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Mutagenesis of Beryllium-Specific TCRs Suggests an Unusual Binding Topology for Antigen Recognition

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Unconventional Ags, such as metals, stimulate T cells in a very specific manner. To delineate the binding landscape for metal-specific T cell recognition, alanine screens were performed on a set of Be-specific TCRs derived from the lung of a chronic beryllium disease patient. These TCRs are HLA-DP2–restricted and express nearly identical TCR Vb5.1 chains coupled with different TCR a-chains. Site-specific mutagenesis of all amino acids comprising the CDRs of the TCRA and TCRB genes showed a dominant role for Vb5.1 residues in Be recognition, with little contribution from the TCR a-chain. Solvent-exposed residues along the a-helices of the HLA-DP2 a- and b-chains were also mutated to alanine. Two b-chain residues, located near the proposed Be binding site of HLA-DP2, played a dominant role in T cell recognition with no contribution from the HLA-DP2 a-chain. These findings suggest that Be-specific T cells recognize Ag using an unconventional binding topology, with the majority of interactions contributed by TCR Vb5.1 residues and the HLA-DP2 b1-chain. Thus, unusual docking topologies are not exclusively used by autoreactive T cells, but also for the recognition of unconventional metal Ags, such as Be. The Journal of Immunology, 2011, 187: 3694–3703.

A conventional immune response is initiated by a b TCR recognition of an MHC molecule with a bound peptide derived from a foreign protein (1). Immune-mediated clearance of unconventional Ags, such as metals, is poorly understood, especially in the context of interactions required for TCR recognition. Yet, understanding these interactions are critical because metal-induced diseases, such as chronic beryllium disease (CBD) and nickel-induced contact hypersensitivity, occur in a significant number of exposed individuals, leading to morbidity and, in the case of CBD, mortality. Metal binding to a peptide–MHC complex (pMHC) adds a third component of complexity for recognition by T cells, with all three components required to elicit T cell activation. For example, in the case of nickel-induced hypersensitivity, the nickel-specific TCR interacts with an antigenic complex formed by nickel, an unknown peptide, and HLA-DR52c (2).

CBD is a granulomatous lung disorder characterized by the accumulation of Be-responsive CD4+ T cells in the lung (3, 4). These Be-specific T cells express a polarized Th1 phenotype and recognize Be in an HLA-DP–restricted manner (5, 6). Genetic susceptibility to Be-induced disease has been linked to MHC class II (MHC II) molecules. In particular, HLA-DP alleles possessing a glutamic acid at the 69th position of the b-chain (bGlu69) are strongly linked to disease susceptibility (7–11). The HLA-DP molecules that present Be to T cells match those implicated in the genetic susceptibility, suggesting that the HLA contribution to disease is based on the ability of those molecules to bind and present Be to T cells (6, 12, 13). We have recently crystallized HLA-DP2, and its unique structural features, including surface exposure of bGlu69, provide an explanation of the genetic linkage between bGlu69-containing HLA-DP alleles and Be-induced disease (14).

Of the ~20 TCR–pMHC structures solved to date, analyses show a distinction in T cell recognition of foreign- versus self-derived Ags. The TCR generally uses a conserved binding topology for recognition of foreign-derived Ags, including a diagonal orientation, conserved polarity, and a centered position over the pMHC complex (15, 16). This conserved polarity places the TCR b-chain in close contact with the a helix of MHC and the TCR a-chain over the a2 helix of MHC class I or b1 helix of MHC II. Essential contacts are made with both a-helices of the MHC, generating optimal binding interactions between the T cell and the APC. In contrast, unconventional binding interactions have been reported for the majority of human autoimmune complexes solved to date (17–19). The docking footprint for these TCRs is shifted laterally or tilted over the pMHC complex, creating suboptimal binding interactions. It has been postulated that these unusual binding features may allow these low-affinity self-reactive T cells to escape negative selection in the thymus (20). In this regard, it remains unknown how Be-specific TCRs recognize this unconventional Ag in the context of HLA-DP2–peptide.

Using a set of Be-specific Vb5+ TCRs derived from the lung of a subject with CBD, we show that these TCRs recognize the HLA-DP2–peptide–Be complex using an unusual binding topology. The atypical docking footprint for recognition of the Be Ag was characterized by residues in the Vb5 chain playing a dominant role in Be Ag recognition, with no contribution from germline-
derived CDR1 and CDR2 residues of the TCR α-chain. Selected mutations of solvent-exposed residues of the HLA-DP2 α-helices confirmed these findings and showed little contribution from the α1 helix of HLA-DP2. Taken together, our findings suggest that the Be-specific TCRs are centered directly over the putative Be binding site of HLA-DP2, with residues of the TCR Vβ domain and the β1 helix of DP2 contributing to binding and recognition of the Be Ag.

