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Identification of Multiple Public TCR Repertoires in Chronic Beryllium Disease

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Chronic beryllium disease (CBD) is a granulomatous lung disease characterized by the accumulation of beryllium (Be)-specific CD4+ T cells in bronchoalveolar lavage. These expanded CD4+ T cells are composed of oligoclonal T cell subsets, suggesting their recruitment to the lung in response to conventional Ag. In the current study, we noted that all bronchoalveolar lavage–derived T cell lines from HLA-DP2–expressing CBD patients contained an expansion of Be-responsive Vβ5.1+ CD4+ T cells. Using Be-loaded HLA-DP2–peptide tetramers, the majority of tetramer-binding T cells also expressed Vb5.1 with a highly conserved CDR3β motif. Interestingly, Be-specific, Vβ1.1-expressing CD4+ T cells displayed differential HLA-DP2–peptide tetramer staining intensity, and sequence analysis of the distinct tetramer-binding subsets showed that the two populations differed by a single conserved amino acid in the CDR3β motif. TCR Va-chain analysis of purified Vβ5.1+ CD4+ T cells based on differential tetramer-binding intensity showed differing TCR Vα-chain pairing requirements, with the high-affinity population having promiscuous Vα-chain pairing and the low-affinity subset requiring restricted Vα-chain usage. Importantly, disease severity, as measured by loss of lung function, was inversely correlated with the frequency of tetramer-binding CD4+ T cells in the lung. Our findings suggest the presence of a dominant Be-specific, Vβ5.1-expressing public T cell repertoire in the lungs of HLA-DP2–expressing CBD patients using promiscuous Vα-chain pairing to recognize an identical HLA-DP2-peptide/Be complex. Importantly, the inverse relationship between expansion of CD4+ T cells expressing these public TCRs and disease severity suggests a pathogenic role for these T cells in CBD. The Journal of Immunology, 2014, 192: 4571–4580.

The ag-specific T cell repertoire is shaped by binding of the TCR hypervariable region to a diverse array of short processed peptides bound to MHC molecules. To ensure an adequate immune response to a vast number of potential Ags, the total theoretical T cell repertoire is extremely diverse and estimated at 2.5 × 107 T cells in an individual at any given time (1–5). Hence, it is surprising that nearly identical Ag-specific or public T cells have been identified in multiple individuals (1, 3, 6). Public T cells are characterized by the expression of identical TCR Vα and/or Vβ genes that are present in the majority of subjects and dominate the response to a specific epitope. Private repertoires are those Ag-specific T cells bearing TCRs that are unique to an individual. Despite public repertoires being restricted in nature, they are typically dominant and dictate disease severity (7–11). Most studies of public repertoires have involved MHC class I–restricted CD8+ T cells (1, 3, 6). Conversely, public repertoires have rarely been identified in the CD4+ T cell subset due, in most cases, to unknown stimulatory Ags and the unavailability of optimal tools such as MHC class II–peptide tetramers.

Chronic beryllium disease (CBD) is a granulomatous lung disease that occurs in genetically susceptible subjects exposed to beryllium (Be) in the workplace (12, 13). The onset of CBD is associated with the accumulation of Be-specific, Th1 cytokine–secreting CD4+ T cells in the lung (14, 15). With a known Ag and access to pathogenic CD4+ T cells from the lung, CBD is an important organ-specific immune-mediated disease, characterized by a CD4+ T cell alveolitis and lung fibrosis. Genetic susceptibility to CBD is strongly linked to HLA-DP alleles that contain a glutamic acid at the 69th position of the β-chain (βGlu69) (16–23), with the majority of CD4+ T cells recognizing Be in an HLA-DP–restricted manner. Importantly, the HLA-DP molecules that mediate Be presentation match those implicated in disease susceptibility, confirming that the mechanism of HLA contribution to disease susceptibility depends on Be presentation to pathogenic CD4+ T cells (24, 25). Previous characterization of a Be-responsive Vβ5.1/Vα22 TCR expressed on CD4+ T cells derived from the lung of an HLA-DP2–expressing CBD patient showed that Be-specific T cells recognized Ag using an unconventional binding topology, with the majority of interactions occurring between TCR Vβ5.1 residues and the HLA-DP2 β1-chain (26). We have recently identified mimotopes and self-peptides (e.g., those derived from plexin A4) that in the presence of Be complete the αβTCR ligand for the Vβ5.1/Vα22 TCR.

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The TCRα and TCRβ sequences presented in this article have been submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under accession numbers KJ026955–KJ026960.

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Abbreviations used in this article: BAL, bronchoalveolar lavage; Be, beryllium; CBD, chronic beryllium disease; Fcε, forced expiratory volume in 1 s; FVC, forced vital capacity; MECV, murine ecotropic virus.

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(27). In addition to anchoring to HLA-DP2 and interacting with TCR, our findings suggested that Be-dependent peptides play a novel role in metal ion coordination (27). Using Be-loaded HLA-DP2–mimotope-2 and plexin A4 tetramers, CD4+ T cells specific for these ligands were identified in the bronchoalveolar lavage (BAL) fluid of all HLA-DP2+ CBD patients analyzed (27).

