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Research Article

Molecular Identification of an MHC Class Ib (H2-Q9) Restricted T Cell Receptor Specific for a Mouse Polyoma Virus Peptide VP2.139

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Abstract

The murine major histocompatibility complex (MHC) class Ib region encodes more than 20 proteins with diverse biological functions, and many have not been characterized. Evidence emerging in recent years, indicates that MHC class Ib-restricted CD8 T cells can play an important role in antiviral immunity. We molecularly cloned an α/β T cell receptor (TCR) from a T cell hydridoma cell line, that is restricted by a MHC class Ib molecule H2-Q9, and specifically recognizes the VP2.139 peptide epitope from mouse polyoma virus (PyV). We further demonstrated that the cloned TCR, using TCRα10 and TCRβ4 genes, is functional and mediates the development of Q9/VP2.139-restricted CD8 T cells *in vivo*. The cloning of a H2-Q9-restricted, PyV-specific αβ TCR provides a useful tool for future study of the potential role of MHC class Ib restricted CD8 T cells in antiviral immunity.

Keywords: MHC Class Ib; Qa-2; H2-Q9; CD8+ T cell; TCR; Polyomavirus

Introduction

Major histocompatibility complex (MHC) class I proteins play an important role in adaptive immunity [1,2]. They provide a mechanism of immunosurveillance by presenting intracellular antigens from either self or pathogenic origin to CD8 T cells, natural killer (NK) cells and/or natural killer T (NKT) cells [1,3-5]. The functions of the highly polymorphic classical MHC class I (MHC class Ia) molecules, including H2-K, -D and -L in mouse and HLA-A, -B and -C in human, are well established [2,6]; however, most of the non-classical MHC class I (MHC class Ib) molecules, with limited polymorphism, are not well studied [6,7]. The Qa-2 antigen (encoded by the H2-Q6, 7, 8, 9) is one of the best characterized class Ib molecules in mouse (possibly homologous to HLA-G in human). H2-Q9 encodes two types of Qa-2 isoforms, membrane-bound and soluble, through alternative splicing of transcripts [8]. Multiple biological functions of Q9, as well as that of HLA-G in human, have been documented, such as participating in preimplantation embryo development [9,10], tumor immunity [11,12], and antiviral immunity [13]. However, the selection of CD8+ T cells by Q9 and the characteristics of Q9-restricted CD8+ T cells are poor understood.

Cytotoxic CD8+ T cells directly eradicate or control intracellular pathogens by killing infected cells or by releasing cytokines to inhibit further spread of infection [14]. The selection and development of antiviral CD8+ T cells by class Ia molecules has been well documented [1,2]. However, the role of class Ib-restricted antiviral CD8+ T cells needs further investigation. The potential mechanisms of control of viral infection by class Ib-restricted CD8+ T cells are unclear and the development pathways for this subset of CD8+ T cells are poorly defined. Recently, CD8+ T cells have been identified that can recognize viral epitopes presented by MHC class Ib molecules [15-17], and there is evidence that class Ib-restricted CD8+ T cells contribute to the control or eradication of virus infection [13,18,19].

Polyomaviruses are double-stranded DNA viruses, persistently infecting humans or animals, and potentially inducing tumors in immuno compromised individuals [20]. Since the 1970s, at least 6 polyomaviruses, including BK virus, JC virus, KI polyoma virus, WU polyoma virus, Merkel cell polyoma virus and trichodysplasia spinulosa virus, have been identified in humans, [20-24]. Mouse polyomavirus (PyV) was the first identified polyomavirus [20,25] and it can induce tumors in immunocompromised mouse strains [25,26]. T antigens of PyV can induce class Ia-restricted CD8+ T cell responses [27,28]. Recently, Swanson et al. successfully identified MHC class Ib, Q9-restricted CD8+ T cells that responded to a PyV-derived epitope VP2.139 [13], which provided the first solid evidence demonstrating that class Ib-restricted CD8+ T cells might play a role in controlling persistent viral infection.

In the current study, we molecularly identified a T cell receptor (TCR) from Q9/VP2.139-specific CD8+ T cells and confirmed its function in transduced cells as well as in retrogenic mice. To our knowledge, this was the first identified MHC class Ib-restricted TCR that responds to viral infection.

Materials and Methods

Mice

C57BL/6J and Rag1$^{-/-}$ mice were purchased from the Jackson Laboratory. OT-I TCR transgenic mice, with specificity to OVA357-364/K^{b} (OVA-I/K^{b}), have been previously described [29]. K$^{b-/-}$D$^{b-/-}$ CIITA$^{-/-}$ mice were generated in our laboratory as previously described [30]. Eight- to 12-week-old mice were used in the experiments. Mice were bred and housed in a specific pathogen-free facility at the University of Utah and were handled according to the Institutional Animal Care and Use Committee policies.

