

Distinct T Cell Interactions with HLA Class II Tetramers Characterize a Spectrum of TCR Affinities in the Human Antigen-Specific T Cell Response¹

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The polyclonal nature of T cells expanding in an ongoing immune response results in a range of disparate affinities and activation potential. Recently developed human class II tetramers provide a means to analyze this diversity by direct characterization of the trimolecular TCR-peptide-MHC interaction in live cells. Two HSV-2 VP16_{369–379}-specific, DQA1*0102/DQB1*0602 (DQ0602)-restricted T cell clones were compared by means of T cell proliferation assay and HLA-DQ0602 tetramer staining. These two clones were obtained from the same subject, but show different TCR gene usage. Clone 48 was 10-fold more sensitive to VP16_{369–379} peptide stimulation than clone 5 as assayed by proliferation assays, correlating with differences in MHC tetramer binding. Clone 48 gave positive staining with the DQ0602/VP16_{369–379} tetramer at either 23 or 37°C. Weak staining was also observed at 4°C. Clone 5 showed weaker staining compared with clone 48 at 37°C, and no staining was observed at 23°C or on ice. Receptor internalization was not required for positive staining. Competitive binding indicates that the cell surface TCR of clone 48 has higher affinity for the DQ0602/VP16_{369–379} complex than clone 5. The higher binding affinity of clone 48 for the peptide-MHC complex also correlates with a slower dissociation rate compared with clone 5. *The Journal of Immunology*, 2000, 165: 6994–6998.

The human Ag-specific T cell response is extremely heterogeneous. Even within a single peptide-MHC recognition specificity, variation in TCR gene usage permits a range of diverse affinities and a corresponding variation in activation profiles. Identifying T cells that illustrate this diversity has been a challenging task. Recently, soluble MHC class I tetramers have provided a new tool for counting and sorting of Ag-specific CD8⁺ CTLs (1–9). In a similar approach, recombinant MHC class II tetramers with antigenic peptides covalently linked to the β -chain have been used to stain Ag-specific CD4⁺ Th cells (10–12). Recently, we described human MHC class II tetramers in which the peptide is loaded into the binding groove after formation of the class II α/β -heterodimer (13, 14).

The relationship between MHC tetramer binding of T cells and T cell activation by an APC presenting the same epitope is not clear. Whelan and co-workers, working with MHC class I-restricted CTLs, suggested that there might be experimental conditions where staining of a T cell clone with a particular MHC tetramer can occur, while the same epitope presented on an APC cannot induce a response in the same T cell clone (15). To address the relationship among tetramer staining intensity, TCR affinity, and T cell proliferation stimulated by the cognate MHC/peptide complexes, we analyzed two HLA-DQ0602-restricted CD4⁺ T

cell clones, directed against the same HSV-2 VP16 epitope, but which use different V α - and V β -chains. We show that the two T cell clones have different affinities for the peptide-MHC complex and that this correlates well with staining properties using the specific MHC class II tetramer.

Materials and Methods

Cell lines and donors

Bare lymphocyte syndrome (BLS)³ 1 DQ0602 is an HLA-DQ0602-transfected EBV-transformed B lymphoblastoid cell line (B-LCL) that has been previously described, in which DQA1*0102 and DQB1*0602 are the only HLA class II genes expressed (16, 17). Two distinct VP16_{369–379}-specific, DQ0602-restricted T cell clones (clones 5 and 48) were isolated from the same HSV-infected patient and were described previously (14). Clone 5 uses a TCR gene from the V β 6 family, while clone 48 uses a V β 13. BLS-1 DQ0602 was propagated in RPMI medium containing 10% FCS, 25 mM HEPES, 50 U/ml penicillin, and 50 μ g/ml streptomycin (all from Life Technologies, Gaithersburg, MD). The T cell clones were propagated in the same medium lacking FCS and supplemented with 12% pooled human serum and 64 U/ml human IL-2 (Hemagen, Waltham, MA).