In the current study, we hypothesized that these peptides may be one of the immunodominant epitopes in the lung of HLA-DP2+ CBD patients to select T cells expressing a public Vβ5.1+ TCR repertoire. We show that a Be-loaded, HLA-DP2–mimotope-2 tetramer predominantly stained Vβ5.1-expressing CD4+ T cells in the lung of HLA-DP2–expressing CBD patients, and sequencing of the TCR genes identified multiple oligoclonal T cell populations bearing a public Vβ5.1+ TCR repertoire. This public T cell repertoire exhibits extremely limited variation in CDR3β expression and distinct Vα-chain pairing requirements. Thus, the conserved elements of the TCR CDR3β of this public T cell repertoire suggests that the generation of Be-responsive CD4+ T cells specific for this potential immunodominant Ag allows promiscuous Vα-chain pairing to maintain Be specificity. In addition, the dominance of these Vβ5.1-expressing public T cells in the immunopathogenesis of CBD is further supported by an inverse relationship between the expansion of these T cells and lung function.

Materials and Methods

Study population

Experiments performed in the current study used CD4+ T cells derived from BAL of HLA-DP2–expressing CBD patients. The diagnosis of CBD was established using previously defined criteria (28, 29), including a history of Be exposure, the presence of granulomatous inflammation on lung biopsy, and a positive proliferative response of blood or BAL T cells to BeSO4 in vitro. Pulmonary function testing and exercise physiology were performed as part of the subject’s clinical evaluation (30). Informed consent was obtained from these subjects, and the protocol was approved by the Human Subject Institutional Review Board at the University of Colorado Anschutz Medical Campus and National Jewish Health.

Identification of Be-responsive T cell expansions using dual intracellular cytokine and TCR Vβ staining

Be-specific T cell lines were derived from BAL cells obtained from CBD patients as previously described (26). TCR Vβ expansions in Be-responsive, IFN-γ-expressing CD4+ T cells were identified in these T cell lines using dual staining with mAbs specific for IFN-γ and the most prevalent human TCR Vβ-chains. A minimum of 5 × 10^5 T cells were stimulated with 100 μM BeSO4 for 6 h in the presence of an equal number of autologous EBV-transformed lymphoblastoid cells. After 1 h, brefeldin A (10 μg/ml) was added, and cells were incubated at 37°C in a humidified 5% CO2 atmosphere for the remaining 5 h. Washed cells were stained with an anti-CD4-PerCP mAb (BD Biosciences) and an FcR blocking reagent (Miltenyi Biotec) and transferred to a 96-well round-bottom microtiter plate for incubation with 24 PE- and FITC-conjugated anti-TCR Vβ mAbs (IOTest β Mark; Beckman Coulter). The Arden nomenclature system was used to designate TCR Vβs (31). This panel of anti-TCR Vβ mAbs was used to stain TCR Vβ repertoire in healthy subjects. Cells were incubated for an additional 30 min at 4°C. Washed cells were fixed, permeabilized, and stained with anti-IFN-γ–allophycocyanin mAbs (Invitrogen) for 30 min at room temperature. The lymphocyte population was identified using forward and 90° light scatter patterns, and fluorescence intensity was analyzed using an LSRII flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star).

Analysis of TCRBV5S1 gene expression in BAL-derived T cell lines and ex vivo BAL CD4+ T cells

Be-specific T cell lines and ex vivo BAL CD4+ T cells were sorted based on dual staining with a Be-loaded HLA-DP2–mimotope-2 (FWIDLFETIG) tetramer (27) and an anti-TCR Vβ5.1 mAb. T cells were stained with 20 μg/ml PE-labeled tetramer in medium containing an anti-human Fc blocking Ab for 2 h at 37°C. Cells were stained with mAbs directed against CD3–Texas Red, CD4-PerCP–Cy5.5, and TCR-Vβ5.1–allophycocyanin.

A FITC-conjugated dump gate included mAbs directed against CD8, CD14, and CD19. Cells were stained for 30 min at 4°C, washed with 0.5% BSA-containing PBS, and sorted using an FACSAria flow cytometer (BD Immunocytometry Systems).

Sorted T cells were harvested, and RNA was isolated using a Qiagen RNeasy kit according to the manufacturer’s instructions (Qiagen). cDNA was prepared, and TCRB gene fragments were amplified using a TCRBV5S1 primer (5′-ATACCTCAGTGAACACAGAGAAAC-3′) and a TCRB primer (5′-TTCCTGATGCTCAAAACAC-3′). PCR products were purified using a DNA binding membrane spin column (Qiagen), ligated into the pcR2.1 TOPO cloning vector (Invitrogen), and transformed into DH5α competent cells. Purified plasmid DNA was isolated from bacterial colonies containing appropriate inserts and sequenced with an M13 reverse-sequencing primer.

In select experiments, single cells from a BAL-derived CD4+ T cell line were sorted, and TCRαV and BV gene expression was determined using a 5’ RACE and nested PCR method as previously described (32, 33). Briefly, T cells were stained with the PE-labeled HLA-DP2–mimotope-2/Be tetramer and anti-TCR Vβ5.1 mAb as described above and sorted as described above directly into a reverse-transcription buffer.

