Metal-specific CD4\(^+\) T-cell responses induced by beryllium exposure in HLA-DP2 transgenic mice

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Chronic beryllium disease (CBD) is a granulomatous lung disorder that is associated with the accumulation of beryllium (Be)-specific CD4\(^+\) T cells into the lung. Genetic susceptibility is linked to HLA-DP\(B1\) alleles that possess a glutamic acid at position 69 (\(\beta\)Glu69), and HLA-DP\(B1^{*02:01}\) is the most prevalent \(\beta\)Glu69-containing allele. Using HLA-DP2 transgenic (Tg) mice, we developed a model of CBD that replicates the major features of the human disease. Here we characterized the T-cell receptor (TCR) repertoire of Be-responsive CD4\(^+\) T cells derived from the lungs of Be oxide–exposed HLA-DP2 Tg mice. The majority of Be-specific T-cell hybridomas expressed TCR V\(\beta\)6, and a subset of these hybridomas expressed identical or nearly identical \(\beta\)-chains that were paired with different \(\alpha\)-chains. We delineated mimotopes that bind to HLA-DP2 and form a complex recognized by Be-specific CD4\(^+\) T cells in the absence of Be. These Be-independent peptides possess an arginine at p5 and a tryptophan at p7 that surround the Be-binding site within the HLA-DP2 acidic pocket and likely induce charge and conformational changes that mimic those induced by the Be\(^{2+}\) cation. Collectively, these data highlight the interplay between peptides and Be in the generation of an adaptive immune response in metal-induced hypersensitivity.

INTRODUCTION

Chronic beryllium disease (CBD) is a classic example of a human disorder resulting from gene-by-environment interactions. Genetic susceptibility to CBD is linked to HLA-DP alleles that contain a glutamic acid at the sixty-ninth position of the \(\beta\)-chain (\(\beta\)Glu69),\(^1\) with the prevalence of \(\beta\)Glu69-expressing HLA-DP alleles ranging from 73 to 95% of CBD patients.\(^2,4\) In addition, the probability of CBD increases with HLA-DP \(\beta\)Glu69 copy number and increasing workplace exposure to beryllium (Be).\(^7\) Depending on genetic susceptibility and exposure, CBD develops in up to 18% of Be-exposed workers.\(^1,8-10\) The disease is characterized by granulomatous inflammation and an influx of T helper type 1–polarized CD4\(^+\) T cells that express a differentiated memory T-cell phenotype\(^1,11-13\) and an oligoclonal T-cell receptor (TCR) repertoire.\(^14,15\) Importantly, the vast majority of Be-specific CD4\(^+\) T cells recognize antigen in an HLA-DP-restricted manner,\(^1,6,17\) and the HLA-DP molecules that mediate Be presentation match those implicated in disease susceptibility. These findings confirm that the mechanism of HLA contribution to disease susceptibility is based on the ability of those molecules to bind and present Be to pathogenic CD4\(^+\) T cells.\(^1,6,17\)

We recently developed an HLA-DP2 transgenic (Tg) murine model of Be-induced disease.\(^18\) Intratracheal exposure of these mice to Be oxide (BeO) induced mononuclear cell infiltrates in the lung and a Be-specific adaptive immune response in the lung and spleen that was CD4-dependent and HLA-DP2-restricted. Using Be-loaded HLA-DP2 tetramers expressing either mimotope 2 or plexin A4 peptides,\(^19\) we identified a population of lung CD4\(^+\) T cells in HLA-DP2 Tg mice that recognized the same TCR ligands as CD4\(^+\) T cells derived from the bronchoalveolar lavage (BAL) of CBD patients. Thus this murine model replicates the major features of the human disease and proves that expression of a single major
histocompatibility complex (MHC) class II (MHCII) molecule in a previously resistant mouse drives disease development upon environmental exposure to Be.

Here we determined the TCR gene sequences expressed by Be-responsive CD4⁺ T cells derived from the lungs of BeO-exposed HLA-DP2 Tg mice and investigated the spectrum of peptides that permit Be recognition by a subset of these TCRs. The TCR repertoire of these Be-responsive clones was dominated by the expression of Vβ6 TCRs that were composed of identical or related complementarity determining region 3 (CDR3) sequences. We utilized a positional scanning peptide library19–22 to screen a Vα4/Vβ6-expressing T-cell transfectedoma to determine whether the peptides required to complete the ab TCR ligand possessed similar Be-coordinating amino-acid residues as was previously observed for human Be-specific TCRs.19 As opposed to the identification of Be-dependent mimotopes,19 we identified mimotopes that stimulated the Be-specific T-cell transfectedomas in the absence of Be. These Be-independent mimotopes expressed arginine (R) and tryptophan (W) residues at the p5 and p7 positions of the peptide, respectively. We speculate that these distinct amino acids near the Be-binding site induce similar changes in charge and conformation to the HLA-DP2-peptide complex as those induced by the addition of the Be2⁺ cation.