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Cell lines and culture conditions

The Q9/VP2.139-specific C1-21 T cell clone and T cell hybridoma lines C3K and 3H6, and the recipient lines Z6.21-3B2.E7 and BWZ were provided by Dr. Aron Lukacher (Emory University). The recipient cell lines 58α-/β- and 5KC/CD4 were provided by Dr. Piotr Kraj (Georgia Regents University) and maintained as previously described [31]. 3H6 T cell hybridoma was further sorted into 3H6 (+) and (-), based on the presence or absence of surface expression of TCR Vα2. 3H6(-) cells, which did not produce IL-2 upon stimulation of VP2.139 peptides, were used as recipient cells in retroviral transduction experiments. The retroviral packaging cell line Plat-E was described previously [32]. The C1-21 T cell clone was maintained in IMDM with 10% FBS (Hybrid), 10 U/ml of mouse IL-2, 10 uM of VP2.139 peptides and irradiated $K^{b-/-}D^{b-/-}CIITA^{-/-}$ mouse splenocytes as antigen presenting cells (APCs). C3K and 3H6 T cell hybridomas, recipient cell lines and Plat-E packaging cell lines were maintained in DMEM with 10% FBS, supplemented with 100 U/ml of penicillin, 100 mg/ml of streptomycin, 292 mg/ml of L-glutamine, 100 mM of non-essential amino acids, 1 mM of sodium pyruvate, and 55 uM of 2-ME (Invitrogen), as complete DMEM as previously described [18]. Cells were maintained in a humidified 37°C incubator supplied with 5% CO_2.

Generation of soluble H2-K^b, H2-Q9 proteins and Q9/VP2.139 tetramer

The peptides L19 (ILMEHIHKL), VP2.139 (HALNVVHDW), OVA-I (SIINFEKL) were synthesized, and substitute L19-H5C, VP2.139-V5C and OVA-I-N4C peptides were synthesized and biotinylated at the core facility of University of Utah. Soluble H2-K^b and H2-Q9 proteins were purified from *E. coli* (BL21)-derived proteins and refolded with the appropriated peptides in a 1:1 molar ratio to produce Q9/L19, Q9/VP2.139 or K^b/OVA-I monomers, using our previously described method [33]. To generate Q9/VP2 tetramers, refolded Q9/VP2.139 monomer was biotinylated and tetramerized with streptavidin-APC (Invitrogen) in a 4:1 molar ratio as previously described [13,34]. Peptide dissociation rates and MHC-peptide complex half-lives were measured as previously described [33,35]. Briefly, Q9/L19, Q9/VP2.139 or Kb/OVA-I complex (50 nM) was incubated with biotin-L19, biotin-VP2.139 or biotin-OVA-I (1 μM) peptide, respectively, in PBS with 0.01% Nonidet P-40 at RT for overnight. Then, unlabeled L19, VP2.139 or OVA-I peptide (200 μM), respectively, was added and incubated at 37°C for the indicated lengths of time. The remained biotin-peptide that bound to the MHC class I molecules was measured by Eu-ELISA using an anti-β2m capture mAb [35].

Abs and flow cytometry

Fluorophore-conjugated mAbs to mouse CD4 (H129.19), CD8 (RPA-T8), CD62L (MEL-14), Vα2 (B20.1), Vα3.2 (B21.14), Vα8.3 (RR3-16), Vα11 (RR8-1), isotype-matched control mAbs, and a mouse Vβ TCR Screening Panel (557004) were purchased from BD Pharmingen. Cell surface staining and Q9/VP2 tetramer staining were performed according to the standard procedures. Stained cells were analyzed on a FACS can flow cytometer (BD Bioscience) and data were analyzed using FlowJo 8.4 software.

Cloning of the TCR α chain and β chain gene and generation of expression constructs

Total mRNA of C3K hybridoma cells was extracted using the RNeasy Kit (Qiagen) and the cDNA was reversely transcribed, with the SuperScript™ III Reverse Transcriptase Kit (Invitrogen) following the manufacturer's instructions, for further cloning. The TCR Express™ mouse TCR Vα screening kit was purchased from Biomed Immunotech. TCR variable region prediction and open reading frame analysis were performed using the IMGT website for mouse TCR analysis (http://www.imgt.org/IMGT_vquest/), as well as the sequences previously published [36-38]. The primers used for cloning of the full length TCRα10 and TCRβ4 cloning were listed as below:

TCRα10 forward (TCRVα10f-F): 5'-GTGCGGCCGCGTTTAAACATGAAGAGGCTGCTGTGCTCTCTGC-3', TCRα10 reverse (TCRCαf-R): 5'-TTCCTGCAGGGTTTAAACTCAACTGGACCACAGCCTCAG-3'; TCRβ4forward (TCRVb4f-F): 5'-GCTTAATTAAGTTTAAACATGGGCTCCATTTTCCTCAGTTG-3', TCRβ4 reverse (TCRCb1fR-R): 5'- ATGGCGCGCCGTTTAAACTCATGAATTCTTTCTTTTGACCATAGCC-3'. To co-express TCRα10 and TCRβ4, we fused full-length TCRα10 (excluding stop codon) and TCRβ4 genes with T2A (GAGGGCAGAGGAAGTCTTCTAACATGCGGTGACGTGGAGGAGAATCCCGGCCCT) sequence [39], using primers TRA10-2A-R (5'-CTCCTCCACGTCACCGCATGTTAGAAGACTTCCTCTGCCCTCGTTAACACTGGACCACAGCCTCAGCG-3') and 2A-TRB4-F (5'-CTTCTAACATGCGGTGACGTGGAGGAGAATCCCGGCCCTATGGGCTCCATTTTCCTCAGTTGC-3'), paired with TCRVα10f-F or TCRCβ1fR-R, respectively, in two rounds of PCR amplification, to generate a TCRα10-T2A-β4 (C3K) construct. All of these PCR products were amplified with the Platinum® Taq DNA Polymerase High Fidelity PCR Kit (Invitrogen) following the manufacture's instruction and cloned into the pCR2.1 vector using the TOPO® TA Cloning Kit (Invitrogen). The DNA sequences were determined by sequencing (the GenBank accession number: KJ755332). Then, the TCRα10-T2A-β4 construct in the pCR2.1 vector were digested with PmeI (Roche) and furthercloned into a retroviral vector MigR1 as previously described [40,41]. The OT-I TCR construct TCRα2-T2A-β5.2 in MigR1, used as a positive control in generation of the retrogenic mice, was a gift from Dr. Kate Vignali (St. Jude Children's Research Hospital).

Retroviral transfection and retrogenic mouse generation

Retroviral supernatants were generated by transfection of Plat-E cells with MigR1-TCRα10-T2A-β4 (C3K) or MigR1-TCRα2-T2A-β5.2 (OT-I) as previously described [32,41,42]. The C3K and OT-I retrogenic mice were generated as previously described [43]. Briefly, $5x10^7$ bone marrow cells were harvested from 6-week-old $Rag1^{-/-}$ mouse and cultured in 15 ml of complete DMEM with 20% FBS, supplemented with IL3 (20 ng/ml), IL-6 (50 ng/ml) and SCF (50 ng/ml) (R&D systems, Inc) for 48 hours. The infected bone marrow cells were re-infected by adding 5 ml of fresh C3K or OT-I retroviral supernatants produced from Plat-E transfectants. Four days post-tranduction, semi-lethally irradiated (450 rad) $Rag1^{-/-}$ recipient mice were reconstituted with the transduced bone marrow cells, following the previously described protocol [43]. Five-weeks post reconstitution, peripheral blood samples were collected and analyzed for GFP expression and cell surface staining. Six weeks post-reconstitution, retrogenic mice were sacrificed for analysis.

T cell activation assay and measurement of IL-2 production

To stimulate C1-21 T cells, 3H6 and C3K T cell hybridoma cells, or the TCR transductant, 2x10^5 cells (per well in a 96-well plate), with 2x10^5 of irradiated K$^{b-/-}$D$^{b-/-}$CIITA$^{-/-}$ splenocytes, which only expresses MHC class Ib [30], as APCs, and 0, 1, or 10 uM of VP2.139, OVA-I or L19 peptides, were added. For retrogenic mice T cell activation assay, 4x10^5 cells per well of C3K retrogenic or OT-I TCR transgenic mouse splenocytes were cultured with 0, 0.5, 1.0, 2.0, or 4.0 uM of VP2.139, OVA-I or L19 peptides. Eighteen hours later, the supernatants were harvested and measured for mouse IL-2 production by the standard IL-2 ELISA procedure (eBioscience).

Results

Characterization of Q9/VP2.139 complex stability and T cell specificity

It has been shown previously that VP2.139 peptide bound to Q9 can stimulate specific CD8+ T cells during mouse polyomavirus infection [13]. In order to further characterize the VP2.139 peptide binding capacity with Q9 and to generate a Q9/VP2.139 tetramer reagent for staining of T cells, soluble recombinant Q9 protein (α1 to α3 domains without the transmembrane and cytoplasmic domains) was expressed in *E. coli* and the inclusion body was purified and refolded with VP2.139 peptide or L19 peptide (as a control), a known Q9 bound endogenous peptide derived from mouse 60S ribosomal protein [44, 45]. In addition, soluble K^b [33] was prepared using the same method (Figure 1A). Interestingly, the Q9/VP2.139 complex has relatively lower complex stability with a half-life of 248 min, compared with the complex of Q9/L19 or Kb/OVA-I with half-life of 1732 min or 1155 min, respectively (Figure 1B), which is consistent with previously observations [46].