Materials and Methods

Study population

Experiments performed in the current study used CD4+ T cells derived from the lung of patients with CBD. The diagnosis of CBD was established using previously defined criteria (11, 21), including a history of Be exposure, the presence of granulomatous inflammation on lung biopsy, and a positive proliferative response of bronchoalveolar lavage (BAL) T cells to BeSO4 in vitro. Informed consent was obtained from these subjects, and the protocol was approved by the Human Subject Institutional Review Board at the University of Colorado Denver and National Jewish Health.

Immunofluorescence staining and analysis of intracellular cytokine expression

BAL-derived, Be-specific T cell lines were derived as previously described (6, 12) and maintained in culture by cycles of stimulation (every 2 to 3 wk) with 100 μM BeSO4-pulsed autologous EBV-transformed lymphoblastoid cell lines and further expanded with rIL-2. For stimulation of cytokine secretion, the CD4+ T cell line (2.5 × 10^6 cells) was stimulated with either medium or 100 μM BeSO4 using autologous lymphoblastoid cell lines (2.5 × 10^6 cells) as APCs. Cells were incubated for 6 h at 37°C in a humidified 5% CO2 atmosphere with 10 μg/mL brefeldin A added after the first hour of stimulation (5, 22). After stimulation, cells were washed and stained with mAbs directed against CD3, CD4 (all from BD Biosciences), and anti-TCR Vβ5.1 (clone LC4) (23). Cells were fixed, permeabilized, and stained with an anti–IFN-γ mAb (BD Biosciences) for 30 min at 4°C. The lymphocyte population was identified using forward and 90° light scatter patterns, and fluorescence intensity was analyzed using an FACSCalibur flow cytometer (BD Biosciences) (5, 22). Data files were analyzed using FlowJo software (Tree Star).

Analysis of TCRBV551 expression by BAL CD4+ T cell clones

Because the majority of Be-responsive CD4+ T cells in this line expressed Vβ5.1, we sorted CD4+Vβ5.1+ T cells using an FACSAria flow cytometer (BD Immunocytometry Systems) and cloned by limiting dilution. Cells from clonal cultures were harvested, and RNA was isolated using a Qiagen RNeasy kit according to the manufacturer’s instructions (Qiagen). cDNA was prepared, and the TCRBC gene segment for each T cell clone was cloned into MSCV plasmids (24). cDNA was prepared, and the TCRBC gene segment for each T cell clone was cloned into MSCV plasmids (24). cDNA was prepared, and the TCRBC gene segment for each T cell clone was cloned into MSCV plasmids (24).

Cloning of TCR and MHC constructs into retroviral vectors

Full-length chimeric TCRs were cloned into a murine stem cell virus (MSCV) plasmid for retroviral transduction of Phoenix 293T cells with MSCV plasmids (25). Phoenix cells were plated at 4.5 × 10^5 cells/well in six-well plates (Corning) coated with 100 μg/mL poly-o-lysine (Sigma-Aldrich). After 24 h, wells were washed with PBS and replenished with IMDM-glutamax (Invitrogen) culture medium without FBS. Cells were transfected with Lipofectamine 2000 (Invitrogen) preincubated with 6 μg MSCV and 1.5 μg pCI-Eco packaging vector, with PBS added after 4 h at 37°C. After 24 h, the cells were replenished with medium containing 10% PBS and incubated for 24 h before collecting retroviruses-containing supernatants.

Expression of TCR variants on the murine T cell hybridoma

Wild-type and HLA-DP2 variants were packaged as retrovirus, as described for the TCR variants, and the retrovirus was used to transduce murine fibroblast line B6DK10, which had previously been transfected with murine B7 and ICAM. Brieﬂy, ﬁbroblasts (5 × 10^5) were adhered to a six-well plate (Corning) in cell-culture medium (IMDM-Glutamax; Invitrogen) and 10% FBS for 1 h at 37°C. Media was removed, and 1 ml filtered viral supernatant and 8 × 10^5 cells were transfected with 1.5 × 10^6 pMSCV and 1.5 μg pCRI-Eco packaging vector, with PBS added after 24 h (37°C). Viral supernatant was removed by washing with PBS and cultures were replenished with medium containing FBS. After 2–4 d, cell-surface expression of HLA-DP was measured by using a conformation-dependent anti–HLA-DP mAb, B7.21 (28). Fibroblasts were stained and sorted as described above, and data analysis was performed using FlowJo software (Tree Star).

T cell hybridoma activation assays

T cell hybridoma cells (1 × 10^5) and murine fibroblasts transduced with wild-type or variant HLA-DP2 were incubated overnight at 37°C with various concentrations of BeSO4 or dengue virus peptide. Supernatants were harvested, and IL-2 was measured using the mouse IL-2 Ready-Set-Go ELISA kit (eBioscience). Activation curves were generated by plotting percentage of maximal IL-2 release, (A450 [sample] − A450 [control])/(Max A450 [sample] − A450 [control]) × 100, against Ag concentration. The concentration of BeSO4 or dengue virus peptide required for half-maximal IL-2 release, or EC50 value, was determined using nonlinear regression (sigmoidal-fit, GraphPad Prism; GraphPad) of the activation curves. Assays using the murine fibroblast line B6DK10 expressing wild-type or HLA-DP2 variants required 1 to 2 × 10^6 APCs/well, whereas for the HLA-DP2-transfected DAP3L fibroblasts, 5 × 10^5 cells were used.