RESULTS
Expansion of Vβ6⁺ CD4⁺ T cells in the lung and BAL of BeO-exposed HLA-DP2 transgenic mice
To characterize the TCR repertoire of CD4⁺ T cells present in the lung and spleen of HLA-DP2 Tg FVB/N mice, we pooled the lung and spleen cells from 10 mice and determined the percentage of CD4⁺ T cells expressing particular TCR Vβ chains using immunofluorescence staining (Figure 1). Because the FVB/N strain is characterized by a large genomic deletion in the TCRB locus,23 we focused on the limited set of expressed Vβs shown in Figure 1a. We noted an increased percentage of CD4⁺ T cells expressing Vβ6 in the BAL and lung of BeO-exposed mice (Figure 1a,b). For example, compared with 11.1% of CD4⁺ T cells in the lungs of unexposed mice, 13.4% and 16.8% of lung and BAL CD4⁺ T cells, respectively, expressed Vβ6 (Figure 1b). In addition, when gating on blasting (large forward scatter) T cells compared with a small lymphocyte gate (Figure 1c), the
frequency of CD4⁺ T cells expressing Vβ6 increased from 13.7% to 22.5% of BAL cells from 20 BeO-exposed HLA-DP2 Tg mice (Figure 1c,d). This distribution between blasting and small lymphocytes contrasted to other TCR Vβ subsets that either decreased in frequency or remained unchanged (Figure 1c,d). Collectively, these findings suggested that Vβ6⁺ CD4⁺ T cells are expanded in the lung and BAL after BeO exposure and may contain Be-responsive T cells.

**TCR gene expression of Be-specific T-cell hybridomas derived from the lungs of BeO-exposed HLA-DP2 Tg mice**

We have previously shown that the Be-specific adaptive immune response in BeO-exposed HLA-DP2 Tg mice is CD4⁺ T cell dependent. To characterize this Be-specific T-cell population, the lungs from 10 BeO-exposed HLA-DP2 Tg mice were harvested, and the pooled cells were stimulated in vitro with 10 μM BeSO₄. Blasting T cells were purified and expanded in interleukin (IL)-2 prior to fusing with TCR α/β⁺ BW5147 thymoma cells. Of 90 T-cell hybridomas screened for Be specificity, 29 were Be-responsive and secreted IL-2 after BeSO₄ exposure using an HLA-DP2-expressing murine fibroblast cell line as antigen-presenting cells (data not shown).

To determine TCR gene usage for each of the hybridomas, panels of TCRαV and TCRβV primers were used with TCR constant region primers to screen hybridoma cDNA by PCR. As shown in Figure 2a, TCRαV4 and TCRβV6 were the most commonly utilized V gene segments, being expressed in 45% and 55% of the Be-responsive hybridomas, respectively. TCRαV and TCRβV gene segment usage and CDR3 amino-acid sequences of all of the Be-responsive T-cell hybridomas are displayed in Figure 2b. Among the TCR Vβ6-expressing hybridomas, a CDR3β motif was evident in a subset of five hybridomas, utilizing different nucleotides to encode identical or nearly identical amino-acid sequences with conserved CDR3 length and TCRβJ usage (Figure 3). As some of the Vβ6-expressing hybridomas expressed multiple TCR α-chains (Figure 2b) and considering the genetic instability of the hybrids, we generated immortalized T-cell transfecomas for each of the TCRs shown in Figure 3. For transfecoma LB9-18, the accompanying α-chain that conferred Be specificity was TCRαV4S2 while the correct α-chain for LB10-17 was TCRαV1S1 (see Supplementary Figure S1 online). Similar to Be-specific TCRs derived from the lungs of CBD patients, the oligoclonal Vβ6 chains were paired with different α-chains (Figure 3). Using these transfecomas in T-cell-stimulation assays, their ability to respond to BeSO₄ in vitro was equivalent to that of a Be-specific TCR (AV22) derived from the lungs of a CBD patient (Figure 4a).

We have previously shown that TCR recognition of the HLA-DP2-peptide/Be complex by human CD4⁺ T cells requires glutamic acid (E) residues at positions 26, 68, and 69 of the HLA-DP2 β-chain. For the human TCR AV22 and five murine T-cell transfecomas, amino-acid substitutions at these positions of the HLA-DP2-peptide/Be complex abrogated Be-induced IL-2 secretion (Figure 4b). Thus these Be-specific TCRs derived from BeO-exposed HLA-DP2 Tg mice recognize the HLA-DP2-peptide/Be complex in a similar manner as TCRs derived from humans with CBD. The only difference was that AV22 had a partial reduction in IL-2 secretion with the EA68 HLA-DP2 β-chain variant while this mutation abolished the response of the murine TCRs (Figure 4b).

**TCR gene expression of Be-specific T-cell hybridomas that recognize the HLA-DP2-PLXNA4/Be complex**

We previously demonstrated that a subset of lung CD4⁺ T cells from BeO-exposed mice recognized Be-loaded HLA-DP2 tetramers with either mimotope 2 or plexin A4 peptide in the peptide-binding groove, the same TCR ligands found in CBD patients. Interestingly, all of the TCRs expressed on the Be-specific hybridomas in the current study, with the exception of LB10-13, failed to bind either of these tetramers (data not shown). Using a T-cell transfeccoma expressing the AV22 TCR as a positive control for tetramer co-staining, Figure 5 shows that LB10-13 bound to the HLA-DP2-plexinA4/Be tetramer but not to a Be-loaded HLA-DP2 tetramer expressing the highly related peptide, mimotope 2. The amino-acid sequence of the plexin A4 epitope is FVDDLFTET while that of mimotope 2 is FWIDLFELIG. Conversely, LB11-3 did not bind either Be-loaded HLA-DP2-peptide tetramer. Thus only one of the 29 Be-responsive T-cell hybridomas derived from the lung of BeO-exposed HLA-DP2 Tg mice was specific for the HLA-DP2-plexinA4/Be complex.