Generation of T cell hybridomas expressing Be-specific TCRs

T cell hybridomas were cloned into a murine stem cell virus (MSCV) plasmid for retroviral transduction into a murine T cell αβ+ T cell hybridoma line that expresses human CD4 (designated 5KC-9C5), as described previously (26, 34). PCR fragments encoding the extracellular domains of the TCR α- and β-chains identified from each T cell were cloned into separate MSCV plasmids that encode an internal ribosomal entry site, GFP reporter for selection and either a murine Cx or CBβ domain. Full-length chimeric TCRα and TCRB gene constructs were packaged as retrovirus by transient transfection of Phoenix 293T cells with the MSCV plasmids as described previously (26). 5KC-9C5 cells were transduced with filtered viral supernatant using a spin-infection protocol as previously described (35). Positively staining cells were sorted as described above.

T cell hybridoma activation assays and HLA-DP2 tetramer staining

T cell hybridoma clones (1 × 10^5) and murine fibroblasts transfected to express HLA-DP2 (2.5–5.0 × 10^6) were incubated overnight at 37°C with various concentrations of BeSO4 and 500 nM mimotope-2 peptide, and IL-2 was measured in supernatants using the mouse IL-2 Ready-Set-Go ELISA kit (eBioscience) as described previously (26). Activation curves were generated by plotting percentage of maximal IL-2 release—[A450 (sample) − A450 (control)] / [maximum A450 (sample) − A450 (control)] × 100—against Ag concentration. The concentration of BeSO4 required for half-maximal IL-2 release, or EC50 value, was determined using nonlinear regression (sigmoidal fit; GraphPad Prism; GraphPad) of the activation curves.

In separate experiments, T cell hybridomas were stained with Be-loaded HLA-DP2–mimotope-2 (FWIDLFETIG) and Be-loaded HLA-DP2–plexin A4 (FVDDLFETIF) tetramers as previously described (27). An HLA-DP2–mimotope-2 tetramer that had not been pulsed with Be was used as an negative control staining reagent. In select experiments, an mAb specific for the mouse TCR CBβ domain (clone H57-597) was added at 1 μg/ml to aggregate cell-surface TCR prior to staining with the Be-loaded HLA-DP2–mimotope-2 tetramer (36).

Statistical analysis

ANOVA analysis was used to calculate the significant difference between samples tested. A p value < 0.05 is considered statistically significant. A Spearman correlation was used to compare the frequency of tetramer-binding CD4+ T cells with markers of lung function.

Results

Be-responsive Vβ5.1+ CD4+ T cell expansions in T cell lines derived from CBD patients

We have previously shown an increased frequency of Be-responsive Vβ5.1+ CD4+ T cells in the lung of HLA-DP2–expressing CBD patient 1332 (26). To determine if Be-responsive Vβ5.1+ CD4+ T cell expansions exist in other CBD patients, we stimulated BAL-derived CD4+ T cell lines from two additional CBD patients with BeSO4 and identified TCR Vβ expansions in the Be-responsive, IFN-γ–expressing T cell populations (Fig. 1). We focused on IFN-γ because it is the predominant cytokine expressed by Be-specific
CD4+ T cells (14, 15). Similar to patient 1332, CD4+ T cell lines derived from the BAL of HLA-DP2-expressing CBD patients. Data are expressed as the percentage of IFN-γ (black bars) or IFN-γ− (white bars) CD4+ T cells expressing a particular Vβ. An asterisk (*) is used to identify expanded Vβ subsets in the Be-responsive, IFN-γ-expressing CD4+ T cell subset in individual patients.

CD4+ T cells (14, 15). Similar to patient 1332, CD4+ T cell lines derived from BAL of patients 1056 and 1435 also expressed Vβ5.1+ T cell expansions as measured by an increased frequency (10–30%) of IFN-γ− producing Vβ5.1+ cells compared with non–IFN-γ− producing cells (<6%) (Fig. 1). Other TCR Vβs were also expanded in the IFN-γ− CD4+ T cell subsets. For example, Be-responsive expansions of CD4+ T cells expressing Vβ1, Vβ2, and Vβ13.6 were seen in patients 1332, 1056, and 1435, respectively (Fig. 1). These findings demonstrate the presence of shared as well as unique Be-responsive TCR Vβ expansions in the BAL of CBD patients.

Be-loaded HLA-DP2–mimotope-2 tetramer predominantly stains Vβ5.1+ T cells from ex vivo BAL cells of CBD patients. Using a Be-loaded HLA-DP2–mimotope-2 tetramer, we have previously shown a high frequency of CD4+ T cells in the BAL of all HLA-DP2-expressing CBD patients that bind to this peptide–MHC class II/Be complex (27). To determine whether the TCR repertoire of Be-responsive T cells specific for this ligand is restricted or diverse, we stained ex vivo BAL cells from four HLA-DP2+ CBD patients. For example, Be-responsive expansions of CD4+ T cells expressing Vβ1, Vβ2, and Vβ13.6 were seen in patients 1332, 1056, and 1435, respectively (Fig. 1). These findings demonstrate the presence of shared as well as unique Be-responsive TCR Vβ expansions in the BAL of CBD patients.