Results

Characterization of Be-responsive T cell clones derived from the lung of a CBD patient

We have previously identified a large number of Be-responsive CD4+ T cells compartmentalized to the BAL of CBD patients (5). With repeated cycles of BeSO4 stimulation, a Be-responsive CD4+ T cell line was developed from one CBD patient (designated 1332). Using intracellular cytokine staining as a measure of T cell response, 40% of the CD4+ T cells were Be-responsive and expressed IFN-γ (Fig. 1A). Interestingly, the predominant TCR Vβ expansion comprising this line was Vβ5.1 (data not shown), and the vast majority of Vβ5.1-expressing CD4+ T cells were Be...
specific (81% in the density plot shown compared with 8.3% in the Vb5.1 T cell subset) (Fig. 1A). To determine whether this expansion of Vb5.1-expressing CD4+ T cells was composed of a single T cell clone or related clones, we sorted CD4+Vb5.1+ T cells and cloned via limiting dilution. RNA and cDNA were prepared from each clonal isolate, and the TCRB genes were amplified and sequenced. We identified three Be-specific T cell clones that express TCRBV5S1 and nearly identical CDR3 sequences (Fig. 1B). Two of the T cell clones, 1332-28 and 1332-2, used different nucleotide combinations in the nBDn region and TCRBJ2S5 to encode identical amino acids. The third T cell clone, 1332-22, used TCRBJ1S4, resulting in a 2 aa difference in the TCRB gene sequence (serine at position 99 and lysine at position 101). Interestingly, the three T cell clones used different TCRAV genes coupled with TCRBJ28. As a result, these TCRs have different germline-derived CDR1α and CDR2α despite encoding nearly identical somatically rearranged CDR3α (except for 2 aa differences, one encoded by germline and the other by an N-region modification) (Fig. 1B). These findings raise the possibility that the TCR Vβ domain of the Be-specific TCR is playing the dominant role in recognition of the HLA-DP2–peptide–Be complex, whereas the contribution of the Vα domain is limited to CDR3α.

**Generation of T cell hybridomas expressing Be-specific TCRs**

Because it is difficult to perpetuate BAL-derived, Be-specific T cell clones indefinitely, we generated T cell hybridomas that express the TCR of these Be-specific T cell clones. Hybridomas have several advantages compared with the T cell clones from which their TCRs are derived. They can be repeatedly grown to large numbers, and their stimulation is independent of the functional state of the parental T cell clone. For 1332-28, -22, and -2, gene fragments of the variable domains of each clones’ TCRα and TCRβ genes were cloned into retroviral vectors encoding murine TCR constant domains and expressed on the surface of the murine T cell hybridoma line 5KC (25). Thus, these chimeric TCRs consist of human variable and murine constant domains. The 5KC T cell line no longer expresses murine CD4 and has been transduced with human CD4 (Fig. 2A). Fig. 2B shows the ability of these three Be-specific hybridomas to secrete IL-2 in response to various concentrations of BeSO4 presented by murine DAP3.L cells transfected with HLA-DP2. This Ag-specific response was completely blocked by the addition of an anti–HLA-DP–specific mAb (Fig. 2C).
Mapping T cell recognition of the Be Ag in the context of HLA-DP2

To determine whether TCRs recognize Be in a similar manner as other Ag-specific TCRs, we mutated TCR residues of the 1332-28 T cell clone at potential Ag-contact sites, with the aim of mapping T cell recognition of the Be Ag. We initially focused on 1332-28 because a crystal structure of an autoimmune Vo22/Vb5.1 TCR (same as that expressed by 1332-28) bound to a myelin basic protein (MBP) self-peptide in the context of HLA-DR2α exists (DRA1*0101/DRB5*0101; Protein Data Bank [PDB] identification code 1ZGL) (19). Each TCRA and TCRB CDR residue was separately mutated to alanine using site-directed mutagenesis, and each mutant TCR was expressed on the surface of 5KC. Using a mouse anti-TCR Cβ mAb, similar levels of TCR expression were documented for each cell line (Supplemental Fig. 1). The T cell hybridoma response to BeSO₄ was measured using IL-2 secretion, and activation curves were generated by plotting percentage of maximal IL-2 release against BeSO₄ concentration (Fig. 3A, 3B). Surprisingly, none of the alanine mutations in the germline-derived CDR1α and CDR2α of 1332-28 had any effect on Be-induced IL-2 secretion, with EC₅₀ values (defined as the BeSO₄ concentration required for a half-maximal T cell hybridoma response) similar to the wild-type TCR (Fig. 3A, top and middle panels). In contrast, mutation of the tyrosine at position 95 of the somatically rearranged CDR3α to alanine (Y95A) abolished Be-induced T cell activation (Fig. 3A, bottom panel). This tyrosine is derived from the TCRAJ28 gene segment. To quantitatively display the EC₅₀ differences of the individual mutant TCRs, the overall EC₅₀ fold-change difference (mean ± SEM) for each TCR compared with the wild-type TCR for three separate experiments is shown (Fig. 3C). Thus, only 1 aa of the TCR α-chain is critical for functional recognition of the HLA-DP2–peptide–Be complex.