To identify additional CD4⁺ T cells specific for this αβTCR ligand, lung cells from BeO-exposed HLA-DP2 Tg mice were stained with the HLA-DP2-PLXNA4/Be tetramer, and positively staining cells were sorted for direct TCR sequencing. As shown in Figure 6a, we identified αβTCR pairs for three CD4⁺ T cells. Interestingly, two of the three were highly related to LB10-13 that was identified in our initial screen, and both of these Vβ16⁺ TCRs (scg-1 and scE-1) utilized TCRαV4S9 with differing n and Jβ regions (Figure 6a). To confirm antigen...
### Table: Gene Expression Profiles

| Hybridization | BV | Vβ | NDβN | Jβ | BJ | AV | Vα | N | Jα | AJ |
|---------------|----|----|------|----|----|----|----|---|----|----|
| LB12-17       | 1S1 | CASS | QLTDS | YAEQFFG | 2S1 | 4S9 | CAL | SST | ASLGKLFQG | 24 |
| LB10-7        | 1S1 | CASS | QTNF | YEQYFG | 2S7 | 4.93 | CAP | PNNAGAKLTFG | 39 |
| LB9-13        | 2S1 | CSAD | HGGL | YAEQFFG | 2S1 | 13S1 | CAAS | SV | YNVLTYFG | 21 |
| LB12-3        | 2S1 | CSAD | HRGG | AETLYFG | 2S3 | 13S1 | CAAS | S | NYNLTYFG | 21 |
| LB12-4        | 2S1 | CTCS | AAGGAR | AETLYFG | 2S3 | 4S2 | CAL | GEG | DSGTYQRFG | 13 |
| LB11-3        | 3S1 | CASS | LGRA | NTLYFG | 1S3 | 4S9 | CAL | SL | NGNYKVYFG | 40 |
| LB9-17        | 6S1 | CASS | LNA | NTEVFFG | 1S3 | ND | 10S3 | CAM | E | NMGKLFQG | 9 |
| LB11-4        | 6S1 | CASS | RTNS | YNSPLYFA | 1S6 | 10S3 | CAM | DR | DTNYKVIFG | 30 |
| LBM9-5        | 6S1 | CASS | RGQL | NSPLYFA | 1S6 | 10S3 | CAM | DR | DTNYKVIFG | 30 |
| LB11-12       | 6S1 | CASS | STGWD | AEQFFG | 2S1 | 4S11 | CAVL | V | GTGKYKVF | 12 |
| LB9-18        | 6S1 | CASS | ILGG | QNTLYFG | 2S4 | 4S2 | CAL | Q | SSGSWQLIFG | 22 |
| LB10-9        | 6S1 | CASS | ILGG | QNTLYFG | 2S4 | 13S1 | CAAR | RR | NNYAQGLFG | 26 |
| LB10-12       | 6S1 | CASS | ILGG | QNTLYFG | 2S4 | 4S2 | CAL | Q | SSGSWQLIFG | 22 |
| LBM10-10      | 6S1 | CASS | ILGG | QNTLYFG | 2S4 | 13S1 | CAAR | RR | NNYAQGLFG | 26 |
| LB11-9        | 6S1 | CASS | ILGG | QNTLYFG | 2S4 | 4S9 | CAL | S | SSGSWQLIFG | 22 |
| LB10-17       | 6S1 | CASS | ILGG | QNTLYFG | 2S4 | 4S9 | CAL | S | SSGSWQLIFG | 22 |
| LBM12-1       | 6S1 | CASS | TRLGG | QNTLYFG | 2S4 | 1S3 | CAVS | P | SSGWHQLIFG | 22 |
| LBM11-8       | 6S1 | CASS | RLG | QDTQYFG | 2S5 | 1S3 | CAVS | T | TGGADRFTG | 45 |
| LBM11-1       | 6S | CASS | IRGGY | QDTQYFG | 2S5 | 3rd | CAVS | P | MGYKLYFG | 9 |
| LBM9-1        | 6S1 | CASS | RGGG | DTQYFG | 2S5 | 10nd | CAM | L | SSGSWQLIFG | 22 |
| LB9-5         | 6S1 | CASS | RGQ | DTQYFG | 2S5 | 4S9 | CAL | SP | SGGSNALKLFTG | 42 |
| LB9-16        | 6S1 | CASS | WTP | DTQYFG | 2S5 | ND | 10S6 | CARL | SNTDKVF | 34 |
| LBM9-18       | 14S1 | CAVS | PGTN | SNTLYFG | 1S3 | ND | 1S3 | CAVS | P | SSGWHQLIFG | 22 |
| LB9-14        | 15S1 | CGAR | GTGS | SQNTLYFG | 2S4 | 4S11 | CVL | GERA | GNTKFLIFG | 37 |
| LB10-12       | 15S1 | CGAA | GTQL | QDTQYFG | 2S5 | 4.93 | CAL | GA | YAQLTGF | 26 |
| LBM9-13       | 16S1 | CASS | LKGG | QNTLYFG | 2S4 | 4S9 | CAL | TS | NGYQNFY | 49 |
| LBM10-13      | 16S1 | CASS | LGG | QTQYFG | 2S5 | 4S9 | CAL | SP | NTGQNFY | 49 |
| LB11-8        | 17S1 | CASS | PRGG | YNSPLYFA | 1S6 | 3nd | CAVS | A | YGGSNKLIFG | 32 |
| LBM9-4        | 20nd | CSS | PRGGWD | TENVFFG | 1S1 | 4S2 | CAL | R | ATGGNKLFTG | 56 |
| LBM10-21      | ND | 8S3/S9 | CALS | DGG | GSALGRHLFG | 18 |
specificity, we expressed these TCRs on an αβ-immortalized cell line. Each of the TCRs was confirmed to be Be-specific and recognized the HLA-DP2-plexinA4/Be complex as demonstrated by tetramer staining (Figure 6b) and IL-2 secretion (Figure 6c). Similar to LB10-13, none of these transfectomas bound the HLA-DP2-mimotope-2/Be tetramer (data not shown). Finally, the Be-specific TCR (scC-6) that stained with the highest affinity (highest mean fluorescent intensity) also responded to the addition of PLXNA4/Be with greatest IL-2 secretion (i.e., lowest EC50 value, defined as the peptide concentration inducing a half-maximal IL-2 response) (Figure 6b,c).