HLA-DQ0602 tetramers

Peptide-loaded HLA-DQ0602 tetramers were produced as described previously (14). Briefly, recombinant DQA1*0102 and DQB1*0602, in which the transmembrane domain was replaced by a leucine zipper, were produced in *Drosophila* S2 cells. BirA was used to biotinylate the specific sequence contained in the DQB1*0602 construct. The resulting biotinylated heterodimers were loaded with peptides for 3 days at 37°C. PE-labeled streptavidin was used to produce fluorescent peptide-loaded DQ0602 tetramers. Peptides used corresponded to the sequence of the HSV-2 VP16 protein residues 369–388 (NNYGSTIEGLLDLPDDDDAP; VP16_{369–388}) and the HSV-2 VP16 protein residues 369–379 (NNYGSTIEGLL). The HSV-2 VP16 peptide, protein residues 40–50 (VP16_{40–50}; RLSQAQLMPSP), and class II-associated invariant chain peptide (CLIP) peptide, invariant chain residues 91–103 (MRMATPLLMQALP), were used as irrelevant peptides for

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³ Abbreviations used in this paper: BLS, bare lymphocyte syndrome; B-LCL, B lymphoblastoid cell line; CLIP, class II-associated invariant chain peptide; FSN, fluorescence signal to noise ratio.

a negative control. The peptides were synthesized on a peptide synthesizer (Perkin-Elmer/Applied Biosystems, Foster City, CA).

T cell proliferation assay

HSV-2 VP16₃₆₉₋₃₇₉ was used in T cell proliferation assays. The assays were performed in triplicate as 150- μ l reactions in 96-well plates. BLS-1 DQ0602 was used as the APC and was irradiated with 30,000 rad gamma radiation and used as a stimulator to responder ratio of 1:1. APCs were preincubated with 10, 1, and 0.1 μ M VP16₃₆₉₋₃₇₉ for 2 h and 10,000 T cells were added without removal of peptide. No IL-2 was added. After a 3-day incubation at 37°C, 1 μ Ci of [³H]thymidine/well was added. Cells were harvested, and tritium uptake was measured on a 1450 Microbeta Plus liquid scintillation counter (Wallac, Turku, Finland) after 15-h additional incubation.

DQ0602 tetramer staining analysis

T cells were stained with PE-DQ0602 tetramers, which were loaded with an HSV-2 VP16₃₆₉₋₃₈₈ or VP16₃₆₉₋₃₇₉ peptide in 50- μ l reactions containing 16 μ g/ml DQ0602 tetramers under various conditions. The conditions of tetramer staining are described in more detail in *Results*. As negative control for specific peptide-loaded DQ0602 tetramers, DQ0602 tetramers loaded with either another VP16 peptide (VP16₄₀₋₅₀) or CLIP were used. All staining reactions were conducted in staining buffer 1 \times PBS containing 0.02% NaN₃ and 0.2% FCS. The flow cytometry was conducted on a BD FACScalibur cell sorter (Becton Dickinson, Franklin Lakes, NJ). For TCR-binding competition experiments, PE-labeled DQ0602 tetramers at a final concentration of 8 μ g/ml were mixed with unlabeled DQ0602 monomers

at final concentrations of 40, 80, 160, 240, 320, 400, 600, and 800 μ g/ml. T cells were incubated with the tetramer/monomer mixtures for 3 h at 37°C and were analyzed by flow cytometry. For DQ0602 tetramer dissociation studies, T cells were incubated for 30 min in PBS containing 0.5% para-formaldehyde at ambient temperature, washed in staining buffer, and stained for 3 h at 37°C. T cells were washed in cold staining buffer, re-suspended on ice in staining buffer containing 100 μ g/ml of a blocking pan-DQ Ab (SPVL3; DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA), and incubated for various periods on ice before analyzing the staining intensity by flow cytometry as described for the off-rate of an I-E^k/TCR complex (18).

Results

Clones 48 and 5 are distinct DQ0602-restricted, CD4⁺ human T cell clones isolated from the same individual and directed against the same HSV-2 VP16 epitope (14). Staining of the clones with peptide-loaded, PE-labeled DQ0602 tetramers, however, revealed differences between these two clones that indicate a wide disparity in binding and activation properties. Incubation of T cells for 3 h with VP16₃₆₉₋₃₈₈ peptide-loaded, PE-labeled DQ0602 tetramers on ice gave only a very weak staining with clone 48 (Fig. 1*a*). Incubating clone 48 with DQ0602 tetramers at 23°C for 3 h gave rise to an intensive staining (Fig. 1*b*). The mean staining intensity was even higher when incubation was conducted at 37°C (Fig. 1*c*). In contrast to these results, clone 5 showed no staining with

