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Quantification of a selective expansion of T cell receptor Vβ by superantigen using real time PCR

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Summary

Selective expansion of T cells bearing specific T cell receptor Vβ segments is a hallmark of superantigens. Analyzing Vβ specificity of superantigens is important for characterizing newly discovered superantigens and understanding differential T cell responses to each toxin. Here, we described a real-time PCR method using SYBR green I and primers specific to Cβ and Vβ genes for an absolute quantification. The established method was applied to quantify a selective expansion of T cell receptor Vβ expansion by superantigens and generated accurate, reproducible, and comparable results.

Keywords

superantigen; T cell receptor Vβ; quantitative real time PCR

1. Introduction

The T cell receptor (TCR) is a α and β chain heterodimer that recognizes antigen-derived peptides presented by the major histocompatibility complex II (MHC II) molecules on antigen presenting cells, thereby triggering a clonal expansion of T cells [1]. The TCR consists of a variable and constant region, where the variable region interacts with peptide and MHC II, thus determining the antigen specificity of T cells [2,3]. A high diversity of the variable region of TCR is generated by a somatic recombination of variable (V), diversity (D), and joining (J) genes with a constant (C) gene and imprecise joining of VDJ segments during thymic development [4]. Cloning and sequencing human Vβ genes have identified 49 functional Vβ subgroup genes. Based on the sequence similarity, these genes were grouped in 24 different Vβ groups. Due to the sequence variations, some Vβ groups contains multiple subgroups [2,5,6].

Staphylococcal enterotoxins (SEs), SE-like toxin (SEl), and toxic shock syndrome toxin-1 (TSST-1) are prototypical microbial superantigens (SAg). Thus far, 23 SEs and SEls including SEA through X, excluding F, and TSST-1 have been characterized in *Staphylococcus aureus* [7]. Unlike conventional antigens, most SAgs directly bind to the specific variable regions of TCR β chain (Vβ) and outside of the peptide binding groove of

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MHC II. This binding triggers a clonal expansion of T cells bearing specific TCR Vβ segments, leading to a massive production of proinflammatory cytokines and chemokines [8]. Thus, SAgs induce antigen-independent, Vβ-dependent T cell proliferation. The shared biological properties of SAg such as Vβ specificity and cytokine responses were closely correlated with the amino acid sequence and 3 dimensional structure of SAgs. Therefore, the analysis of Vβ specificity of SAg is important for characterizing newly discovered SAg and understanding T cell responses to the SAg.

Several approaches are used to analyze the Vβ specificity of SAg including northern blotting, semi-quantitative PCR [9], and flow cytometry using monoclonal antibodies specific to Vβ segments [10,11]. Shortcomings such as a lack of available reagents limit the practical application of these approaches. We developed a quantitative real time PCR (qRT-PCR) method using SYBR green I and primers specific to 22 Vβ groups and constant β chain (Cβ) genes [12]. The specificity of established method was verified by sequencing PCR amplification products showing that 36 out of 49 functional Vβ subgroup genes were successfully amplified. Standard curves for primers to Cβ and Vβ genes were generated to allow an absolute quantification. The established method was applied to assess the Vβ specificity of SAgs and showed reproducible and comparable results from previous approaches.

2 Materials

2.1 Preparation and stimulation of enriched human lymphocytes

1. BD Vacutainer Saftey-Lok Blood collection set

2. Blood collection tube containing Heparin

2. Phosphate buffered saline (PBS)

3. Ficoll-Hypaque plus solution (density 1.077g/liter)

4. Hanks balanced salt solution (HBSS)

5. Complete RPMI1640 medium: RPMI medium supplemented with 2 % FBS, 100 U penicillin G, and 100 μg/ml streptomycin.

7. Beckman GPR centrifuge with GH-3.7 horizontal rotor (or equivalent temperature-controlled centrifuge)

8. Endotoxin free SAg

9. Murine monoclonal antibody (mAb) specific to human CD3 (Sigma-Aldrich)

2.2 RNA extraction

1. RNeasy Mini Kit (Qiagen)

2. RNase free TURBO DNase I (Ambion)

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3. 70% ethanol

2.3 cDNA synthesis

1. DNA, RNA-free 0.2 ml PCR tube

2. Thermocycler

3. dNTP mix (10 mM each, Life technologies)

4. oligo$(dT)_{20}$ (0.5μg/μl, Life technologies)

5. Superscriptase III first strand synthesis kit (Life technologies)

2.4 Quantitative real time PCR

1. Applied Biosystems 7500 real time PCR system or equivalent real time PCR thermal cycler

2. MicroAmp Optical 96-well reaction plate (Life Technologies)

3. MicroAmp Optical plate seals (Life Technologies)

4. Power SYBR Green PCR Master mix (Life Technologies)

5. Primers specific to the Cβ and Vβ gene

3. Methods

3.1 Preparation and stimulation of enriched human lymphocytes.

1. Collect whole blood (10 –20 ml) from the healthy donor by venipuncture using BD Vacutainer Saftey-Lok Blood collection set (21G needle) and Blood collection tube containing Heparin (14U/ml blood).