Identification of TCR ligands using a decapeptide positional scanning library

As the specific peptides required for T-cell recognition of Be remained unknown for the vast majority of murine Be-specific TCRs, we utilized an unbiased decapeptide positional scanning library to systematically assess all peptides of a given length in a standard T-cell activation assay. We focused on LB9-18 as a representative of the set of Be-specific Vβ6+ T-cell transfectomas expressing related oligoclonal TCRs (Figure 3). A surprisingly limited number of mixtures with particular amino acids fixed at certain positions of the peptide stimulated the LB9-18 TCR above background (Figure 7a). Specifically, the peptide mixtures that induced > 20 pg ml⁻¹ IL-2 secretion in the presence of BeSO4 contained a glycine (G) at the p2 position, methionine (M) at p3, serine (S) at p4, arginine (R) and leucine (L) at p5, leucine (L) at p6, tryptophan (W) at p7, and tyrosine (Y) at p8 (Figure 7a). No definitive selection of amino acids was seen at the p1, p9, and p10 positions, although a possible preference for nonpolar amino acids (isoleucine (I) and valine (V)) was observed at p9.

Based on the preferences of the LB9-18 TCR for particular amino acids at each position of the peptide (Figure 7b), we synthesized a set of 14 mimotopes, also choosing to include mimotopes with a phenylalanine (F) at p1 as this amino-acid is a preferred anchor residue for HLA-DP2 at this position (Figure 8a). These peptides were tested for their ability to stimulate the Be-specific T-cell transfectomas. In Figure 8a, mimotopes were ranked based on IL-2 secretion by transfectoma LB9-18 with mimotopes 6, 5, 13, 7, 9, and 1 stimulating T-cell activation and IL-2 secretion. However, only mimotopes 5, 6, and 13 induced IL-2 secretion at the lowest peptide concentration tested (0.5 µM) (Figure 7a). Interestingly, these mimotopes were Be independent as they resulted in a half-maximal T-cell transfectoma response (µM) for each transfectoma were determined by nonlinear regression of the activation curves using Prism software (GraphPad). The AV22 transfectoma, expressing a human Vβ6 +/Vγ22+ TCR derived from lung of a chronic beryllium disease patient is shown for comparison. Dose–response curves are representative of three independent experiments carried out in triplicate. (b) Be-induced IL-2 secretion (pg ml⁻¹) by AV22 and the five murine Vβ6+ T-cell transfectomas in response to BeSO4 presentation by fibroblasts expressing either wt HLA-DP2 or HLA-DP2 variants with alanine (A), glutamine (Q), or lysine (K) substitutions at positions 26, 68, and 69 of the β-chain is shown.
the right; \( EC_{50} = 26 \pm 8.0 \mu M \) (Figure 8c), and LBM11-8 and LBM12-1 did not respond at all (data not shown).

To determine the critical amino acids for either mimicking Be or interacting with the TCR, alanine (A) substitutions of mimotope 6 at positions p2, p3, p4, p5, p7, and p8 were synthesized and tested in the absence of Be. We chose not to alter p1 and p6 as these are known anchor residues\(^{19,28}\) and we could directly compare mimotopes 6 and 5 (T vs. F at p1). In addition, as no obvious amino-acid selection was seen at p9 and p10 and as we have previously shown that Be-specific TCRs do not contact these positions,\(^{19,29}\) we did not further investigate p9 and p10. Using T-cell transfectoma LB9-18, an F-to-T substitution at p1 of the peptide shifted the dose–response curve to the right, resulting in an 11-fold increase in the \( EC_{50} \) value (Figure 9, upper panel). Alaines at the p3, p5, or p7 positions abrogated T-cell recognition of the HLA-DP2-peptide/Be complex while A at p2, p4, or p8 had minimal effects on activation of the LB9-18 transfectoma (Figure 9, upper panel). LB9-18 and LB10-10 had a similar response to the alanine variants (Figure 9, middle panel). Conversely, LB10-17 had a 14-fold increase in \( EC_{50} \) values for mimotope 6 and the variants and did not recognize the mimotope 6 A4 variant (Figure 9, lower panel).