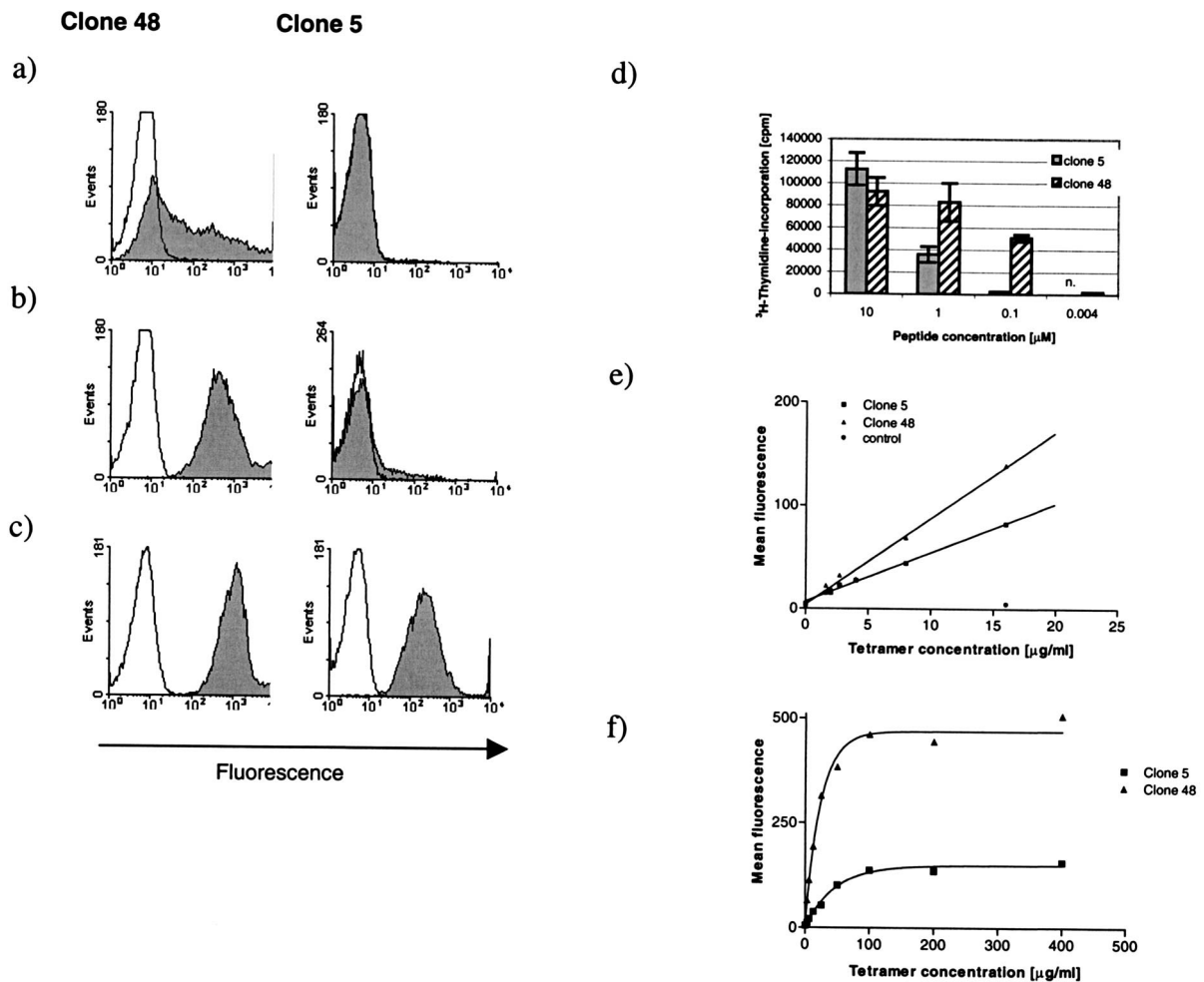


FIGURE 1. DQ0602 tetramer staining patterns of clones 5 and 48 at various incubation temperatures. *a*, Incubation on ice; *b*, incubation at 23°C; *c*, incubation at 37°C. The staining was conducted with nonfixed cells and an incubation time of 3 h. *d*, Thymidine incorporation of clones 5 and 48 after stimulation with the VP16₃₆₉₋₃₇₉ peptide. The stimulation was conducted with peptide left in for 3 days. *e* and *f*, Influence of DQ0602 tetramer concentration on staining intensity. Staining of nonfixed cells was performed for 3 h at 37°C at different DQ0602 tetramer concentrations. DQ0602 tetramers loaded with an irrelevant peptide were used as a negative control at a concentration of 16 μ g/ml.