Importantly, CD4+ T cells expressing Vβ5.1 comprised the predominant tetramer-binding population. For example, 95, 70, 76, and 50% of tetramer-binding CD4+ T cells expressing Vβ5.1 in the four HLA-DP2+ CBD patients was 73 ± 9.2%, suggesting that the predominant Vβ subset on CD4+ T cells specific for this particular ligand is Vβ5.1 (Fig. 2B). Similar findings were also seen when using the Be-loaded HLA-DP2–plexin A4 tetramer (data not shown). None of the other Vβs analyzed in Fig. 2B were expressed on <2% of the tetramer-binding CD4+ T cell subset (Fig. 2B). Using an irrelevant IAβ-insulin10–23 tetramer (38), nonspecific tetramer staining was not observed (data not shown). Thus, with either IFN-γ expression as a measure of overall Be responsiveness (Fig. 1) or HLA-DP2–mimotope-2 tetramer binding as a measure of epitope specificity (Fig. 2), CD4+ T cells expressing Vβ5.1 are present in all HLA-DP2+ CBD patients analyzed to date, suggesting that this Be-specific T cell subset is responding to an immunodominant Ag in the lung and...
may represent a public T cell repertoire. As a result, we initially focused on those Be-specific CD4+ T cells expressing Vβ5.1.

Vβ5.1+ T cells express a conserved CDR3β motif and represent a public TCR repertoire

Based on the presence of Be-specific Vβ5.1+ T cell expansions in multiple CBD patients, we next determined if these expansions consisted of a public TCR repertoire having a conserved CDR3β motif. We were especially interested to see if the related CDR3β sequences identified in patient 1332 (26) were present in other CBD patients. To examine the repertoire of Vβ5.1-expressing T cells, a BV5S1 primer was used to PCR-amplify cDNA generated from CD4+ T cells derived from three BAL T cell lines and four ex vivo BAL samples from CBD patients. The PCR products from each sample were cloned, and bacterial isolates were selected and sequenced to determine the nucleotide and deduced amino acid sequences of the CDR3β. A highly related CDR3β motif was evident in all patients studied (Fig. 3). This conserved CDR3β motif consists of an identical length, conserved J region expression (BJ2S5 or BJ1S4) and homologous amino acid residues surrounding an essential glutamine (Q) residue at position 97 of the β-chain (Fig. 3). For example, surrounding the Q in the NDβN of these related CDR3βs, small, noncharged amino acids such as alanine (A), glycine (G), and serine (S) were preferred (Fig. 3).

Two predominant CDR3β sequences were present in all CBD patients described in this study, consisting of either AQGG or QQGG in the NDβN and using BJ2S5 (Fig. 3). Fig. 4 shows examples of identical or nearly identical β-chain amino acid sequences in different patients that were encoded by different nucleotides (i.e., expressed by different Vβ5.1+ T cell clones), thus precluding the possibility of a PCR contamination or artifact. The nucleotides that comprise the predominant AQGG and QQGG populations shown in Fig. 4 are primarily encoded by germline BV5S1 (highlighted in blue) and BDI1 (red). The complete BV5S1 gene is expressed in these public β-chains with the last nucleotide (guanine) of the BV5S1 gene dictating the expression of an A or G at position 1 of NDβN. Few N-region additions are used to generate the conserved XQGG motif in NDβN with ~50% of the unique sequences having as few as two to three N-region nucleotide additions (black) (Fig. 4). Taken together, these findings provide strong evidence for the selection and expansion of particular TCRβ gene–expressing T cells in response to the same antigenic stimulus found in the lung of all HLA-DP2–expressing CBD patients.

Be-loaded HLA-DP2–mimotope-2 tetramer detects distinct Vβ5.1 clonal populations of T cells

Using the Be-loaded HLA-DP2–mimotope-2 tetramer, we noted two distinct Vβ5.1+ tetramer-binding populations. As shown in Fig. 5A, tetramer-binding Vβ5.1+ CD4+ T cells from T cell lines derived from CBD patients 1435 and 1056 could be divided into high- and low-intensity (tethi and tetlo) groups based on tetramer staining. Similar to the ex vivo BAL cells shown in Fig. 2A, the Be-loaded tetramer stained predominantly Vβ5.1+ CD4+ T cells (e.g., 82 and 86% for the T cell lines derived from CBD patients 1435 and 1056, respectively) (Fig. 5A). The tethi and tetlo populations from patient 1435 were isolated by FACS sorting, and junctional region nucleotide sequencing of the BV5S1 PCR products revealed subtle amino acid differences in the conserved CDR3β motif (Fig. 5B). Variability in the amino acid composition between the tethi and tetlo populations was mainly found at position 96 of the conserved XQGG motif in the CDR3β (Fig. 5B). The tethi T cells predominantly expressed an A at this position, whereas tetlo T cells expressed a G or S residue (Fig. 5B). For example, in patient 1435, the predominant AQGG motif in the CDR3β of the tethi population was observed in 86% of the sequenced bacterial clones, whereas SQGG was expressed in 11% of clones tested (Fig. 5B). Both of these CDR3β–chains were paired with BJ2S5. The CDR3βs comprising the tethi population expressed a more diverse set of amino acids, with 50% of the sequences expressing SQGG, 24% MGQGG, and 15% GQGG, coupled with either BJ2S5 or BJ1S4 (Fig. 5B).

Similar to patient 1435, the tethi population from patient 1056 exclusively expressed an AQGA sequence (Fig. 5C). The tethi population expressed a diverse set of Vβ5.1 sequences with ~50% of the Vβ5.1 chains expressing SQGG and 24% QQGG. Our findings suggest that the high-frequency clonotypes in the tethi and tetlo sorted populations are the predominant T cell populations. Whether the low-frequency TCRβBV sequences represent a true subset or the result of cross-contamination resulting from cell sorting of closely related populations is unknown.