Compared with Figure 4b where glutamic acids at positions 26, 68, and 69 of the HLA-DP2 \( \beta \)-chain were required for Be-induced T-cell activation of LB9-18 and LB10-10, the addition of the Be-independent mimotope 6 eliminated the requirement for an E at position 26 (see Supplementary Figure S2). In addition, the presence of the isomorphic Q at this position enhanced IL-2 secretion by the transfectomas as compared with an A. Importantly, an E at positions 68 and 69 remained required even in the presence of mimotope 6 (see Supplementary Figure S2).
DISCUSSION

CBD results from Be exposure in the workplace and the subsequent development of an adaptive immune response to the metal.30 Our previous studies have characterized the TCR repertoire14,15 and binding topology24,25 of Be-specific CD4⁺ T cells derived from the blood and BAL of humans with CBD. In addition, recent analyses of the structure and function of HLA-DP2 in the context of CBD have shown that glutamic acid residues at positions 26, 68, and 69 of the HLA-DP2 β-chain are required for successful Be presentation to T cells,27 and the function of these amino acids is to capture and coordinate the Be²⁺ cation.29 Although Be does not directly interact with the TCR, the addition of the Be²⁺ cation induces both charge and conformational changes to the HLA-DP2-peptide complex that are subsequently recognized as a neoantigen.29 Here we characterized the TCR repertoire of Be-responsive CD4⁺ T cells in an HLA-DP2 Tg murine model of CBD18 and identified CD4⁺ T cells specific for the HLA-DP2-PLXNA4/Be complex. In addition, we identified Be-independent mimotopes for a set of highly related Vβ6-expressing CD4⁺ T cells derived from the lungs of BeO-exposed HLA-DP2 Tg mice. Collectively, our findings expand our understanding of the interplay between Be and HLA-DP2-bound peptide in the generation of Be-induced hypersensitivity.

The ability of murine T cells to fuse with an immortal thymoma line enabled the characterization of TCRs derived from 29 Be-specific T cells, a quantity that far exceeds the total number of Be-specific T-cell clones derived from CBD patients. Using an unbiased combinatorial peptide library approach, we identified mimotopes that stimulated Be-specific Vβ6⁺ CD4⁺ T-cell transfecomas in the absence of Be. In contrast to previously identified Be-dependent mimotopes that possessed negatively charged aspartic and glutamic acid residues at the p4 and p7 positions of the peptide,19 these mimotopes were composed of a positively charged R at p5 and a bulky W at p7. Despite the differences, similar features included the preference for F at p1 and the absence of any significant selection for particular amino acids at the carboxy end of the peptide. An F at p1 has been shown by our group19 and others28 to be the preferred anchoring amino-acid in the P1 pocket of HLA-DP2 while the lack of selection at the peptide carboxy end is likely due to the focus of the TCR on the center of the HLA-DP2-peptide complex.29

Figure 7 Response of T-cell transfecomas LB9-18 to 200 mixtures of decapeptide positional scanning library TPIMS 2040 in the presence of BeSO₄ (75 µM). (a) At each position of the peptide (p1–p10, see histograms), individual mixtures were composed of peptides having one defined amino-acid (20 amino acids, labeled on x-axis in single letter code) and randomized amino acids at the remaining 9 peptide positions. T-cell transfecoma activation was assessed by interleukin (IL)-2 secretion (enzyme-linked immunosorbent assay) after overnight culture of transfecoma cells with mixtures (200 µg ml⁻¹) and BeSO₄ presented by DP2.21 cells in protein-free media. A positive IL-2 response to particular mixtures demonstrates the presence of active peptides within the mixture and provides insight into which amino acids are preferred at a given position of the peptide. Data show IL-2 (mean ± s.e.m.) of three separate experiments carried out in duplicate. IL-2 produced in wells not containing mixtures was below limits of detection. (b) Summary of the most active mixtures from positional scanning library TPIMS 2040. Mixtures were grouped by their ability to stimulate transfecoma LB9-18 by determining net IL-2 secretion (subtracting the amount of IL-2 secreted in wells not containing mixtures from IL-2 values obtained from wells that included mixtures). Bolded amino acids indicated those that were chosen for the generation of mimotopes.
The recently published structure of the HLA-DP2-mimotope 2/Be complex showed that binding of the Be\(^{2+}\) cation reduced the electrostatic surface potential and changed the surface topology of the HLA-DP2-peptide complex where the AV22 TCR CDR3 interacts. Both of these changes must contribute to generation of the AV22 TCR ligand as this TCR is not stimulated by mimotope 2 without Be. In the absence of an HLA-DP2-mimotope 6 structure and insight into the actual Be-dependent epitope for the LB9-18 TCR, we can only speculate on how this Be-independent mimotope might imitate