For the TCR β-chain analysis of 1332-28, several mutations resulted in either reduced or abolished T cell hybridoma response

![FIGURE 3. Contact sites of the Be-responsive TCR, 1332-28, with the HLA-DP2–peptide–Be complex. Be-specific response of T cell hybridoma 1332-28 expressing wild-type TCR and TCR variants with single-site alanine mutations at each amino acid position of the CDR1 (upper panel), CDR2 (middle panel), and CDR3 (lower panel) of Vo22 (A) and Vb5.1 (B). TCRs were expressed on 5KC and stimulated with various concentrations of BeSO₄ presented by HLA-DP2–transfected murine fibroblasts. IL-2 secretion was measured by ELISA and plotted as percent maximal IL-2 secretion against BeSO₄ concentration. Activation curves are representative of three independent experiments, and EC₅₀ values (the concentration of BeSO₄ required for a half-maximal T cell hybridoma response) were determined by nonlinear regression of the activation curves using GraphPad Prism software (GraphPad). The mean ± SEM EC₅₀ values for the wild-type TCR and each of the variants are shown. The overall EC₅₀ fold change difference (mean ± SEM) for the TCR α-chain (C) and β-chain (D) variants compared with the wild-type TCR is shown. The dotted line at y = 1 represents the wild-type TCR response.](http://www.jimmunol.org/)

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compared with the wild-type TCR (Fig. 3B, 3D). For example, four mutations including Y49A, F50A, and R55A in CDR2β and Q97A in CDR3β abolished the Be-induced T cell hybridoma response (EC50 values >1000 nM). Several other mutations in the β-chain moderately reduced (increased EC50 values 2–6-fold compared with wild-type), but did not abolish, IL-2 secretion in response to Be (H29A and R30A in CDR1β and L95A and E100A in CDR3β) (Fig. 3B). Similar to the α-chain mutants, the EC50 values were plotted as a fold-difference compared with the wild-type TCR response (Fig. 3D). Overall, these data show that the Vβ domain of 1332-28 TCR plays the dominant role in Be recognition, with no contribution from the germline-derived regions of the Vα22 chain.

To determine whether the other Be-specific Vβ5+ T cell clones, 1332-22 and -2, recognize Ag in a similar manner, we performed site-directed mutagenesis on selected CDR α- and β-chain residues that abolished IL-2 secretion in the 1332-28 TCR. The 1332-22 and -2 TCR variants were expressed on 5KC, and the IL-2 response to various BeSO4 concentrations presented by HLA-DP2–expressing fibroblasts was compared with the wild-type 1332-22 and -2 TCRs. Of note, the three Be-specific TCRs had similar EC50 values, ranging from 1.8–2.3 nM BeSO4 (compare Figs. 3 and 4). Identical to 1332-28, the same five mutations (Y95A in CDR3α, Y49A, F50A, and R55A in CDR2β, and Q97A in CDR3β) in the TCRs of 1332-22 and -2 abolished Be-induced IL-2 secretion (Fig. 4A, 4B). As controls, we also mutated germline-derived CDR1α and CDR2α residues of 1332-22 (S30A in CDR1α and N53A in CDR2α) and 1332-2 (S30A in CDR1α and R53A in CDR2α) at positions that had no effect on Be recognition in 1332-28. As expected, these mutations in the TCRAV gene segments of 1332-22 and -2 also had no effect on Be-induced IL-2 secretion compared with the wild-type TCR (Fig. 4A, 4B, top panels).