The C3K T cell hybridoma used for TCR cloning was derived from a previously reported Q9/VP2.139-specific CD8+ T cell clone, C1-21[13], which was also confirmed for its TCR binding specificity using our Q9/VP2.139 tetramer in staining (Figure 1C). When stimulated with the VP2.139 peptide, the C3K T cell hybridoma cells were activated to produce IL-2 cytokine (Figure 1D), indicating that the TCR used in C3K was the same as its parental CTL clone C1-21. Thus the C3K T cell hybridoma line was used to clone the α and β chains of its TCR.

Cloning of the α and β chains of TCR from C3K T cell hybridoma

To identify the α and β chains of TCR used by C3K T cell hybridoma, C3K cells were firstly surface stained for the TCR Vβ usage by using a mouse Vβ TCR Screening Panel. As shown in Figure 2A, Only anti-TCR Vβ4 mAβ positively stained the C3K cells, among all of the 17 anti-TCR Vβ mAβs included in the Screening Panel, but none of the commercially available anti-TCR Vα antibodies, Vα2, Vα3.2, Vα8.3 Vα11, stained the C3K cells. In order to determine the TCRα chain usage of C3K, we next screened the TCR Vα region by PCR using a TCR Express™ mouse TCR Vα screening kit. Initially, several Vα regions were positively amplified (Figure 2B). After sequencing the PCR products, we found that many of the PCR products were from non-specific amplification due to the sequence similarity, such as Vα1, Vα2 and Vα9. Vα5 was another transcript amplified in several different reactions (Vα5, Vα15, Vα16, Vα18 and Vα20), but there was no open reading frame (ORF) predicted at the translation level (Figure 2C). Therefore, we concluded that TCRα10 and TCRβ4 were used in C3K.

Next, the full-length genes of both TCRα10 and TCRβ4 were

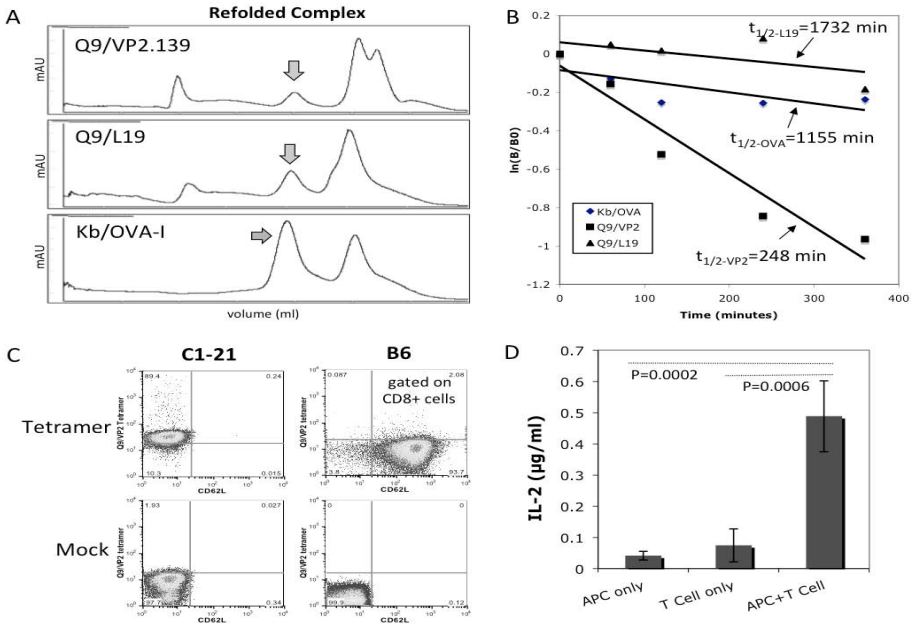
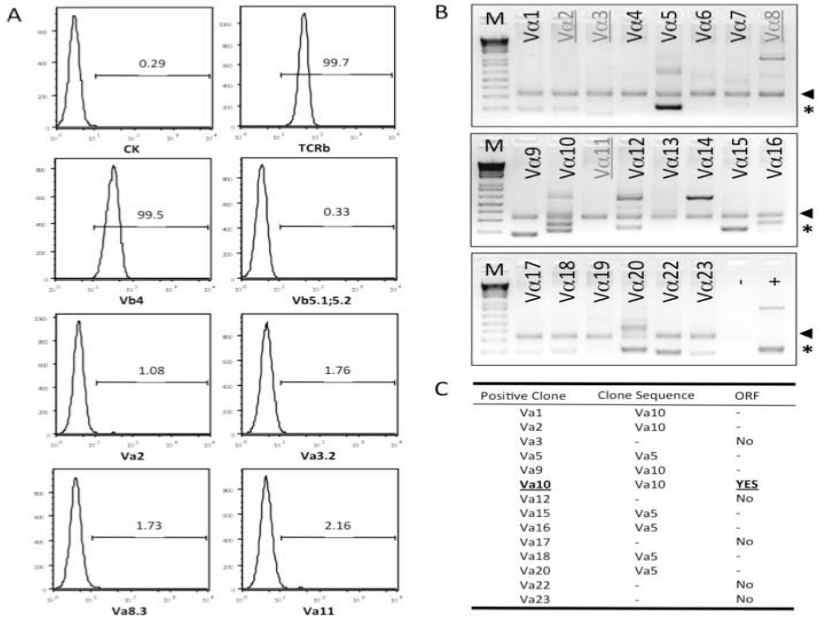
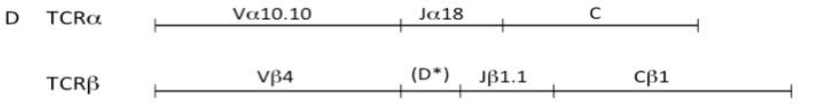