**Figure 8** Beryllium (Be)-specific T-cell transfectomas recognize mimotopes in a beryllium (Be)-independent manner. (a) The sequence of each mimotope was based on the results of the decapeptide positional scanning for LB9-18. Equal numbers of LB9-18 cells and DP2.21 antigen-presenting cells were mixed with crude preparations of mimotopes (0.5, 5.0, and 50 \(\mu\)g ml\(^{-1}\)) and BeSO\(_4\) (75 \(\mu\)M). Interleukin (IL)-2 secretion was measured by enzyme-linked immunosorbent assay after 22 h of culture. Mimotopes are ordered by LB9-18 response to 50 \(\mu\)g ml\(^{-1}\) of peptide. Hyphens indicate no IL-2 secretion above background levels in response to mimotope. (b) T-cell transfectoma LB9-18 response to select mimotopes in the presence and absence of BeSO\(_4\) (75 \(\mu\)M) and highly purified peptides with single alanine substitutions at positions 2, 3, 4, 5, 7, and 8 of mimotope 6. Interleukin (IL)-2 secretion was measured by enzyme-linked immunosorbent assay after 22 h of culture, and data are plotted as the percentage of maximum IL-2 secretion against peptide concentration in the presence of BeSO\(_4\). EC\(_{50}\) values (mean \(\pm\) s.e.m., \(\mu\)M) for mimotope 6 and each variant peptide from four independent experiments are shown. ND, not determined.
the Be-specific TCR ligand. For example, the positively charged R at the p5 position of the peptide may substitute for Be\(^{2+}\) and reduce the electrostatic surface potential of the complex by partially neutralizing the solvent-exposed glutamic acids on HLA-DP2. In addition, the long side chain of R together with the bulky, hydrophobic W at p7 may create a conformation that mimics the conformation induced by the addition of Be\(^{2+}\) and Na\(^+\) to the MHCII–endogenous peptide complex. 29 Of note, mutating E at positions 26, 68, and 69 of the HLA-DP2 β-chain abrogates Be-specific activation and IL-2 secretion of T-cell clones and transfectomas. 17,26,29 However, in the presence of Be-independent mimotope 6, fibroblasts expressing a variant HLA-DP2 molecule containing an isomorphic Q substitution at position 26 of the HLA-DP2 β-chain induced equivalent IL-2 secretion by the Be-specific T-cell transfectoma, LB9-18, compared with fibroblasts expressing native HLA-DP2. Thus our findings raise the possibility that the amino-acid composition of the Be-independent mimotopes mimics the charge and structural changes induced by the addition of Be\(^{2+}\) and Na\(^+\) to the HLA-DP2-peptide complex.

Metal-independent mimotopes have been identified in other metal-induced hypersensitivities. For example, in subjects with nickel (Ni)-induced hypersensitivity, Ni-independent mimotopes were identified that replaced both the self-peptide and the Ni\(^{2+}\) ion bound to HLA-DR52c. 31 These mimotopes share a lysine (K) at the p7 position of the peptide. Structural analysis of a Ni-specific TCR interacting with the HLA-DR52c-peptide complex showed that the interface was dominated by TCR Vβ CDR3 interacting with the exposed p7 K. 31 These findings suggest that the Ni\(^{2+}\) cation directly contributes to the TCR interaction, which would contrast with the indirect effects of the Be\(^{2+}\) cation in altering both conformation and charge of HLA-DP2. Thus the Ni\(^{2+}\) and Be\(^{2+}\) cations may generate an adaptive immune response and metal-induced hypersensitivity via different mechanisms, with Ni\(^{2+}\) acting as a hapten and directly participating in TCR engagement while Be\(^{2+}\) indirectly induces a postranslational alteration in an endogenous HLA-DP2-peptide complex. Collectively, these studies highlight the complexity of TCR recognition of metal-containing ligands.

When T cells derived from the site of pathology express the same Vβ and share a CDR3β motif, such as the Vβ6\(^+\) Be-specific TCRs investigated in the current study, it is usually inferred that these TCRs respond to the same antigen. Thus, although mimotope 6 was identified through an unbiased screen using the Be-specific T-cell transfectoma LB9-18, several of the other T-cell transfectomas were stimulated by this peptide. T-cell transfectomas LB10-10 and LB10-17 responded to mimotope 6 presented by HLA-DP2 with varying affinities while LB11-8 and LB12-1 did not recognize it at all. LB9-18 and LB10-10 recognized this MHCII–peptide complex with lower EC\(_{50}\) values compared with LB10-17. Thus, as the TCR β-chains of these three TCRs are identical, the differences in TCR affinity are due to the different α-chains expressed by these cells. In this regard, while both LB9-18 and LB10-10 express V\(\alpha4\) and J\(\alpha22\), LB10-17 expresses V\(\alpha1\) and J\(\alpha45\) and has a 5–10-fold higher EC\(_{50}\) than the other two Be-specific TCRs. In addition, the inability of LB11-8 and LB12-1 to respond to mimotope 6 likely stems from a combination of an R at position 95 of the TCR β-chain and expression of V\(\alpha1\). Thus the response to mimotope 6 clearly relates to similarities of the recognizing TCRs (e.g., LB9-18 ≥ LB10-10 > LB10-17 > LB11-8/LB12-1).

In conjunction with characterizing the overall T-cell response to Be, we further analyzed murine-derived CD4\(^+\) T cells specific to the HLA-DP2-PLXNA4/Be complex, a T-cell specificity found in all HLA-DP2\(^+\) CBD subjects. 19 In the initial analysis, only 1 of the 29 Be-specific hybridomas stained with the Be-loaded HLA-DP2-PLXNA4 tetramer. Thus we confirmed our previous finding that these T cells are present in BeO-exposed HLA-DP2 Tg mice, 18 although they do not represent a major specificity. Further analysis of single-cell sorted tetramer-binding T cells enabled the identification of the TCR genes expressed by three additional T-cell clones. Two of these clones expressed homologous V\(\beta16^+\)/V\(\gamma4^+\) TCRs that closely matched the sequence of the LB10-13 TCR while the third clone expressed a completely unrelated V\(\beta6^\) TCR. Similar to LB10-13, none of these TCRs recognized the HLA-DP2-mimotope 2/Be tetramer. Thus tetramer co-staining patterns at the clonal level verified our previous findings of bulk BAL T cells derived from CBD patients. 19 In essence, subsets of T cells exist that either co-stain with mimotope 2 and plexin A4 tetramers or only bind to the tetramer presenting the endogenous antigen (plexin A4/Be). These differences likely reflect dissimilarities in TCR sequence among the antigen-specific population and are an important consideration when using a mimotope selected by a single TCR.