FIGURE 4. Vβ5+ TCRs recognize the HLA-DP2–peptide–Be complex using identical contact sites. A and B, Stimulation of Be-specific T cell hybridomas, 1332-22 and -2, expressing selected mutations in the CDRs of the TCR α- and TCR β-chains with HLA-DP2–expressing DAP.3 fibroblasts and various concentration of BeSO4. T cell secretion of IL-2 was measured by ELISA and plotted as described in Fig. 3. Activation curves are representative of three independent experiments. The mean ± SEM EC50 values for the wild-type TCR and each of the variants are shown. C, A color-coded summary of T cell activation data for the stimulation of T cell hybridomas 1332-28, -22, and -2. CDR CDR amino acid sequences for each of the three T cell hybridomas are aligned with the amino acid sequence number shown above. Residues are highlighted based on their effect when mutated to alanine, in comparison with the wild-type response. Residues highlighted in white indicate that an alanine mutation had no effect on T cell response, with EC50 values identical to the wild-type response. Residues highlighted in yellow indicate a mutation that had a moderate effect on T cell hybridoma response, a 2–6-fold reduction in response compared with wild-type. Residues highlighted in red indicate the absence of a hybridoma response when mutated to alanine.
Even though the Vβ5.1\(^*\) T cell clones express nearly identical TCR β-chains, minor differences exist between 1332-28 and -22, with variations at positions 99 and 101 of CDR3\(\beta\) (see Fig. 1B). To determine if the S99 and K101 in the CDR3\(\beta\) of 1332-22 have an influence on Be recognition, we also mutated these residues to alanine. Similar to 1332-28, the CDR3\(\beta\) S99A mutation had no effect on the 1332-22 T cell hybridoma response (Fig. 4A, bottom panel). In contrast to mutation of the threonine of the CDR3\(\alpha\) residue at position 101 of 1332-28, which had no effect on Be-induced T cell activation, a moderate decrease was seen in the 1332-22 response with the CDR3\(\beta\) K101A mutation (Fig. 4A, bottom panel). The EC_{50} fold-differences for each variant compared with the wild-type 1332-22 TCR. As shown in Fig. 5 (bottom panel), the mutant TCR had a slightly increased Be-induced response with a 5-fold decrease in the EC_{50} value compared with the wild-type TCR. These differences are likely the result of a neutral threonine versus a positively charged lysine at position 101 of the β-chain of 1332-28 and 1332-22, respectively. Although the changes were subtle, the optimal combination of α- and β-chains for Be-induced T cell activation was the Vβ5.1 chain from 1332-28 and the Vα8.1 chain from 1332-22. Taken together, these findings confirm our alanine scan of three Be-specific TCRs, showing that the β-chain of the TCR plays the dominant role in binding to the HLA-DP2–peptide–Be complex and that coupling with any of the TCR Vα-chains will result in Be-induced T cell activation as long as the tyrosine (Y) at position 95 of the CDR3\(\alpha\) is present.

**Effect of HLA-DP2 variants on the Be-induced T cell response**

The predominant role of the TCR β-chain in Be recognition with a limited requirement for the Vα domain suggests an unconventional binding topology for these Be-specific TCRs. To further characterize T cell recognition of the Be Ag in the context of HLA-DP2, we performed site-directed mutagenesis on HLA-DP2. We have recently crystallized HLA-DP2 (PDB identification code 3LQZ) (14) and used this structure to delineate solvent-exposed residues most likely to impact T cell recognition when mutated and not affect stability or expression of the MHC II molecule. Amino acid residues that spanned the length of HLA-DP2 α1 and β1 helices were chosen for mutagenesis, and their location on the α-helices of HLA-DP2 is shown in Fig. 6A. The following mutations were introduced in the HLA-DP2 α1 helix—E55A, Q57A, L60A, A64Q, N68A, and N71A—and β1 helix—Y58A, D64A, E67A, A71Q, R75A, and H79A. We chose to place the alanine at positions 64 of the α1 helix and 71 of the β1 helix with a glutamine to disrupt TCR binding through steric hindrance (29). Wild-type and variants of HLA-DP2 were expressed on the surface of the murine fibroblast line, B6DK10, and all APC lines expressed similar levels of HLA-DP2 as shown by staining with a conformation-dependent anti–HLA-DP mAb (B7.21) (data not shown). The Be-specific T cell hybridoma 1332-28 was stimulated with various concentrations of BeSO_{4} added to the fibroblasts expressing the HLA-DP2 variants, and activation curves were generated as described above (Fig. 6B). Surprisingly, not a single mutation in the HLA-DP2 α1 helix reduced the T cell hybridoma response as indicated by similar EC_{50} values of the variants compared with wild-type DP2 (Fig. 6B, left panel). In contrast, several mutations in the α-helix of the DP2 β-chain either abolished or reduced the 1332-28 T cell hybridoma response. For example, two DP2 β1 helix mutations (D64A and R75A) abolished 1332-28 hybridoma response, as indicated by no IL-2 secretion in response to up to 1000 \(\mu\)M BeSO_{4} (Fig. 6B, right panel). In addition, β1-Y58A moderately reduced the response as indicated by a 2.6-fold increase in the EC_{50} compared with wild-type HLA-DP2 (Fig. 6B, right panel). A summary of the EC_{50} fold differences for the 12 variants compared with wild-type DP2 is shown in Fig. 6C, with only two of these upward-facing, solvent-exposed residues of HLA-DP2 representing major sites for either direct or indirect contact with the Be-specific 1332-28 TCR.
FIGURE 6. Identification of HLA-DP2 contacts for Be-specific, Vβ5.1+ T cells. A. Crystal structure of HLA-DP2 with a self-derived HLA-DRα peptide (PDB identification code 5LQZ). The α1- and β1-domains of HLA-DP2, along with the peptide, are shown in green. Solvent-exposed, upward-facing residues of the HLA-DP2 α1 and β1 α-helices subjected to mutagenesis are shown in blue and red, respectively. Cyan residues denote three glutamic acids proposed to contribute to Be binding. B, HLA-DP2 molecules with single-site mutations introduced along both α-helices were expressed on the surface of murine fibroblasts and used to stimulate 1332-28 TCR. Representative activation curves for the α-chain (left panel) and β-chain (right panel) variants are plotted as percent maximal IL-2 release against BeSO4 concentration. Data are representative of at least three separate experiments. C, Shown are EC50 fold change differences (mean ± SEM) for hybridoma 1332-28 induced by the HLA-DP2 variants compared with wild-type HLA-DP2 for three separate experiments. The dotted line at y = 1 represents the wild-type TCR response.