Figure 1: Generation of Q9/VP2.139 peptide complexes and characterization of C3K, a VP2.139 peptide specific CD8 T cell hybridoma line. (A) Refolding of soluble Q9 with a polyomavirus-derived VP2.139 peptide, or L19 peptide, and of K^b with OVA-I peptide as a positive control. The arrows indicated the refolded MHC-I/peptide complexes. (B) Comparison of peptide dissociation rates. The arrows show the calculated half-lives ($t_{1/2}$) of biotin-labeled peptides dissociated from the MHC-I/peptide complexes. (C) Cell surface staining of Q9/VP2.139-specific CD8 T cell clone by Q9/VP2 tetramers. Non-biotinylated Q9/VP2.139 monomers mixed with SA-APC, and CD62L mAb matched isotype control, were used for the mock staining. (D) T cell activation assay by measuring the secreted IL-2 cytokine for C1-21-derived C3K T cell hybridomacells stimulated with 2x10^5 cells per well, using K$^{b-/-}$D$^{b-/-}$CIITA$^{-/-}$splenocytes as APCs and 10 uM of VP2.139 peptides.



C

Positive Clone	Clone Sequence	ORF
Va1	Va10	-
Va2	Va10	-
Va3	-	No
Va5	Va5	-
Va9	Va10	-
Va10	Va10	**YES**
Va12	-	No
Va15	Va5	-
Va16	Va5	-
Va17	-	No
Va18	Va5	-
Va20	Va5	-
Va22	-	No
Va23	-	No



(D*) A short version of Dβ1 with only 4 nucleotides (acag) left and one N-nucleotide (t) insertion, between Vβ4 and Jβ1.1.

TCR	V region	N	D region	J region
TCRα10	tgtgct.....	tc		.agatagaggttcagccttagggaggctgcatttt
TCRβ4	tgtgccagcagccaa..	t	...acag.....	....cacagaagtcttcttt

Figure 2: Cloning of T cell receptor from C3K T cell hybridoma. (A) Cell surface staining to screen usage of TCRα and TCRβ in C3K T cell hybridoma. (B) PCR screening of the variable region(s) of TCRα chain expressed in C3K T cell hybridoma. The arrows show the internal control designed in the TCR PCR screening kit (BioMed Immunotech). The asterisks indicate the positive PCR products. All of the positive bands were purified and cloned into a TA cloning vector and the sequence was determined by sequencing. The signs of (–) and (+) indicate the negative and positive PCR controls. (C) Identification of Vα10 as the variable region used in C3K T cell hybridoma by sequencing analysis and open reading frame (ORF) prediction. Some of the amplified bands were proved to be non-specific PCR amplification products, confirmed by sequencing (-) and without ORF (no). (D) The detailed usage of V, D, J and C regions in full length of TCRα and TCRβ chains cloned from C3K T cell hybridoma, showed in upper panel. The lower panel shows the information of P- and N-nucleotides and the V and J regions coding the CDR3 region of the TCRα10 and TCR β4 chains.

cloned from C3K cDNA. Sequence analysis confirmed that the variable region of C3K TCRα contained Vα10.10 and Jα18, and that of C3K TCRβ contained Vβ4, Dβ1 and Jβ1.1 with the constant region of Cβ1. Interestingly, only a very short D region of Dβ1 (acag) with one N-nucleoside (t) insertion was detected between Vβ4 and Jβ1.1. Both TCRα10 and TCRβ4 from C3K showed open reading frames in the correct frame (Figure 2D), with a 14aa of coding complementarity determining region 3 (CDR3) sequence, CASDRGSALGRLHF, for TCRα10, and a 12aa of CDR3 sequence, CASSQYSTEVFF, for TCRβ4, respectively.