Subtle differences in CDR3β composition of the Vβ5.1 chain affect Vα-chain requirements and maintenance of Be specificity

Next, we queried whether differences in TCR Vα-chain usage could account for differing HLA-DP2 tetramer binding affinities
FIGURE 4. Oligoclonal expansions of nearly identical TCRBV5S2 chains expressed in multiple patients diagnosed with CBD. Shown are nucleotide and deduced amino acid sequences of two related sets of CDR3β expressing nearly identical TCRBV5S2 chains that, in most cases, differed by a single amino acid at the first position of the NDβN region. The number of identical sequences (defined at the nucleotide level) is shown over the total number of sequences analyzed. The cysteine (C) of the β-chain is designated as position 91 with the essential glutamine (Q) at position 97. Boldface amino acids are generated by either all nontemplate-encoded nucleotides or a combination of nontemplate- and germline-encoded nucleotides. Nucleotides highlighted in blue, red, green, and white are encoded by all nontemplate- and germline-encoded nucleotides. Boldface amino acids denote those that are encoded by all nontemplate nucleotides or a combination of nontemplate and germline-encoded nucleotides.

FIGURE 5. Distinct TCR VB5.1 CDR3β sequences in tetβ and tetβ CD4⁺ T cell populations from CBD patients. (A) Density plots of HLA-DP2–mimotope-2/Be tetramer staining of CD4⁺ T cells from BAL-derived T cell lines of multiple CBD patients (Fig. 4). Single-cell PCR on sorted tetβ and tetβ VB5.1⁺ CD4⁺ T cell populations from patient 1435 was used to determine the accompanying native Vα-chains. We identified Vα1 chain pairing with the tetβ VB5.1 (AQGG) chain and Vα8 chain pairing with the varying VB5.1 chains isolated from the tetβ population (Fig. 6). Both the Vα1 and Vα8 TCRs used the Jα28 gene segment that encodes an essential tyrosine (Y) at position 95 of the CDR3α (bolded in Fig. 6) (26). We have previously shown that a Be-specific, VB5.1/Vα22-expressing T cell hybridoma with a CD3β chain could pair with multiple Vα-chains, including Vα22, Vα8, and Vα9, with the sole α-chain requirement being a Y expressed by Jα28 (26). We sought to determine the extent of Vα-chain cross-pairing for the AQGG and GQGG CD3β VB5.1 chains isolated from patient 1435. These VB5.1 chains were paired with Vα22, Vα8, Vα9, or Vα1 (CRD sequences shown in Supplemental Table I), and the resultant TCRs were expressed on the surface of an αβ⁻ murine T cell hybridoma in equivalent amounts (Supplemental Fig. 1). The hybridomas were stained with PE-labeled, HLA-DP2–mimotope-2/Be and HLA-DP2–plexin A4/Be tetramers (Fig. 7A). Although tetramer staining intensity varied, T cell hybridomas expressing VB5.1 with the AQGG NDβN region stained with both tetramers when paired with all of the Vα-chains tested (Fig. 7A). Conversely, the VB5.1 chain expressing the GQGG NDβN region could only bind to the Be-loaded HLA-DP2–mimotope-2 tetramer when paired with its native Vα8 chain (Fig. 7A), and none of the GQGG-expressing hybridomas were capable of binding to the HLA-DP2–plexin A4/Be tetramer.

To confirm the Be specificity observed by tetramer staining, IL-2 secretion by T cell hybridomas expressing different VB5.1/Vα TCR pairs was measured in response to BeSO₄ and an optimal concentration of mimotope-2 using HLA-DP2–expressing fibroblasts as APCs. Similar to HLA-DP2 tetramer staining, all of the T cell hybridomas expressing VB5.1 with the AQGG NDβN region and paired with Vα22, Vα8, Vα9, or Vα1 secreted identical levels of IL-2 in response to Ag exposure (Fig. 7B). In contrast, only the GQGG-expressing VB5.1 chain paired with Vα8 was Be specific and secreted IL-2 after mimotope-2/Be exposure. Finally, the Vα8 chain was the optimal α-chain for pairing with both the AQGG- and GQGG-containing VB5.1 chains, as indicated by a shift in the IL-2 response curve to the left and a 4-fold lower EC₅₀ value (Fig. 7C). Thus, subtle differences in the CDR3β can...
significantly affect the TCR Vα-chain pairing required for an optimal response to Be.