Effect of HLA-DP2 variants on the response of additional HLA-DP2–restricted TCRs

To determine whether the abnormal binding topology shown for the three Vβ5+ TCRs was present in other Be-specific TCRs, we investigated a T cell hybridoma (designated 1041-3.3) expressing a Be-specific TCR derived from the lung of an additional CBD patient. The TCR of the parental T cell clone uses the CDR3α and CDR3β sequences of the Vβ5.1+ T cell clones (compare Fig. 1B and Supplemental Fig. 3). The 1041-3.3 T cell hybridoma was generated in a similar manner as described for the Vβ5.1+ T cell hybridomas and had a >100-fold increase in the Be-induced EC50 value compared with 1332-28, suggesting a significantly decreased binding affinity of this TCR for the HLA-DP2–peptide–Be complex (compare Figs. 3 and 7A). Fibroblasts expressing the HLA-DP2 α1 helix mutations had no effect on the Be-induced IL-2 response of this Vα1/Vβ3 T cell hybridoma (Fig. 7A, left panel). Conversely, two DP2 β1 helix mutations either reduced (Y58A) or abolished (R75A) the Be-induced IL-2 response (Fig. 7A, right panel). These findings suggest that this hybridoma contacts the HLA-DP2–peptide–Be complex in the same unconventional manner as the three related Be-specific TCRs despite expressing distinctly different TCR genes and having a much lower binding affinity for Ag.

To verify that the abnormal binding topology observed for these Be-specific TCRs is unique for this unconventional Ag, we determined how a T cell recognizes a conventional (viral) Ag in the context of HLA-DP2. Thus, we expressed on 5KC a Vα11/Vβ23 TCR derived from a T cell clone specific to an epitope of the NS3 protein of dengue virus (aa 251–264; HTGREIVDLMCHAT) (24). Upon exposure to the dengue virus peptide, hybridoma cells expressing the dengue-specific TCR (designated JK34) secreted IL-2, with an EC50 value ranging between 5.2 and 7.0 μM dengue virus peptide (Fig. 7B). Using fibroblasts expressing single-site variants of HLA-DP2 to map Ag recognition of this dengue virus-specific TCR, the HLA-DP2 α1 helix variants had no affect on the JK34 T cell hybridoma response, as indicated by identical EC50 values for the α1 helix variants compared with the wild-type TCR (Fig. 7B, left panel). Conversely, alanine mutations at positions 64 and 75 (D64A and R75A) of the HLA-DP2 β1 chain abolished the dengue virus-specific T cell response (Fig. 7B, right panel). In addition, two other HLA-DP2 β1-chain mutations (Y58A and E67A) markedly reduced the dengue viral-specific hybridoma response. In Fig. 7C, we used a color-coded scheme to depict the amino acid substitutions and their respective effects on the T cell hybridoma response. Taken together, these findings suggest that these HLA-DP2–restricted TCRs predominantly contact the HLA-DP2 β1 chain with little to no interactions with the α1 chain, raising the possibility that all DP2–restricted TCRs display a binding footprint distinct from those seen with other HLA II–restricted TCRs. Interestingly, the β1 R75A mutation abolished the response for all three T cell hybridomas (Fig. 7C), suggesting that this amino acid is a critical contact site for HLA-DP2–restricted TCRs.
FIGURE 7. Effect of HLA-DP2 variants on the response of other DP2-restricted TCRs. A, Stimulation of a T cell hybridoma expressing a Be-specific, Vα1/Vβ3+ TCR derived from the lung of CBD patient 1041 with HLA-DP2 variants. The T cell hybridoma, designated 1041-3.3, was stimulated with fibroblasts expressing wild-type and α-chain (left panel) and β-chain (right panel) variants of HLA-DP2 and varying BeSO₄ concentrations. Activation curves and EC₅₀ values are representative of three independent experiments. B, Stimulation of cells expressing a dengue virus-specific Vα11/Vβ23 TCR, designated JK34, with fibroblasts expressing HLA-DP2 α-chain (left panel) and β-chain (right panel) variants and presenting a dengue virus peptide. Representative activation curves from three independent experiments are plotted and used to determine EC₅₀ values. C, A color-coded summary shows the effects of HLA-DP2 variants on various DP2-restricted T cell hybridomas, expressing Be-specific TCRs 1332-28 and 1041-3.3 and a dengue virus-specific JK34 TCR. Residues are colored based upon their effect when mutated to alanine in comparison with the wild-type response. Residues highlighted in white indicate that an alanine mutation had no effect on T cell hybridoma response, with EC₅₀ values identical to the wild-type response. Residues highlighted in yellow and blue indicate a mutation that either had a moderate (2–8-fold) or large (50–100-fold reduction) effect on the hybridoma response. Residues highlighted in red abolished the IL-2 response when mutated to alanine.

