonotyping**

Clonotypic analysis of antigen-specific CD8 T cell populations was performed as described previously with minor modifications (26). Briefly, 222-1,071 viable tetramer-labeled CD3^+CD8^+ T cells were sorted directly *ex vivo* into 1.5 mL microtubes (Sarstedt) containing 100 µL RNAlater (Applied Biosystems) using a custom-modified FACSIAria™ II flow cytometer (BD Biosciences). Unbiased amplification of all expressed TRB gene products was conducted using a template-switch anchored reverse transcription-polymerase chain reaction (RT-PCR) with a 3′ constant region primer (5′-TGGCTCAAACAAGGAGACCT-3′). Amplicons were sub-cloned, sampled, sequenced and analyzed as described previously (24). The IMGT nomenclature is used in this report (27).

**Statistical analysis**

Single experimental variables were analyzed using the Mann-Whitney U-test or the Wilcoxon signed-rank test in GraphPad Prism 5 (GraphPad Software). Multivariate analyses of flow cytometric data were performed using the probability binning algorithm in FlowJo version 9.7.2 (TreeStar Inc.).

**RESULTS**

*Ex vivo* identification of β-cell-specific CD8 T cells

To ensure the optimal detection of autoreactive β-cell-specific CD8 T cell populations directly *ex vivo*, we conducted extensive pilot experiments with dasatinib, a reversible protein kinase inhibitor (PKI) that lowers the TCR affinity threshold required for tetramer binding at the cell surface (25). This approach enables the visualization of low avidity CD8 T cells that would otherwise remain undetectable and enhances the intensity of tetramer staining via active inhibition of TCR downregulation. In line with our previous findings, we observed greater frequencies of β-cell-specific CD8 T cells in all test subjects after sample pre-treatment with dasatinib (Figure 1 A-E) and clearer separation of tetramer-labeled events across all specificities (Figure 1 A-G).

Next, we used this approach to compare β-cell-specific CD8 T cell frequencies in newly diagnosed type 1 diabetes patients and healthy non-diabetic controls across five distinct HLA-A*0201-restricted specificities (PPI_{15-24}, InsB_{10-18}, IGRP_{265-273}, IA-2_{797-805} and GAD_{114-123}). Two pHLA-A*0201 tetramers representing immunodominant epitopes from common persistent herpesviruses (CMV pp65_{495-503} and EBV BMLF1_{280-288}) were also included for control purposes (Table 1). Again, increased β-cell-specific CD8 T cell frequencies were observed for the majority of subjects in the presence of dasatinib, reaching statistical significance for most specificities in each subject group (Figure 2). Although less striking, we noted a similar frequency enhancement for CMV pp65_{495-503} tetramer-binding CD8 T cells. However, this most likely reflects improved visualization of non-amplified precursors close to the technical limit of detection rather than a biologically relevant phenomenon within the antigen-experienced pool. Consistent with this interpretation, no such differences were observed for EBV BMLF1_{280-288} tetramer-binding CD8 T cells, which are driven to expand in the majority of HLA-A*0201+ donors as a consequence of high viral prevalence. More importantly, there were no significant differences between type
1 diabetes patients and healthy controls with respect to CD8 T cell frequencies across any of the seven incorporated specificities, either in the presence or absence of dasatinib. Accordingly, we hypothesized that disease-related differences in autoreactive CD8 T cell populations may reflect an antigen-driven inflammatory process that does not manifest in simple numerical terms, at least within the peripheral circulation.

**β-cell-specific CD8 T cells are more differentiated in type 1 diabetes patients**

To examine the phenotypic characteristics of β-cell-specific CD8 T cells, we constructed a polychromatic flow cytometry panel designed to exclude irrelevant events (ViViD, CD14 and CD19), assign lineage (CD3, CD4 and CD8) and define differentiation status (CD27, CD45RO, CD57, CD95 and CCR7). Across all pooled β-cell specificities, we found that the percentage of autoreactive CD8 T cells with a naïve phenotype (T<sub>N</sub> - CD27<sup>+</sup>CD45RO<sup>-</sup>CD57<sup>-</sup>CD95<sup>-</sup>CCR7<sup>+</sup>) was significantly lower in type 1 diabetes patients compared to healthy controls (p<0.0001; Figure 3). This pattern held within individual specificities, reaching significance for PPI<sub>15-24</sub> (p=0.02), IGRP<sub>265-273</sub> (p=0.01) and IA-2<sub>270-285</sub> (p=0.02). Importantly, no such differences were detected between groups for either of the virus-derived specificities. Moreover, total naïve CD8 T cell frequencies were similar in type 1 diabetes patients and healthy controls (Supplementary Figure 3). Of note, a large proportion of CD8 T cells specific for the CMV pp65<sub>495-503</sub> epitope displayed a classical naïve phenotype. In contrast, very few CD8 T cells specific for the EBV BMLF1<sub>280-288</sub> epitope were naïve. These observations substantiate the interpretation above that dasatinib-mediated frequency amplification within the CMV specificity reflects enhanced precursor detection in seronegative individuals.