Identification of an additional Be-specific public T cell repertoire

Although our data clearly show that Vβ5.1+ CD4+ T cells comprise the predominant HLA-DR2-tetramer binding population, other Vβs are also capable of binding to the HLA-DR2-mimotope-2/Be tetramer (Fig. 2A). Approximately 50% of Be-loaded HLA-DR2 tetramer–binding T cells from patient 1234 expressed a Vβ other than Vβ5.1 (Fig. 2A), and the other tetramer-binding Vβ(s) was not identified with our panel of anti-TCR Vβ mAbs (Fig. 2B). Thus, we sorted tetramer+ Vβ5.1+ CD4+ T cells, and the TCR Vα- and Vβ-chains used by these T cells were identified by PCR using a complete set of primers specific to TCRαV and BV genes. We identified BV18S1 sequences in patients 1234 and 3812 expressing a CDR3β motif with a Q residue surrounded by smaller amino acids (Fig. 8A), similar to that described for Vβ5.1. For both 1234 and 3812, the accompanying Vα-chain was AV4S2, with a related CDR3α and AJ38 as shown in Fig. 8A. To demonstrate Be responsiveness of the Vβ18/AV4 tetramers, we expressed each TCR on the surface of a murine T cell hybridoma and measured for IL-2 secretion after BeSO4 stimulation (Fig. 8B). T cell hybridomas expressing Vα4 and Vβ18 chains identified in patients 1234 and 3812 were indeed Be specific and secreted identical levels of IL-2 in the presence of mimotope-2 peptide, BeSO4, and HLA-DP2–mimotope-2–expressing ACPs. As shown in Fig. 8C, both Vβ18-expressing T cell hybridomas also bound the Be-loaded HLA-DR2-mimotope-2 tetramer. Although not examined extensively, our findings of nearly identical Be-responsive Vβ18/Vα4-expressing CD4+ T cells found in the lungs of two CBD patients suggests the presence of an additional public TCR repertoire.

Loss of lung function in CBD patients correlates with increased frequency of Be-loaded HLA-DR2–mimotope-2 tetramer staining of CD4+ T cells

To demonstrate that the public Vβ5.1-expressing T cells are pathogenic in HLA-DR2–expressing CBD patients, we assessed the relationship between frequency of tetramer+ CD4+ T cells in the BAL of seven CBD patients with parameters of lung function. Decreases in lung physiologic measures, such as forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV1), and exercise capacity, as measured by workload, are associated with worsening lung fibrosis (30). As shown in Fig. 9, an inverse correlation ($r = -0.93$, $p = 0.007$) was seen between the percentage of tetramer+ CD4+ T cells in the BAL and FVC (percent predicted). Similarly, an inverse correlation was seen between the frequency of Be-responsive CD4+ T cells specific for this αβ TCR ligand and FEV1 (percent predicted; $r = -0.82$, $p = 0.03$) and work load ($r = -0.65$; $p = 0.04$) (Fig. 9). Conversely, no correlation was noted between tetramer staining and gas exchange (data not shown). Collectively, these data provide a link between the expansion of Be-specific public Vβ5.1-expressing CD4+ T cells in the target organ of CBD patients and disease severity.

Discussion

Pathological immune responses to metal ions such as Be are among the most common causes of T cell–mediated hypersensitivities in humans, yet the nature of Ag presentation and subsequent T cell recognition is poorly characterized. Similar to recognition of self-peptides by autoimmune TCRs (39–43), Be-specific TCRs use an unconventional binding topology to recognize the HLA-DR2–peptide/Be complex (26). We have recently identified mimotopes and self-peptides that complete the αβ TCR ligand for a set of Be-specific TCRs derived from the lung of a CBD patient, and Be-loaded HLA-DR2-mimotope-2–tetramers identified CD4+ T cells specific for this complex in all HLA-DR2-expressing CBD patients (27). In the current study, we identify: 1) an epitope-specific public TCR Vβ5.1 and Vβ18 repertoire expressed on CD4+ T cells derived from the lungs of CBD patients; 2) TCR Vα-chain promiscuity based on Be-loaded HLA-DR2–mimotope-2 tetramer staining affinity; and 3) a link between expansion of CD4+ T cells expressing these public Vβ5.1 TCRs and disease severity, suggesting a pathogenic role for this T cell subset in CBD. Collectively, the identification of public, HLA-DR2–restricted T cell repertoires will aid in our understanding of the role of charged polymorphic amino acids (e.g., βGlu50) in HLA-DR molecules and the generation of immunodominant epitopes in driving the development and progression of CBD.

To date, most public T cell repertoire studies have focused on MHC class I–restricted CD8+ T cells specific for either infectious agents or malignant cells (1, 3, 6), and soluble MHC-based tetramer technology has been used to track Ag-specific CD8+ T cell responses in human disease (44–47). Unfortunately, the use of MHC class II tetramers has lagged due to technical issues in generating the reagents, suboptimal staining procedures, and low frequencies and affinities of Ag-specific CD4+ T cells (36, 48, 49). To our knowledge, this is the first study to use a soluble MHC class II–peptide tetramer to identify and characterize public HLA-DR2–restricted CD4+ T cell repertoires. Our success in detecting ex vivo Be-specific CD4+ T cells in the BAL of CBD patients is likely related to the high affinity of these TCRs for the HLA-DR2–mimotope-2/Be complex, with a $K_D$ of 4.6 μM as measured by surface plasmon resonance (27). This value is at the higher end for most TCR–peptide–MHC interactions, which typically range between 10 and 100 μM (50).