VP16₃₆₉₋₃₈₈ peptide-loaded DQ0602 tetramers when incubation was performed on ice or at 23°C (Fig. 1, *a* and *b*). Incubation at 37°C restored staining with clone 5, although the mean staining intensity was lower compared with that of clone 48 (Fig. 1*c*). Identical staining patterns were observed with VP16₃₆₉₋₃₇₉-loaded tetramers (data not shown). At all three temperatures no binding was observed to either clone with DQ0602 tetramers loaded with an irrelevant peptide.

T cell proliferation assays using peptide-loaded DQA1*0102/DQB1*0602-expressing BLS-1 cells as APCs showed a difference in responsiveness of the two T cell clones when using suboptimal peptide concentrations (Fig. 1*d*). The proliferation of clone 48 was at least 10-fold more sensitive to peptide concentration compared with that of clone 5. Staining for CD3 and the costimulatory molecule CD28 revealed no differences in expression levels for the two clones (data not shown). Clones 5 and 48 were stained for 3 h at 37°C with different concentrations of PE-labeled VP16₃₆₉₋₃₈₈-loaded DQ0602 tetramers (Fig. 1*e*). There was a linear relationship between the mean staining intensity and the tetramer concentration for low tetramer concentrations. The slopes of the line were 4.7 ± 0.1 for clone 5 and 8.3 ± 0.4 for clone 48, meaning that comparable staining intensities of clone 5 compared with clone 48 required a concentration of almost twice as much tetramer. At higher concentrations of PE-labeled tetramers ($>100 \mu\text{g/ml}$) the mean fluorescence of both clones reached saturation (Fig. 1*f*). The mean fluorescence at saturation level for clone 5 was about 150, while that for clone 48 was about 470.

These differences between clones 48 and 5 in sensitivity in proliferation assays and in staining intensities with DQ0602 tetramers could reflect differences in TCR affinity for the DQ0602-peptide complex or variation in the mobility or aggregation of membrane-TCR interactions. These hypotheses were tested in a series of binding studies. Using a suboptimal amount of PE-labeled VP16₃₆₉₋₃₈₈-loaded DQ0602 tetramers ($8 \mu\text{g/ml}$) in the presence of increasing amounts of unlabeled peptide-loaded DQ0602 monomers, T cells of clones 5 and 48 were stained for 3 h at 37°C (Fig. 2). Staining of clone 5 dropped in a steep slope, reaching background levels at an approximately 30-fold excess of monomeric competitor. Staining of clone 48 dropped much more slowly, approaching a plateau at an excess monomer concentration of about 50- to 100-fold. These competition binding profiles showed that while the clone 5 tetramer staining was efficiently competed by peptide-loaded DQ0602 monomers, the clone 48 staining was not, suggesting a high avidity interaction in the latter case.

To evaluate the influence of membrane fluidity, internalization of TCR-DQ0602 complexes, and structural changes in the TCR on staining with DQ0602 tetramers, tetramer binding assays were

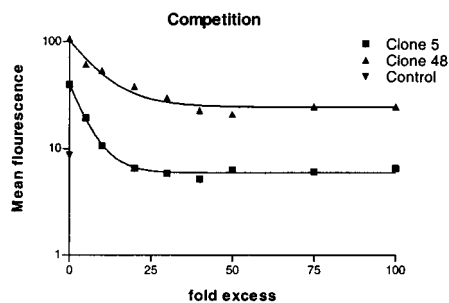


FIGURE 2. TCR binding competition. Cells were incubated for 3 h at 37°C with a suboptimal amount of PE-labeled VP16₃₆₉₋₃₈₈-DQ0602 tetramers and increasing amounts of unlabeled VP16₃₆₉₋₃₈₈-DQ0602 monomers.

compared between paraformaldehyde-fixed cells and nonfixed cells. There was virtually no difference in the tetramer staining of clone 5 using fixed cells, and there was a small decrease in staining of clone 48 (Fig. 3*a*). Interestingly, for incubation times up to 6 h, there was little change in staining intensities for fixed and nonfixed cells, while after a 24-h incubation at 37°C the staining intensities of nonfixed cells were increased relative to staining of paraformaldehyde-fixed cells, suggesting that some internalization may occur with prolonged incubation (data not shown).