In the lungs of CBD patients, we previously identified Be-specific TCRs that were HLA-DP2-restricted, expressed nearly identical TCR V\(\beta5.1\) chains while coupled with different TCR α-chains, 24 and comprised a public TCR repertoire. 25 Results in BeO-exposed HLA-DP2 Tg mouse models has been observed in human disease (i.e., identification of clonally expanded V\(\beta6^+\) Be-specific TCRs expressing identical or nearly identical β-chains coupled with differing TCR α-chains). These findings suggest that the Be-specific TCRs derived from both HLA-DP2-expressing mice and humans recognize the HLA-DP2-peptide/Be complex in a similar manner, with the majority of interactions contributed by TCR Vβ residues and the HLA-DP2 β1-chain. 29 These findings provide further validation for the use of the HLA-DP2 Tg murine model to study the initiation of a Be-specific adaptive immune response.

The identification of Be-independent mimotopes that mimic a Be-modified HLA-DP2–peptide complex raises the possibility that such peptides exist in vivo and can be involved in the maintenance of the Be-specific T-cell response. We found, however, that CD4\(^+\) T cells derived from the lungs of BeO-exposed HLA-DP2 Tg mice and Be-specific CD4\(^+\) T-cell lines derived from CBD patients do not secrete interferon-γ or IL-2 in response to the Be-independent mimotope 6 (data not shown). In addition, preliminary experiments studying adaptively transferred Vβ6\(^+\) CD4\(^+\) T cells derived from the lungs...
of BeO-exposed HLA-DP2 Tg mice showed that this cell population failed to preferentially home to or expand in recipient lungs compared with spleen in the absence of BeO exposure (data not shown). Collectively, these data suggest that T cells reactive to these Be-independent mimotopes do not exist in vivo. It is likely that the persistent inflammation seen in HLA-DP2-expressing mice and CBD patients is secondary to persistent antigen exposure as Be remains within lung granulomas for decades after the cessation of exposure.32

In conclusion, the present work identifies Be-independent mimotopes for a set of oligoclonal TCRs derived from the lungs of BeO-exposed HLA-DP2 Tg mice. The utilization of highly related TCR β-chains paired with different α-chains suggests that TCR interactions with the Be antigen are dominated by the TCR β-chain, regardless of species. Although the Be-independent mimotopes likely do not have in vivo relevance, they further our understanding of Be-induced alterations in the topology of the HLA-DP2-peptide complex. Future studies utilizing TCR Tg mice expressing HLA-DP2–plexin A4/Be or Vβ6 Be-specific TCRs will enable us to define mechanisms of disease pathogenesis. Thus HLA-DP2 Tg mice provide a powerful tool for the dissection of the role of Be-specific CD4+ T cells in the generation of Be-induced hypersensitivity.

METHODS

Exposure of mice to Be and preparation of lung cells. HLA-DP2 (HLA-DPAL*0103 and -DPBI*0201) Tg mice on an FVB/N background were generated as previously described.18,33 Mice were bred and maintained in the Biological Resource Center at the University of Colorado Denver in accordance with the National Institutes of Health guidelines for use of live animals. All experiments were conducted with the approval of the Institutional Animal Care and Use Committee of the University of Colorado Denver.

Six- to 8-week-old HLA-DP2 Tg FVB/N mice were anesthetized with isoflurane, and 100 μg BeO (NIST, standard reference material 1877) was administered via oropharyngeal aspiration on days 0, 1, 2, 14, 15, 18, and 19.18 Mice were killed on day 21, and single-cell suspensions of splenocytes were prepared.18 Harvested lungs were minced and digested in tissue culture medium containing 1 mg ml−1 collagenase (Sigma-Aldrich, St Louis, MO). After 30 min at 37°C, digested lung tissue was disrupted, and the lung cells were centrifuged. After lysis of erythrocytes, lung cells were filtered through a 70-μm strainer and resuspended in complete tumor media, consisting of phosphate-buffered saline. T-cell blasts were enriched by density gradient centrifugation (Histopaque, Sigma-Aldrich) and expanded in IL-2 for 3 days. In culture, T-cell blasts were enriched by density gradient centrifugation (Histopaque, Sigma-Aldrich) and expanded in IL-2 for 3 days. T-cell hybrids were established by fusion of T cells to TCR αβ−/β− BW5147 thymoma cells using polyethylene glycol and cloned by limiting dilution under hypoxanthine, aminopterin, and thymidine (Invitrogen, Life Technologies, Grand Island, NY) selection. T-cell hybridoma clones were screened for Be specificity by measuring IL-2 production by enzyme-linked immunosorbent assay (eBioscience, San Diego, CA) after stimulation with 200 μg BeSO4 presented by an HLA-DP2-transfected murine fibroblast, designated DP3802.

Generation of Be-specific T-cell hybridomas. Be-specific T-cell hybridomas were generated as previously described.18 In brief, lung cell suspensions from 10 mice were pooled and restimulated in vitro at 2 × 106 ml−1 with 10 μg BeSO4 in complete tumor media. After 4 days in culture, T-cell blasts were enriched by density gradient centrifugation (Histopaque, Sigma-Aldrich) and expanded in IL-2 for 3 days. T-cell hybridomas were established by fusion of T cells to TCR αβ−/β− BW5147 thymoma cells using polyethylene glycol and cloned by limiting dilution under hypoxanthine, aminopterin, and thymidine (Invitrogen, Life Technologies, Grand Island, NY) selection. T-cell hybridoma clones were screened for Be specificity by measuring IL-2 production by enzyme-linked immunosorbent assay (eBioscience, San Diego, CA) after stimulation with 200 μg BeSO4 presented by an HLA-DP2-transfected murine fibroblast, designated DP3802.