Discussion

CD4⁺ T cells are intimately tied to the pathogenesis of CBD (4). Progression from Be sensitization to disease is characterized by the presence of granulomatous inflammation and the accumulation of Be-specific, Th1-polarized CD4⁺ T cells in the lung (5, 11, 30, 31). These lung T cells are composed of oligoclonal TCR Vβ expansions that include Vβ5.1 (32, 33). These expansions are specific for CBD patients, compartmentalized to lung, and persist at high frequency in patients with active disease (32). Thus, it was of interest when a large population of Be-responsive CD4⁺ T cells isolated from the lung of an HLA-DP2–expressing CBD patient was shown to express nearly identical TCR Vα1/Vβ5.1 chains asso-
ciated with different Vβ5.1 usage. In the current study, we used site-directed mutagenesis to determine how this set of related, Be-responsive TCRs recognize Be in the context of HLA-DP2–Be complex.

A crystal structure exists of an autoimmune TCR (3A6) expressing the same Vα22/Vβ5.1 pairing as our Be-specific TCR 1332-28. The 3A6 TCR is specific for a MBP self-peptide presented by HLA-DR2a (PDB identification code 1ZGL) and has a CDR footprint that is shifted toward the N terminus of the bound peptide and the MHC II β1 helix (19). Our alanine scan of the CDRs of 1332-28 showed a dominant role for the TCR Vβ5.1 chain in the recognition of Be/DP2, with several mutations in the CDR1β, CDR2β, and CDR3β abolishing the T cell hybridoma response, whereas only one mutation in the Vα domain (Y95A in CDR3α) affected recognition of Be/DP2. Importantly, these findings were corroborated in two other Vβ5.1-expressing, Be-specific TCRs that used different TCR Vα chains. In addition, the Vβ5.1⁺ TCRs maintained Be specificity even in the presence of a noncognate α-chain as long as the tyrosine (Y) at position 95 was present. Consistent with our findings that the CDR2α of 1332-28, -2, and -22 had no significant interactions with Be/DP2, the CDRαs of 3A6 also made no contact with the MBP/DR2a, being too short to interact with either DR2a or MBP (16, 19). Whereas the highly conserved tyrosine residue at position 30 of CDR1α of 1332-28 played no role in binding to Be/DP2, this residue in 3A6 strongly interacts with numerous DR2a β1 helix residues (16, 19). Comparing the TCR Vα chain contacts of 3A6 and 1332-28, eight Vα residues of 3A6 interact with MHC II compared with one for 1332-28. Accordingly, our findings suggest that the docking footprint of this set of Be-specific TCRs is more profoundly shifted than that described for 3A6, using mainly Vβ5.1 residues for the recognition of Be/DP2 with no contribution from germline-derived CDR1α or CDR2α.

Previous mutagenesis and structural studies show that both α-helices of the MHC contribute to TCR recognition. Germline-derived CDR1 and CDR2 residues from both TCR α- and
β-chains of the murine 2C TCR contributed energetically to rec-
ognition of MHC class I Ld and Kd, which was confirmed by the
solved structures (34–36). In addition, residues spanning both α-helices of murine I-Ek contributed to the recognition by the
mitochondrial C-specific TCR 2B4 (37). Similar results were
shown with a variety of T cells specific for 3K/I-Ak, with mu-
tations along both α-helices of I-Ak reducing TCR binding affinity
(29). Conversely, the present findings show that the α1 helix of
HLA-DP2 plays a minor role in interacting with the Be-specific
TCRs, with the β1 helix contributing the majority of the contact
sites. Interestingly, the two HLA-DP2 β1-chain mutations that
abolished the Be-specific T cell hybridoma response (D64A and
R75A in the β1 helix) surround the putative Be binding site on
HLA-DP2, involving glutamic acid residues at positions 26, 68, and
69 (Fig. 8). Site-directed mutagenesis of each of these three
negatively charged amino acids was previously shown to abrogate
T cell recognition of Be/DP2 (14). Thus, the docking footprint
used by Be-specific TCRs for the recognition of antigenic HLA-
DP2 is skewed, being dominated by the TCR β-chain and β1 helix
residues of DP2. To date, there is only one reported case in which
an unconventional binding topology has been used by a TCR for
the recognition of a foreign-derived Ag (38). Because we do not
know the peptide(s) that complete the Be-specific TCR ligand for
this set of TCRs, it remains unknown how peptide contributes to
TCR binding.