The low affinity of clone 5, as shown in Fig. 2, could reflect a rapid dissociation of the TCR-tetramer complex with different off-rates of the DQ0602-tetramer complex binding to clones 5 and 48. To test this, paraformaldehyde-fixed T cells of clones 5 and 48 were stained with DQ0602 tetramers for 3 h at 37°C, cooled on ice, and washed with ice-cold staining buffer. Dissociation of DQ0602 tetramers was conducted in the presence of $100 \mu\text{g/ml}$ of a neutralizing pan-DQ Ab in staining buffer to prevent dissociated DQ0602 tetramers from rebinding. DQ0602 tetramer staining intensities were measured by flow cytometry after incubation on ice for 5, 10, 15, 30, 60, 90, and 120 min (Fig. 3*b*). For clone 5 additional time points after an incubation with neutralizing pan-DQ Ab for 1, 2, 3, and 4 min were measured. After only a 5-min incubation of clone 5 with the neutralizing Ab, staining intensity had dropped to a plateau of about 20% of the original staining level. Dissociation of bound DQ0602 tetramers from clone 48 was much slower, as staining intensities fell gradually below 50% of the original staining intensity after approximately

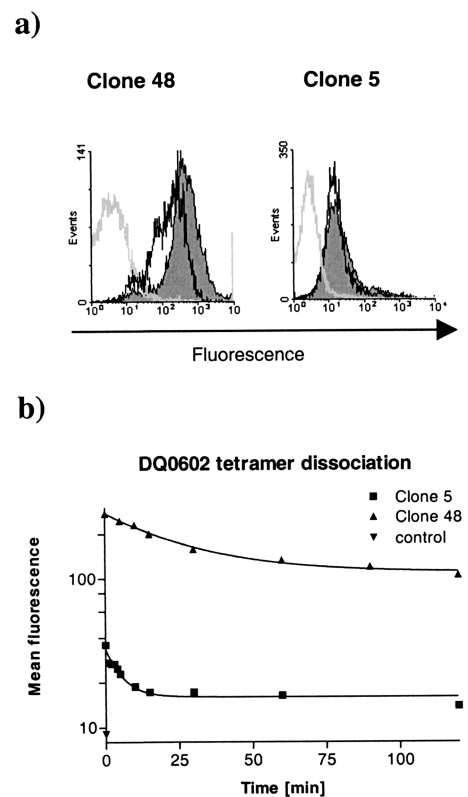


FIGURE 3. Time course of DQ0602 tetramer staining and dissociation of TCR-peptide-DQ0602 tetramer complexes. *a*, Three-hour staining of clones 48 and 5 with nonfixed cells (gray areas), and paraformaldehyde-fixed cells (black line) at 37°C. Gray line, staining with DQ0602 tetramer loaded with irrelevant peptide. *b*, Stained cells were incubated with an excess of neutralizing DQ Ab, and DQ0602 staining intensity was analyzed at different time points.

45-min incubation with the DQ Ab. The decrease in DQ0602 tetramer staining in clone 48 reached a plateau at 40% of the original staining level after 90 min. These results show that while most of the TCR-DQ0602-VP16₃₆₉₋₃₈₈ complexes of clone 5 dissociate in <5 min, the interaction of the TCR of clone 48 with DQ0602 VP16₃₆₉₋₃₈₈ is much more stable.