Verification of the function of cloned TCRα10 and TCRβ4 in cell lines

In general, a given T cell expresses only one TCR α chain and β chain pair. However, a single T cell can express more than one α chain [47-49]. To test whether the cloned C3K TCR α and β chains indeed pair with each other and are functional, we tried to express the construct of TCRα10-T2A-β4-MigR1, which also contained an eGFP reporter driven by an IRES (Figure 3A), in several cell lines, including 58α-/β-, 5KC/CD4, Z6.21-3B2.E7 and BWZ. Although pairing and surface expression of the TCR was observed in several of these cell lines, responsiveness to the VP2.139 peptide was not conferred (data not shown).

The non-clonal T cell hybridoma 3H6 is derived from another Q9/VP2.139-responsive T cell clone, C3-8 [13], but this hybridoma actually includes at least two populations, denoted as 3H6(-) and 3H6(+), sorted by the expression of TCR Vα2 (data not shown). The 3H6(-) cells did not express Vβ4 nor respond to VP2.139 peptide stimulation (Figure 3B, left panel, and Figure 3C). When 3H6(-) cells were retrovirally transduced with the same TCRα10-T2A-β4-MigR1 construct, as shown in Figure 3B, the cloned TCR could be expressed on the cell surface, as stained by anti-TCR Vβ4 mAb. Upon stimulation by VP2.139 peptides, the transduced 3H6(-) cells specifically produced IL-2 in a dose dependent manner. No IL-2 was produced when stimulated with either L19 or OVA-I (OT-I TCR specific) control peptides (Figures 3C and 3D). We also separately expressed the cloned TCRα10 or β4 single chain in 3H6(-) cells. As

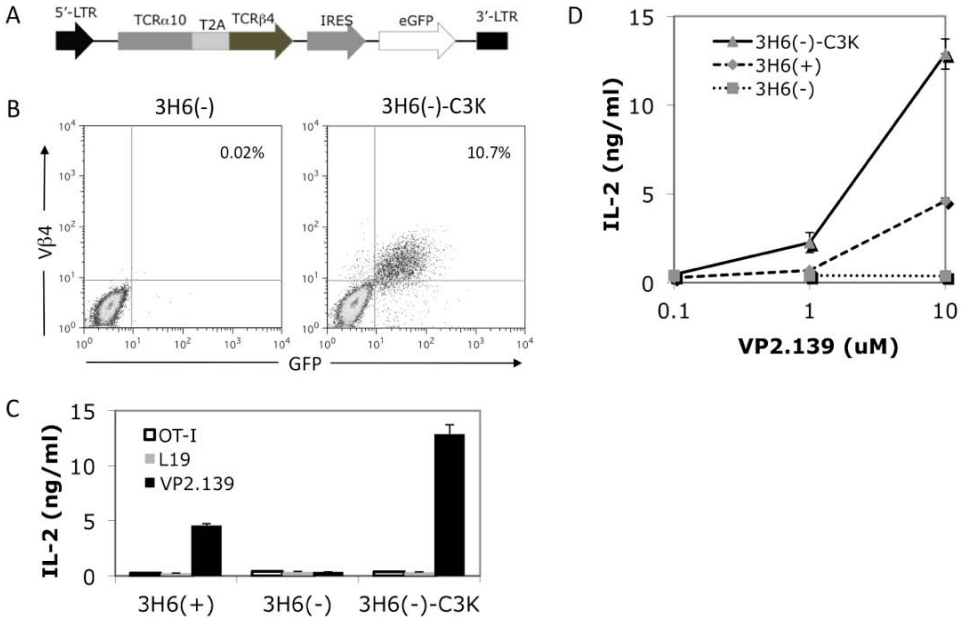


Figure 3: Expression and function of the cloned TCR in a T cell hybridoma line. (A) The diagram shows the construct used in retroviral transfection of 3H6(-) to express TCRα10 and TCRβ4 fused by T2A sequence with eGFP as a reporter driven by IRES. (B) Cell surface staining of TCR Vβ4 expression on transduced 3H6(-) cells. 3H6(-)-C3K represent the 3H6(-) cells transfected with TCRα10-β4 genes cloned from C3K T cell hybridoma. (C) IL-2 production of untransfected or transfected 3H6(-) and 3H6(+) cells stimulated with different peptides at 10 uM, and (D) dose responses of IL-2 production of untransfected or transfected 3H6(-) and 3H6(+) cells upon stimulation with 0 uM, 1 uM or 10 uM. Both (C) and (D) use $K^{b-/-}D^{b-/-}CIITA^{-/-}$ splenocytes as APCs. The recipient cell line, 3H6(-), was isolated from another non-clonal VP2.139 peptide responsible T cell hybridoma 3H6, which has different TCR usage (data not shown) from the C3K T cell hybridoma. The population of Vα2 positive, 3H6(+), respond to VP2.139 stimulation, but not the population of Vα2 negative, 3H6(-).

expected, none of the cells transfected with single chain of α10 or β4 can be stimulated with VP2.139 peptides, while the cells sequentially transfected with both TCRα10 and β4 (in separate constructs) can be stimulated (data not shown). These results indicated that the TCRα10 and TCRβ4 genes cloned from C3K T cell hybridoma were functional, conferring specificity for the VP2.139 peptide. However, the possibility remained that an unknown factor expressed in 3H6(-) cells was required for the ability to respond to Q9/VP2.139.