Collectively, these data suggest that recent onset type 1 diabetes is characterized by antigen-driven expansion of β-cell-specific CD8 T cells into more differentiated compartments, likely facilitated by tissue-specific inflammatory processes. Consistent with this notion, greater proportions of CD8 T cells with a stem cell memory phenotype (T<sub>SCM</sub> - CD27<sup>+</sup>CD45RO<sup>-</sup>CD95<sup>+</sup>CCR7<sup>+</sup>) (28) were present in type 1 diabetes patients compared to healthy controls across all pooled β-cell specificities (p=0.025), as well as individually within the autoreactive populations specific for PPI<sub>15-24</sub> (p=0.05) and I<sub>8</sub>b<sub>10-18</sub> (p=0.029). Moreover, single marker analyses revealed that β-cell-specific CD8 T cells expressed higher frequencies of CD57 (p=0.0002) and CD95 (p<0.0001) in type 1 diabetes patients compared to healthy controls (p<0.0001). These surface proteins demarcate terminal differentiation and memory status, respectively (16; 29). Conversely, β-cell-specific CD8 T cells less frequently expressed CD27 and CCR7 in type 1 diabetes patients compared to healthy controls (p=0.0002 and p=0.005, respectively). These markers classically delineate naïve and early memory T cells (30; 31). No single marker differences between subject groups were observed for either of the virus-derived specificities or CD8 T cells as a whole.

To extend these findings, we used frequency difference gating and probability binning to conduct multivariate analyses across concatenated datasets (32; 33). A phenotypically distinct CD27<sup>intermediate</sup>CD95<sup>+</sup> population of CD8 T cells was identified in type 1 diabetes patients at significantly higher frequencies compared to healthy controls for the PPI<sub>15-24</sub> (p<0.01), IGRP<sub>265-273</sub> (p<0.01) and pooled β-cell (p<0.01) specificities (Figure 5). The
majority of these cells within the PPI\textsubscript{15-24} specificity coexpressed CD45RO and CCR7, whereas greater variability was apparent for the IGRP\textsubscript{265-273} and pooled \(\beta\)-cell specificities. Expression of CD57 was heterogeneous in all cases. These data confirm the preceding observations and define a \(\beta\)-cell-specific early memory phenotype associated with type 1 diabetes.

**Ex vivo repertoire analysis of \(\beta\)-cell-specific CD8 T cells**

To complement our flow cytometric studies, we examined the TCR repertoire of \(\beta\)-cell-specific CD8 T cell populations using a template-switch anchored RT-PCR to amplify all expressed TRB gene rearrangements in an unbiased and quantitative manner (24; 26). The PPI\textsubscript{15-24} specificity was considered representative for this purpose on the basis of consistent phenotypic differences between subject groups across all analyses. Sufficient numbers of cognate tetramer-binding CD8 T cells were isolated by ultrapure flow cytometric sorting from three type 1 diabetes patients and two healthy controls to enable this analysis. In all cases, the *ex vivo* repertoires were oligoclonal and highly skewed towards one or more dominant clonotypes (Table 2). No consistent amino acid patterning or length bias was apparent across the third complementarity-determining region (CDR3) and TRB gene usage was heterogeneous. Thus, the repertoire of CD8 T cells specific for PPI\textsubscript{15-24} is unpredictable and private (34).

**DISCUSSION**

In this study, we combined high definition polychromatic flow cytometry with ultrasensitive pHLAI tetramer staining to define the magnitude and differentiation status of \(\beta\)-cell-specific CD8 T cell populations in type 1 diabetes patients and healthy controls. Moreover, we achieved sufficient resolution with this approach to enable direct *ex vivo* analysis of the autoreactive TCR repertoire in a subset of individuals. In contrast to healthy controls, patients with newly diagnosed type 1 diabetes displayed hallmarks of antigen-driven expansion within the \(\beta\)-cell-specific CD8 T cell compartment. No such differences were observed between subject groups for persistent viral specificities or CD8 T cells globally. Collectively, these data identify phenotypic biomarkers of disease-relevant CD8 T cell-mediated autoimmunity in type 1 diabetes.