Determination of TCRα and TCRβ gene usage. To determine TCRβ gene usage, total RNA was isolated using a Qiagen RNeasy Kit (Qiagen, Valencia, CA), and cDNA was prepared as previously described.14 TCRβ gene fragments were PCR-amplified using a 3′ TCRβC primer and a panel of BV primers (n = 13) to screen for TCRBV gene usage of each of the hybridomas. An identical PCR screening methodology was employed to determine TCRαV gene segment usage, using a 3′ TCRαC primer and panel of AV primers (n = 22). Upon determination of specific BV and AV gene segment usage, larger-scale PCR reactions were completed, and products were purified using a DNA binding membrane spin column (Qiagen) and sequenced (Applied Biosystems, Carlsbad, CA).

Transfectomas expressing particular murine Be-specific TCRs. Because T-cell hybridomas created by fusing murine T cells with BW5147 cells are tetraploid and genetically unstable, TCRs expressed by select Be-specific hybridomas were cloned into expression vectors and stably transfected into the mouse hybridoma cell line 54C, which expresses human CD4,19,22,24,25,36 as described in the Online Supplement.

Decapeptide positional scanning library and peptide synthesis. The decapeptide library used for this study (designated TPIMS 2040) is a synthetic N-acetylated, C-terminal amidated, 4-aminobutyric acid combination peptide library organized in a positional scanning format. This library consists of 200 Mixtures prepared in an OX9 format where each Ox represents a specific amino-acid at a defined position and X represents an equimolar mixture of all natural amino acids (except cysteine) in each of the remaining 9 positions. Each Ox9 mixture consists of 3.2 × 1011 different decapeptides, and the total number of peptides in the library is 6.4 × 1012.22,37 T-cell transfectoma response to HLA-DP2-expressing fibroblasts presenting peptide mixtures in the presence of Be was assessed by IL-2 secretion. The amount of IL-2 secreted in wells not containing mixtures was subtracted from IL-2 values obtained from wells that included mixtures to yield net IL-2 (pg ml−1).

Individual candidate peptides (mimotopes), selected based on analysis of positional scanning results, were synthesized using the PEPscreen 96-well array (Sigma). Peptides chosen for further studies were synthesized on a larger scale and tested at 95% purity (CPC Scientific, Sunnyvale, CA). All peptides were first dissolved in dimethyl sulfoxide at high concentration prior to making a working stock in phosphate-buffered saline.

HLA-DP2–PLXNA4/Be tetramer staining and cell sorting. Preparation of soluble, Be-loaded HLA-DP2-covalent plexin A4 peptide (PLXNA4) tetramers was performed as previously described.18 For sorting of HLA-DP2 tetramer-binding CD4+ T cells, lung cells (5–10 × 106 cells per mouse) from BeO-exposed HLA-DP2 Tg FVB/N mice were incubated in 25 μl of medium containing 5 μM sodium azide, an excess of FcR-specific blocking monoclonal antibody (mAb), and the phycoerythrin-labeled HLA-DP2–PLXNA4/Be tetramer (20 μg ml−1). After 2 h at 37°C, fluorescently conjugated mAbs to murine CD3 (PE-Cy7), CD4 (AF700), CD11c (APC-eFluor780), B220 (eFluor 450), F4/80 (eFluor 450), CD8 (eFluor 450), and CD44 (PerCP-Cy5.5) were added, and cells were incubated at 4°C for an additional 30 min. After washing, cells were analyzed on a FACSAria flow cytometer (BD Immunocytometry Systems, San Jose, CA). Cells staining with mAbs directed against B220, F4/80, CD8, and CD11c were included in a dump gate and excluded from analysis. Using forward and side scatter parameters, dump-negative, CD3+CD4+ T cells were selected and sorted based on tetramer staining and high CD44 expression.

Cloning of PLXNA4/Be-specific TCRs from CD4+ T cells isolated by single-cell sorting. A single-cell suspension was prepared from the lungs of BeO-exposed HLA-DP2 Tg mice as described previously.18 Cells were stained with mAbs directed against CD4 (AF700), CD3 (PE-Cy7), CD8 (eFluor 450), B220 (eFluor 450), F4/80 (eFluor 450), and CD11c (APC-eFluor780), and the phycoerythrin-labeled HLA-DP2–PLXNA4/Be tetramer (20 μg ml−1). After 2 h at 37°C, fluorescently conjugated mAbs to murine CD3 (PE-Cy7), CD4 (AF700), CD11c (APC-eFluor780), B220 (eFluor 450), F4/80 (eFluor 450), CD8 (eFluor 450), and CD44 (PerCP-Cy5.5) were added, and cells were incubated at 4°C for an additional 30 min. After washing, cells were analyzed on a FACSAria flow cytometer (BD Immunocytometry Systems, San Jose, CA). Cells staining with mAbs directed against B220, F4/80, CD8, and CD11c were included in a dump gate and excluded from analysis. Using forward and side scatter parameters, dump-negative, CD3+CD4+ T cells were selected and sorted based on tetramer staining and high CD44 expression.
using a FACSAria flow cytometer. Single cell sorted into reverse transcription buffer in a 96-well plate and a nested PCR method as previously described25,38 and as detailed in the Online Supplement.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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DISCLOSURE
The authors declared no conflict of interest.

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