Discussion

Two distinct DQ0602-restricted T cell clones directed against the same epitope (clones 48 and 5) were used in these studies to explore the range of TCR-peptide-MHC interactions that occur within a single individual during an Ag-specific immune response. We took advantage of recently developed class II human peptide-MHC tetramers to compare differences in staining intensities with specific peptide-loaded class II tetramers seen between these two clones. While both clones expressed surface TCR at about the same level as measured by CD3 staining, clone 5 required a higher peptide concentration in proliferation assays and stained weakly with DQ0602 tetramers compared with clone 48. Staining intensities increased in both clones for incubation times between 1 and 3 h, arguing in favor of a slow process involved in the TCR binding to the specific soluble MHC class II molecules. It is possible that conformational changes in either the TCR itself or the peptide-MHC complex are essential for TCR-MHC binding/staining with peptide-MHC tetramers. However, major movements of the cell membrane and internalization of the receptors does not seem to be crucial, as paraformaldehyde fixation did not abrogate the staining. Conformational changes in the TCR upon peptide-MHC complexes as well as changes in the MHC molecule upon binding peptide have been observed in crystal structures of a class I TCR/MHC complex (19, 20). Using thermodynamic and kinetic analyses of the binding of the TCR to MHC class I molecules, Willcox et al. (21) argued that binding must indeed overcome significant activation barriers to allow binding, suggestive of conformational changes.

It could be hypothesized that tetramer binding cross-links the TCR and therefore increases the local TCR concentration on the surface of the T cell, such that further tetramer binding would be enhanced. In this case a higher tetramer concentration would yield a significantly higher staining pattern than if tetramers would bind independently from each other. The linearity of the effect of tetramer concentration on staining intensity in both clones, however, indicates that there is no significant cooperativity in tetramer binding.

A high affinity of the interaction between the peptide-MHC and the cognate TCR might cause the simultaneous binding of multiple binding sites within the MHC tetramer. For a low affinity TCR, only one binding site in the MHC tetramer may actually be bound to the T cell at a given time point. In this case the displacement of labeled MHC tetramers from the TCR by unlabeled MHC monomers should be more efficient with a low affinity TCR. The results of our competition experiment with clones 5 and 48, as shown in Fig. 2, fit very well with this view.

If at a given time point, only a very low number of available binding sites in a DQ0602 tetramer are bound to the TCR on clone 5 cells, this would also be reflected in a short half-life of the complex when incubated with neutralizing Ab to prevent rebinding of dissociated DQ0602 tetramers. For clone 5, DQ0602 tetramer staining levels fell almost down to background levels in only 5 min of incubation in the presence of an excess of a pan DQ Ab, while staining intensities with clone 48 were at about 40% of the original staining, which is 5 times higher than the staining intensity of the original staining of clone 5, even after 2-h incubation with an excess of neutralizing Ab.

We conclude that the VP16₃₆₉₋₃₇₉-specific DQ0602-restricted T cell clones 5 and 48 are of relatively low (clone 5) and high (clone 48) affinities and that this is not only reflected by the ability of these clones to proliferate after stimulation with different concentrations of specific peptide, but also by peptide-MHC tetramer binding and dissociation rates with peptide-loaded DQ0602 tetramers. Whelan and co-workers used HLA class I tetramers loaded with altered peptide ligands to stain CTLs at different temperatures (15). They demonstrated that tetramers loaded with lower affinity peptide ligands stained CTLs at 4°C, but not at 37°C. Only high affinity ligands were able to stain the specific CTL at 37°C. These results are in sharp contrast to what we found using peptide-loaded HLA class II tetramers to stain different affinity T cell clones, suggesting that MHC class I/peptide and MHC class II/peptide complexes are differently viewed by the TCR. It is conceivable that different molecular orientation or kinetics are involved in the formation of the TCR class I and class II complexes. CD4 or CD8 molecules may also play different roles in the formation of these complexes. Recently, for example, it has been shown that CD8 contributes to class I tetramer staining (22), although the role of CD4 in class II tetramer staining has not been studied.

Clones 5 and 48 arose within a single HSV-2-infected individual and undoubtedly represent isolated examples within a broad spectrum of TCR specificity and avidity. The major differences described in this study in clones with the same peptide-MHC specificity exemplify the enormous potential diversity within an ongoing immune response. Not only do peptide-loaded class II tetramers allow identification of Ag-specific T cells, but temperature sensitivity and intensity of tetramer staining can also provide an estimate of the relative affinity of the TCR toward its cognate MHC/peptide complex. Molecular modeling of these distinct TCRs in complex with DQ0602 and the specific peptide will give further insights into the differences between these two clones and will enhance the understanding of the interaction between the TCR and its peptide-MHC ligand in general.

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