Although defined criteria have been used to characterize T cell
recognition of foreign-derived Ag, these rules do not apply to human
autoantigen-specific TCRs that exhibit atypical binding interactions,
as discussed above for TCR 3A6. The present study suggests that the
unconventional binding topologies observed for autoimmune TCRs can also be used for recognition of uncon-
ventional Ags such as Be and even HLA-DP2–restricted, an-
tiviral TCRs. There are several interpretations of the unusual binding mechanism adopted by the Be-specific TCRs. For ex-
ample, the docking footprint of the Be-specific TCRs may be shifted
toward the β1 helix of MHC II, with CDRIα and CDRIα making
no significant contacts with HLA-DP2 (Supplemental Fig. 4A). This
shift would position the TCR Vβ domain directly over the
putative Be-binding site of HLA-DP2, located between the peptide
backbone and β1 α-helix. To a lesser degree, similar findings were
reported for human TCRs Ob.1A12 and 3A6, which each recog-
nize multiple sclerosis-derived autoimmune complexes (18, 19).
Alternatively, the Be-specific TCRs may recognize Ag using a
tilted and reversed polarity, with the Vβ-chain contacting the β1
α-helix of MHC and the Vα-chain hovering over the α1 helix of
MHC, yet making no contacts (Supplemental Fig. 4B). As sup-
porting evidence, the docking footprint for the human multiple
sclerosis-derived TCR Hy.1B11 is tilted with interactions between
TCR Vβ and the MHC α1 helix dominating, while maintaining a
conserved docking polarity (17). The former mechanism seems
more plausible because a reversed polarity has not been reported
to date. Ultimate proof requires a structure of TCR-DP2–peptide–
Be complex, which has not been feasible without knowing which
peptide(s) completes the Be-specific TCR ligand.

The binding polarity between TCR and MHC is suggested to be a
function of evolutionarily conserved interactions between these
two molecules. However, whether conserved docking topologies are a consequence of evolutionarily conserved interactions between
germline-derived TCR regions (CDR1 and CDR2) and α-helix
residues of MHC is still unresolved (39). Supporting evidence from
analyses of TCR–pMHC crystal structures show that con-
erved pairwise interactions exist for related TCRs with certain
MHC alleles (16, 40, 41). We suggest that the Be-Ag generates an
alternative binding landscape for recognition by T cells even
though evolutionarily conserved interactions exist. To illustrate,
the majority of MHC II molecules express conserved amino acids
that lack bulky side chains (e.g., alanine) at the center of each
α-helix. These small residues are thought to create a cup that is
used to pivot the TCR for a diagonal binding orientation (16, 41).
For all of the DP2-restricted TCRs tested in this study, substitution
of a large bulky side chain at conserved alanine residues (A64Q in
α1 helix and A71Q in β1 helix; Fig. 6) of HLA-DP2 had no effect on
the T cell response. Even though conserved α-helical residues
exist in HLA-DP2, the Be-specific Vβ5.1 TCRs lack conserved
tyrosine residues in CDR2α and CDR1β, which have been sug-
gested to play a role in how TCR recognizes pMHC (16). As
mentioned, even though 1332-28 expresses a conserved Tyr
in CDR1α, an alanine mutation at this position did not affect
Be-specific T cell activation. It is noteworthy that the mutated
CDR2β-Tyr49 residue abolished the Be-specific T cell response,
because many other TCRs express a conserved Tyr in this region.

From the structural analysis of a Vα22/Vβ5.1 TCR (3A6) and our
TCR mutagenesis studies, it was expected that the Be-specific
Vβ5.1 TCR, 1332-28, would respond in a similar manner to Be
presented by either wild-type DP2 or DP2 with alanine mutations
in selected solvent-exposed α-helix residues. However, it was
surprising that two other DP2-restricted TCRs (a Be-specific Vα1/
Vβ3 TCR derived from the lung of another CBD patient and a
dengue virus-specific Vα11/Vβ22 TCR derived from the blood
of a subject infected with dengue virus) displayed similar results.
These findings raise the possibility that, unlike interactions with
other MHC II molecules, the majority of HLA-DP2–restricted
TCRs use a distinct binding topology in which most of the contact
sites involve the β1 helix of DP2. One possible reason for this
atypical footprint may lie in the unusual structural features of the
HLA-DP2 molecule. For example, HLA-DP2 possesses a widened
distance between the peptide backbone and β1 helix compared
with most other MHC II molecules (14). A recent modeling study
of HLA-DR1 with and without a bound peptide suggested that this
region of the β-chain α-helix may be flexible (42), contributing to
the variation in the width of this part of the binding groove and
possibly to differing TCR footprints.

In conclusion, the present work suggests that the Be-Ag (HLA-
DP2–peptide–Be) generates a unique landscape for recognition by
Be-specific TCRs, dominated by TCR β-chain and β1 α-helix
residues of DP2. Our findings provide additional evidence that
unusual binding topologies are not used exclusively by T cells to
recognize autoimmune complexes, but also for T cell recognition.
of unconventional Ags, such as Be, and DP2-restricted antiviral TCRs.

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Disclosures

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References