Remarkably, we found that \(\beta\)-cell-specific CD8 T cell frequencies in peripheral blood were similar between type 1 diabetes patients and healthy controls. These findings are consistent with some previous studies of presumed autoimmune conditions (35), but discrepant with other reports in the field (6; 17), most likely due to differences in detection sensitivity and cohort composition. Of particular note, the use of dasatinib to enhance tetramer staining thresholds revealed autoreactive CD8 T cells in the present study that were not visible with standard protocols. The reliable identification of naïve precursors in addition to antigen-experienced subsets readily explains the equivalent frequencies of \(\beta\)-cell-specific CD8 T cells between subject groups. It is also important to recognize that peripheral CD8 T cell frequencies do not necessarily reflect the tissue-localized immune cell environment. Indeed, histopathological evaluations of \(\beta\)-cell-specific CD8 T cell populations in the insulitic lesions of patients who died close to the time of diagnosis have demonstrated pronounced...
variability in antigen targeting between islets and individuals (3; 36). Accordingly, it is
difficult to equate quantitative measures of immune autoreactivity in the periphery with CD8
T cell-mediated events in the pancreas.

In contrast to the lack of simple numerical correlates, we found clear phenotypic signatures
of functionally relevant β-cell-derived antigen exposure in type 1 diabetes patients.
Conventional subset analyses revealed fewer T_N cells and greater numbers of T_{SCM} cells
across all pooled β-cell specificities within the CD8 compartment of patients with newly
diagnosed type 1 diabetes compared to healthy controls. Single marker evaluations
confirmed this overall pattern, with reduced expression of CD27 and CCR7 and elevated
expression of CD57 and CD95. The increased prevalence of antigen-specific T_{SCM} cells,
which serve as a reservoir to replenish more differentiated memory subsets (16; 37), could
perpetuate the underlying autoimmune process of β-cell destruction. It is also notable that
granzymes and perforin are strongly coexpressed with CD57, which acts accordingly as a
surrogate marker of cytolytic activity (38; 39). Such highly differentiated cells may therefore
associate with more severe disease manifestations (40-42). Multivariate analyses further
identified a phenotypically distinct CD27_{intermediate}CD95^+ population of β-cell-specific CD8
T cells in type 1 diabetes patients that was significantly less frequent in healthy controls,
again consistent with an antigen-driven disease process as reported previously in a viral
system (43). Importantly, all three analytical approaches yielded significant differences
between subject groups solely within the β-cell-specific CD8 T cell compartment. Thus, the
phenotypic profile of tissue-directed autoreactive CD8 T cell populations may act as a useful
biomarker of disease activity in type 1 diabetes.

In further experiments, we characterized the TCR repertoire of tetramer-binding CD8 T cells
specific for the PPI_{15-24} epitope. This specificity was selected on the grounds that robust
phenotypic differences were detected between type 1 diabetes patients and healthy controls
across all analytical strategies. Moreover, quantitative differences in the cognate CD8 T cell
population have previously been reported to associate with disease activity (17). The
enhanced detection sensitivity afforded by our approach facilitated this analysis, which
represents the first ex vivo characterization of autoreactive CD8 T cell clonotypes. In all
cases, we observed highly skewed oligoclonal repertoires reminiscent of virus-driven
expansions (24). These hierarchical structures could reflect focused autoantigen-specific
priming or cross-reactivity with an environmental trigger in the form of a highly
immunogenic pathogen-derived epitope (44). However, it is important to note that this
clonotypic pattern was not unique to the type 1 diabetes setting. Similar findings in two
healthy controls, associated with a small central memory expansion in an otherwise largely
naïve landscape in one (Control 2) and a highly differentiated predominant memory
phenotype in the other (Control 28), suggest the natural occurrence of heterologous
responses. Thus, oligoclonal β-cell-specific CD8 T cell populations can exist in the memory
pool of healthy individuals without concomitant evidence of disease activity. It is intriguing
to speculate that the highly private nature of these repertoires could provide a molecular
explanation for differential TCR-mediated outcomes in the immunopathogenesis of type 1
diabetes, akin to recent descriptions in viral systems (45-47).
Overall, the present study demonstrates that β-cell-specific CD8 T cells are more differentiated in patients with newly diagnosed type 1 diabetes compared to healthy controls. This key result identifies an immune correlate of disease activity that could be further refined at the clonotypic level to monitor organ-specific tissue damage in the periphery.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**ACKNOWLEDGEMENTS**