Functional CD8+ T cell development mediated by the cloned TCRα10 and TCRβ4 *in vivo*

To further examine the function of the cloned C3K TCRα10 and β4 genes, we generated retrogenic mice expressing the TCRα10-T2A-β4-MigR1 construct to test whether this TCR can specifically support the thymic selection and development of CD8+ T cell, and mediate survival and function of T cells in the periphery. To generate C3K retrogenic mice, semi-lethally irradiated $Rag1^{-/-}$ mice were reconstituted with retroviral transfected $Rag1^{-/-}$ bone marrow expressing the C3K TCR or the OT-I TCR (as a control). The C3K TCR specifically mediate the development of CD8+ T cells in recipient $Rag1^{-/-}$ mice and all of the CD8+ T cells from C3K retroviral mice were TCR Vβ4 and GFP double-positive, as shown by staining of peripheral blood from C3K retrogenic mice at 5 weeks post bone marrow reconstitution (Figure 4A), representing a population of CD8+ T cells that developed from the bone marrow T cell progenitors expressing the transfected C3K TCR. Further comparison of thymocytes and splenocytes in retrogenic mice showed that both the C3K TCR, as well as the control OT-1 TCR, rescued CD8+ T cell development in $Rag1^{-/-}$ mice (Figure 4B). The total thymocyte and splenocyte numbers were comparable between C3K and OT-I retrogenic mice, even though the numbers were lower when compared with wild type B6 or OT-I TCR transgenic mice (Figure 4C). Most importantly, the splenocytes from the C3K retrogenic mice responded specifically to stimulation of VP2.139 peptide to produce IL-2, but not to stimulation by control L19 or OT-I peptides. By contrast, the OT-I retrogenic splenocytes were stimulated by the OT-I peptide, not the L19 or VP2.139 (Figure 4D). These results clearly demonstrated that the cloned C3K TCR genes were able to support selection of CD8+ T cells *in vivo*, and that our cloned TCR genes confer specificity to the VP2.139 peptide.

Discussion

In this study, we molecularly identified a Q9/VP2.139-specific TCR and confirmed its function to respond to mouse PyV derived peptide stimulation. We also showed evidences that this TCR can be expressed *in vivo* and mediate specific CD8 T cell development. Thus we not only generated a useful tool in study of class Ib-restricted CD8 T cells in antiviral immunity, but also provide a unique system (MHC class Ib/peptide/TCR) to be used in the future study, such as the generation of an MHC class Ib restricted TCR transgenic mouse, only a few of which have been generated so far, and co-crystaliztion of MHC class Ib MHC/peptide/TCR for structural analysis.

Intriguingly, we notice a shorter half-life and relative instability of the Q9/VP2.139 complex, compared with a class Ia complex (Figure 1B) and Q9 bound to the L19 peptide, a high affinity endogenous self-peptide [44,50]. This observation provides a new piece of evidence consistent with the previous hypothesis that the dependence of CD28 signaling and continuous CD4 T cell help to expand Q9-restricted CD8 T cells is due to the weak interaction among MHC, peptide and TCR [46]. However, further evidence is required from a direct