Public T cell repertoires are defined by the expression of conserved V, CDR3, and J regions (6). Importantly, this type of TCR bias has been infrequently demonstrated in CD4+ T cells obtained from blood or the target organ of human subjects. In this study, we used Be-loaded HLA-DR2–mimotope-2 tetramers to identify and characterize public Vβ5.1 and Vβ18 T cell repertoires in the lung of HLA-DR2–expressing CBD patients. We showed that nearly

<table>
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**FIGURE 6.** Deduced TCR CDR3 sequences from Vβ5.1+ tet5.1 and tet5.1 CD4+ T cell lines. Single cells of tet5.1 and tet5.1 Vβ5.1+ CD4+ T cell populations isolated from patient 1435 were sorted, and PCR was used to identify paired TCR Vα and BV5.1 chains. Amino acid sequences of both TCR CDR3α and CDR3β are shown for clones identified from both populations. Redundancy refers to the number of independent T cell clones that had identical TCRα and TCRβ gene nucleotide sequences.
identical Vβ5.1+CD4+ T cells exist in ex vivo BAL T cells and long-term T cell lines derived from the lung of HLA-DP2+ CBD patients. The TCR Vβ5.1 chains display TCR bias with expression of highly conserved Vβ5.1, CDR3β, and J regions. The CDR3β core motif consists of 4 aa (XQGG) at the V/D/J junction, where X at position 96 of the TCR β chain represents small, noncharged amino acids such as A, G, and S. This position is encoded by the Vβ5.1 germline nucleotide deletions and nontemplated nucleotide insertions during V–D joining. Interestingly, the entire TCRBV5S1 gene is encoded in the public repertoire, with the last nucleotide of this gene likely providing the first essential nucleotide (guanine) that is required to encode the A and G at position X of the XQGG motif. In addition, both the QG and GQG motifs are encoded by the CDR3β BD1 germline gene segment and the strict requirement for the QG motif in this public repertoire (1, 3). Our previous site-directed mutagenesis study showed the importance of the Q since mutating it to an A abolished the Be-induced T cell response (26). In contrast, small variations in amino acid composition are tolerated at the other positions of the motif, in particular A at positions 98 and 99.

Previous studies have shown that nongermline components of the CDR3β can influence TCR Vα- and Vβ-chain pairing (51, 52). In this study, we showed that a Be-loaded HLA-DP2 tetramer differentially stained distinct CD4+ T cell populations. These T cell subsets vary at the first position of the conserved XQGG motif of the CDR3β loop such that a single non–germline-derived methyl group dictates the extent of Vα-chain cross-pairing needed to maintain Ag specificity. The GQGG+ Vβ5.1 tetβ+ chain requires pairing with the native Vα8 chain to generate a Be-specific response. For pairing with the tetβ Vβ5.1 AQGG chain, the requirement for a specific Vα-chain was more promiscuous, with multiple Vα-chains expressing differing germine CDR1α and -2α loops being sufficient to maintain Be recognition. The only α-chain requirement was a conserved J region (Jα28) with an essential Y at
position 95 of CDR3α (26). Our findings suggest that the tet\(^{th}\) T cells are dominant and have a competitive advantage compared with the tet\(^{th}\) T cells due to their ability to pair with multiple V\(_\alpha\) chains, thus increasing the likelihood of their being highly represented in the repertoire.

Glycine-rich CDR3\(\beta\)s can generate cross-reactive TCRs due to the flexible nature of this loop (53). Increased flexibility guarantees T cell responsiveness and elimination of a wide range of pathogens (5). We suggest that the glycine-rich GQGG\(^+\) CDR3\(\beta\) loop from the tet\(^{th}\) T cell population is more flexible than the tet\(^{th}\) AQGG\(^+\) CDR3\(\beta\) loop due to the absence of the additional methyl group. The GQGG\(^+\)V\(\beta\)5.1\(^+\) chain requirement for V\(\alpha\)8 chain pairing may involve enhanced interchain stabilization from amino acids expressed in the CDR1\(\alpha\) and -2\(\alpha\), which may not be required by the more rigid AQGG\(^+\) CDR3\(\beta\) loop (54). The ability of germline residues in the V\(\alpha\) chain to modify V\(\beta\)\(\beta\) interactions with Ag has been previously reported in the murine IA\(^b\)-3K system (54). Even though the cognate V\(\alpha\)1 chain pairs with AQGG\(^+\) V\(\beta\)5.1, V\(\alpha\)8 pairing provides optimal Be recognition, as evidence by an enhanced T cell response. Perhaps the same CDR1\(\alpha\) and/or -2\(\alpha\) residues in V\(\alpha\)8 have a similar positive effect when paired with the AQGG\(^+\) V\(\beta\)5.1 chain as occurs with the GQGG\(^+\) V\(\beta\)5.1 chain. We know from our previous mutagenesis studies that the V\(\beta\)5.1 chain from patient 1332 dominates in TCR recognition of Be (26). This is also true in this study, in which the tet\(^{th}\) V\(\beta\)5.1 T cells from patient 1435 do not require specific residues from CDR1\(\alpha\) or -2\(\alpha\). Conversely, the tet\(^{th}\) V\(\beta\)5.1 T cells are more dependent on the V\(\alpha\) chain for Be recognition, and this requirement for V\(\alpha\)8 may induce a different binding mode for maintenance of Be specificity.