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Figure 1.
Identification of antigen-specific CD8 T cell populations. A-G: Thawed PBMCs were stained with pHLA-A*0201 tetramers representing PPI\textsubscript{15-24} (A), InsB\textsubscript{10-18} (B), IGRP\textsubscript{265-273} (C), IA-2\textsubscript{797-805} (D), GAD65\textsubscript{114-123} (E), CMV pp65\textsubscript{114-123} (F) and EBV BMLF1\textsubscript{280-288} (G). Gates were set serially on singlets, live CD3\textsuperscript{+}CD14\textsuperscript{−}CD19\textsuperscript{−} cells and lymphocytes prior to Boolean exclusion of dye aggregates and subsequent analysis in bivariate CD8 versus tetramer plots (Supplementary Figure 1 A). Representative paired data in the absence or presence of dasatinib are shown for type 1 diabetes patients and healthy controls. Tetramer-binding CD8 T cell frequencies are indicated.
Figure 2.
Summary of antigen-specific CD8 T cell frequencies. A-G: Thawed PBMCs were stained with pHLA-A*0201 tetramers representing PPI15-24 (A), InsB10-18 (B), IGRP265-273 (C), IA-2797-805 (D), GAD65114-123 (E), CMV pp65495-503 (F) and EBV BMLF1280-288 (G). Graphs show tetramer-binding CD8 T cell frequencies in the absence (empty symbols) or presence (filled symbols) of dasatinib for type 1 diabetes patients (T1D) and healthy controls (CTR). No significant differences across identical comparisons were observed between subject groups. Bars represent median values. Statistical analyses were performed using the Wilcoxon signed-rank test; p values ≤ 0.05 are shown.
Figure 3.
Phenotypic subset analysis of antigen-specific CD8 T cells. A: Pie chart representations of mean subset percentages for pooled β-cell-specific CD8 T cells and individual specificities in type 1 diabetes patients (top row) and healthy controls (bottom row). Subsets are defined and color-coded as follows: green, T_N (CD27+CD45RO−CD57−CD95−CCR7+); yellow, T_SCM (CD27+CD45RO−CD95+CCR7+); blue, T_EFF (CD27−CD45RO−CD95+CCR7−); red, all remaining memory cells. B: Column plots showing T_N and T_SCM subset percentages for pooled β-cell-specific CD8 T cells and individual specificities in type 1 diabetes patients (T1D) and healthy controls (CTR). Representative data are shown in Supplementary Figure 1 B&C. Almost identical results were obtained with the T_N subset defined in the absence of CD57 (CD27+CD45RO−CD95+CCR7+). Bars represent median values. Statistical analyses were performed using the Mann-Whitney U-test; p values ≤ 0.05 are shown.
Figure 4.
Single marker analysis of antigen-specific CD8 T cells. A-E: Percent expression of CD95 (A), CD57 (B), CD27 (C), CCR7 (D) and CD45RO (E) is shown for type 1 diabetes patients (T1D) and healthy controls (CTR) across all CD8 T cells and the indicated specificities. Corresponding data for individual β-cell-derived epitope-specific CD8 T cell populations are shown in Supplementary Figure 4. Bars represent median values. Statistical analyses were performed using the Mann-Whitney U-test; p values ≤ 0.05 are shown.
Figure 5.
Frequency difference gating analysis of antigen-specific CD8 T cells. Overlays of concatenated data from type 1 diabetes patients (cloud plots) and healthy controls (blue dots) are shown for the indicated β-cell specificities across bivariate phenotypic profiles. Populations of CD27^intermediateCD95^+ CD8 T cells, present at significantly higher frequencies in type 1 diabetes patients compared to healthy controls, are displayed as red dots.
## Table 1

### HLA-A*0201-restricted CD8 T cell epitopes

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<th>Epitope</th>
<th>Sequence</th>
<th>Subjects*</th>
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<td>Preproinsulin</td>
<td>PPI15-24</td>
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<td>Insulin B chain</td>
<td>InsB10-18</td>
<td>HLVEALYLV</td>
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<td>Inlet-specific glucose-6-phosphatase catalytic subunit-related protein</td>
<td>IGRP265-273</td>
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*Numbers of healthy controls/type 1 diabetes patients studied for each epitope specificity.*
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<tr>
<th>Subject</th>
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Individual clonotypes are represented in order of relative frequency. Gene usage and CDR3 amino acid sequence are shown in each case.