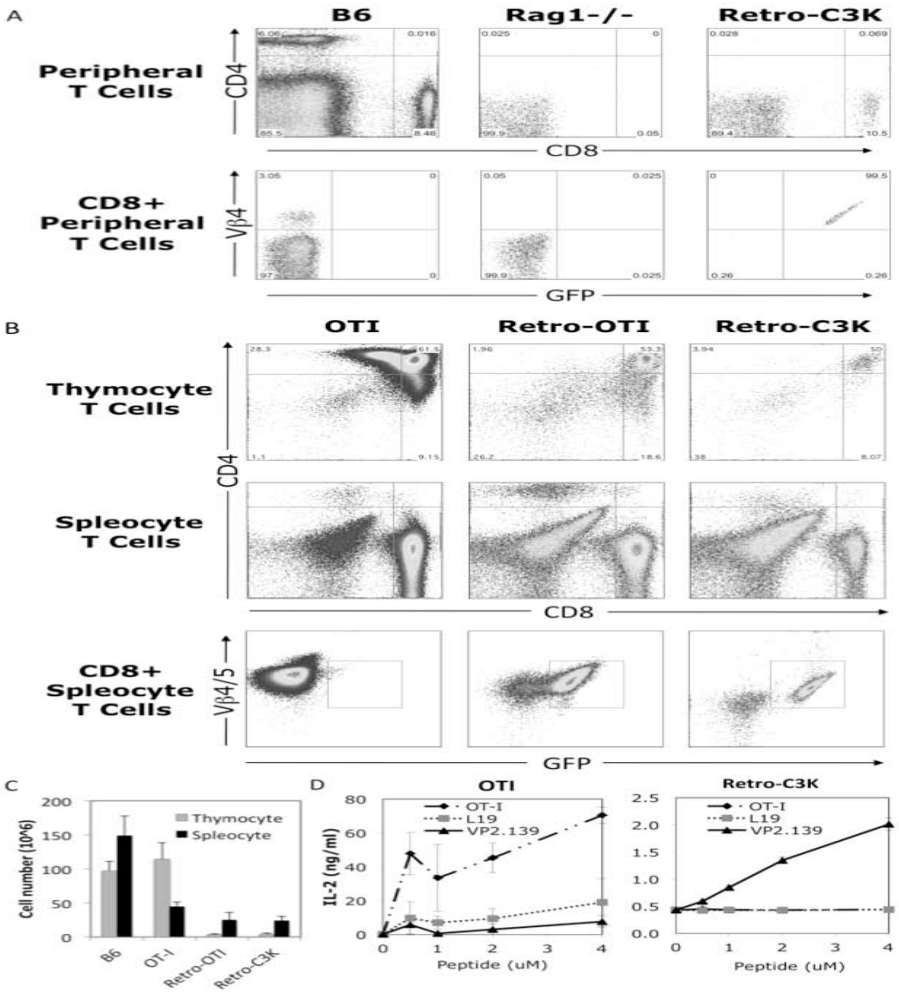


Figure 4: Generation of retrogenic mice expressing the cloned C3K TCR genes. (A) Surface staining of Vβ4 positive CD8+ T cells in peripheral blood of retrogenic mice in 5 weeks post bone marrow reconstitution. (B) Surface staining of Vβ4 positive CD8+ T cells in thymus and spleens of C3K TCR retrogenic, OT-I TCR retrogenic and OT-I transgenic mice (as controls). (C) Total cell number of thymocyte and splenocyte in age-matched wild type B6, OT-I transgenic mice, OT-I and C3K TCR retrogenic mice. The data represents at least 3 mice in each group. (D) IL-2 production from the splenocytes of OT-I transgenic or C3K TCR retrogenic mice *in vitro* stimulated with 0, 0.5, 2 or 4 uM of OT-I, L19 or VP2.139 peptides.

measurement of interaction among Q9, VP2.139 peptide and the cloned C3K TCR. It is plausible that the relative instability of Q9 and VP2.139 peptide might also weaken the interaction between pMHC ligand and TCR, such that it cannot fully stimulate the Q9/VP2.139-specific CD8 T cells. Consistent with this, these CD8 T cells express low level of inhibitory PD-1 but comparable level of activating CD28 [46,51].

It was reported that upon VP2.139 peptide stimulation, the Q9/VP2.139-specific CD8 T cells exhibited functional impairment in IFNγ production [13], in contrast to robust IFNγ production often observed with class Ia-restricted CD8 T cells in antiviral immunity [52,53]. We found that C3K T cells produce significant amount of IL-2 when stimulated with VP2.139 peptides (Figure 1D and Figure 4C). IL-2 is necessary for the growth, differentiation and survival of CD4 and CD8 T cells, especially memory T cell development [54-56]. The observation of impaired production of IFNγ but maintaining IL-2 production has several implications for the potential role of class Ia-restricted CD8 T cells: (1) the intrinsic potency to develop into memory type of CD8 T cell; (2) help in promoting survival and memory development in other CD4 and CD8 T cells; (3) stimulation to prolong the life of effector CD4 helper T cells, and especially promotion of effector MHC class Ia-restricted CD8 T cells with a capacity to control the persistent viral infection. Consistent with these proposed implications, it has been reported [57,13] that Q9/VP2.139-specific CD8 T cells are long lived, and express CD122, a component in CD122-dependent signaling critically involved in CD8 T cell immunity and memory [56,58]. However, further work is required to test these possibilities. Generation of transgenic mice expressing the C3K TCR should provide an opportunity to characterize the developmental pathway of Q9-restricted T cells, the requirements for positive and negative thymic selection, the requirements for expression of Q9 in T cell survival and homing, and the potential for Q9-restricted T cells to mediate viral clearance and establish immunological memory. Transgenic C3K T cells have the potential to provide a valuable tool to explore the potential of MHC class Ib-restricted T cells to participate in the anti-viral immune response.

Disclosures

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