Recent studies have shown that initial recombination events dictate the probability of the occurrence of a public T cell repertoire. These findings are supported by deep sequencing techniques that have identified memory T cell sequences in the naive T cell pool (55), diluting evidence suggesting that thymic selection events dominate in the generation of the memory T cell pool. Studies have suggested various mechanisms that support the role for initial gene recombination events in generating public repertoires. For example, convergent recombination suggests that public repertoires exist due to an increased probability of particular nucleotide sequences occurring in the naive T cell repertoire (1, 3, 45, 55, 56). If the amino acids comprising the CDR3\(\beta\) can be encoded by many nucleotide combinations, there is higher probability of their expression. P-nucleotide additions contributed by the J region during initial recombination events can also generate public repertoires (57). Furthermore, a CDR3\(\beta\) encoded exclusively by germline-derived nucleotides will exist more frequently than one with multiple nucleotide insertions (1, 3). Many of the public V\(\beta\)5.1\(^+\) chains described in this study express as few as two to three nucleotide insertions in the CDR3\(\beta\) and were associated with restricted J\(\beta\)2.5 chain usage. Of the J\(\beta\)2 cluster, J\(\beta\)2.5 was the most likely to preferentially pair with D\(\beta\)1 (58), further supporting a role for biased gene recombination in the generation of this public repertoire. It is also possible that the public V\(\beta\)5.1 repertoire uses promiscuous V\(\alpha\)-chain pairing to maintain TCR diversity in the lung of CBD patients. For example, in murine influenza A virus infection, the biased TCR V\(\alpha\) or V\(\beta\)-chain dictates Ag specificity while the accompanying chain increases diversity to ensure recognition of a wide range of additional Ags (53, 59, 60).

Most TCRs express CDR3\(\alpha\) and CDR3\(\beta\) regions of similar length to recognize Ag with optimal affinity (61). The distance between the conserved cysteine at position 91 of the V-region and the F of the J-region is 12 aa for CDR3\(\beta\) and 14 aa for CDR3\(\alpha\) of the public T cells described in this study. It is likely that the requirement for a Y at position 95 of the CDR3\(\alpha\) selects for an N region of 2 aa to ensure expression of loop size similar to CDR3\(\beta\) as well as to maintain proper positioning of this critical Y residue. In our previous study using site-directed mutagenesis of the amino acids in the CDR3\(\alpha\) region, changing the Y at position 95 to alanine abolished the Be-induced T cell response (26). The Y can be contributed to the TRCA gene either by nontemplated base additions or from the TCRAJ germline. Only 2 of 61 AJ gene segments (AJ28 and AJ45) encode a Y that maintains proper positioning, and usage of AJ28 is clearly the preferred mechanism for generation of these \(\alpha\)-chains. Additionally, the requirement of Y at 95 may explain the stronger restriction of J\(\alpha\) usage compared with V\(\alpha\) usage.

The intensity of MHC tetramer staining has been correlated with T cell affinity and functional avidity (36, 62). An affinity threshold of 1–5 \(\mu\)M exists in which higher affinities no longer enhance functional avidity and tend to increase cross-reactivity with self-derived Ags (62, 63). Although the HLA-DP2–mimotope-2/Be tetramer stained tet\(^{th}\) and tet\(^{th}\) V\(\beta\)5.1\(^+\) populations with varying intensities, these differences did not correspond with functional avidities, as shown by Be-specific T cell hybridoma responses. We observed identical EC\(50\) IL-2 secretion values when stimulating native tet\(^{th}\) V\(\beta\)5.1 (AQGG)/V\(\alpha\)1 and tet\(^{th}\) V\(\beta\)5.1 (GQGG)/V\(\alpha\)8 T cell hybridomas with varying Be concentrations. These findings suggest that the affinities of these two V\(\beta\)5.1 populations for HLA-DP2–mimotope-2/Be are at the higher end of the affinity threshold and thus would not enhance the functional avidity (i.e., decrease the EC\(50\) value) of the tet\(^{th}\) population.

In conclusion, we used a Be-loaded HLA-DP2–mimotope-2 tetramer to identify and characterize a public V\(\beta\)5.1 T cell repertoire, which varies in its V\(\alpha\)-chain pairing requirements. Our findings suggest that the selection mechanism used to generate these public TCRs involves initial gene recombination of primarily germline-encoded genes to generate the V\(\beta\)1D\(\beta\)N1-J\(\beta\)1\(^+\) chain and a requirement of a Y encoded by a restricted J\(\beta\)28 to maintain the length of the V\(\alpha\)-chain. The association between the presence of public V\(\beta\)5.1\(^+\)CD4\(^+\) T cells in the CBD lung and markers of disease severity further supports the pathogenic nature of this Be-specific T cell subset and suggests that quantitation of these T cells by tetramer staining may be used as a marker of disease progression in CBD patients.
Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Table 1: CDRs of Vα chains*

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<th>Clone</th>
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<th>2'CDR1&lt;sup&gt;2z&lt;/sup&gt;</th>
<th>3'CDR2&lt;sup&gt;2f&lt;/sup&gt;</th>
<th>9'CDR3&lt;sup&gt;9g&lt;/sup&gt;</th>
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<td>ATKADDKG</td>
<td>ALS&lt;sub&gt;1&lt;/sub&gt;YSGA&lt;sup&gt;†&lt;/sup&gt;</td>
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</tr>
<tr>
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<td>YFSGDPLV</td>
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*Location of the amino acids corresponding to each of the CDRs is shown.
†The n region of the CDR3α is denoted by an underline.
Supplemental Figure 1. Levels of TCR expressed on the cell surface of murine T cell hybridoma lines, 5KC-9C6. Flow cytometry was used to measure cell surface levels of Vβ5.1+ AQGG or GQGG CDR3β chains paired with multiple Va chains. Positive populations are depicted by a dashed line and represent cells stained with a mAb specific for the murine T cell receptor Cβ domain. The negative population as depicted by a solid line and represents unstained cells.