2. Transfer whole blood into the 50 ml conical polypropylene tube and add an equal volume of PBS. Mix well.

3. Slowly overlay the diluted blood onto the Ficoll-Hypaque plus solution. Use 1 ml Ficoll-Hypaque per 3 ml blood diluted with PBS.

4. Centrifuge at 2000 rpm (900×g), 18 – 20°C for 30 min without brake.

5. Using sterile pipet, carefully remove the upper layer containing the plasma and platelets and discard. Using another pipet, carefully transfer buffy coat that contains the mononuclear cells to the 50 ml conical polypropylene tube.

6. Wash mononuclear cells three times by adding 45 ml of HBSS and centrifuging at 1300 rpm (400×g), 18 –20°C for 10 min (see Note 1).

[1]. If the cell pellet is contaminated with red blood cells, resuspend the cell pellet in 1×ACK lysis buffer (Life technologies, add 1 ml 1 × ACK lysis buffer per 2 ml of original blood volume), incubate at room temperature for 5 min, and add equal volume of PBS.

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7. Resuspend the cells in complete RPMI medium and culture in cell culture Petri dishes (Costar) overnight at 37°C and in 5 % CO_2.

8. The following day, non-adherent, lymphocyte-enriched cells are collected, washed, and resuspended in complete RPMI medium at a final concentration of 2.5×10^6 cells/ml.

9. Each SAg (0.5 μg/ml) is added to lymphocyte-enriched cells. Cells are cultured for 4 days (37°C, 5 % CO_2) and harvested by centrifuging at 1300 rpm (400×g), 4°C for 10 min.

3.2 Total RNA extraction

1. Approximately 5×10^6 cells are resuspended in 350 μl of buffer RLT and homogenized by passing the lysate 10 times through a 20 gauge needle (see Note 2).

2. Centrifuge the lysate at 12000×g for 3 min and carefully transfer the supernatant to a new microcentrifuge tube.

3. Add an equal volume of 70% ethanol and mix by pipetting.

4. Transfer the mixture to the RNeasy spin column and centrifuge at 8000×g for 15 sec and discard the flow through.

5. Add 700 μl Buffer RW1 to the RNeasy spin column and centrifuge at 8000×g for 15 sec and discard the flow through.

6. Add 500 μl Buffer RPE to the RNeasy spin column and centrifuge at 8000×g for 15 sec and discard the flow through.

7. Add 500 μl Buffer RPE to the RNeasy spin column and centrifuge at 8000×g for 2 min and discard the flow through.

8. Place the RNeasy spin column in a new microcentrifuge tube and add 50 μl RNase-free water directly to the spin column membrane. Centrifuge at 8000×g for 1 min.

9. Add RNase-free water to make the volume up to 89 μl.

10. Add 10 μl 10× TURBO DNase Buffer and 1 μl TURBO DNase (2U/μl)

11. Incubate at 37 °C for 30 min.

12. Add 350 μl Buffer RLT and mix well by pipetting.

13. Add 250 μl 100% ethanol and mix well by pipetting.

14. Transfer the mixture to a new RNeasy spin column and centrifuge at 8000×g for 15 sec and discard the flow through.

[2]. The cell pellet or lysate can be stored in – 80°C for a month. Frozen samples should be completely thawed and continue with step 3.

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15. Add 500 μl Buffer RPE to the RNeasy spin column and centrifuge at 8000×g for 15 sec and discard the flow through.

16. Add 500 μl Buffer RPE to the RNeasy spin column and centrifuge at 8000×g for 2 min and discard the flow through.

17. Place the RNeasy spin column in a new microcentrifuge tube and add 50 μl RNase-free water directly to the spin column membrane. Centrifuge at 8000×g for 1 min.

18. Determine the quantity and quality of RNA using Nanodrop and adjust the quantity to 1 μg/5 μl (see Note 3).

3.3 cDNA synthesis

1. Add the following in a 0.2 ml PCR tube:
   - 5 μl total RNA (1 μg)
   - 1 μl dNTP mix (10 mM each dATP, dGTP, dCTP, and dTTP)
   - 1 μl oligo$(dT)_{20}$ (500 ng)
   - 7 μl RNase-free water

2. Incubate for 5 min at 65°C, then place on ice for 2 min

3. Add the following cDNA Synthesis Mix to the tube.
   - 4 μl 5 × Reverse Transcriptase Buffer
   - 1 μl 0.M DTT
   - 1 μl Superscriptase III RT (200 units/μl)

4. Incubate for 50 min at 50°C

5. Incubate for 5 min at 85°C to terminate the reactions, then place on ice

6. Dilute cDNA with 980 μl DNase-free water and store at – 80°C until used.

3.4 Primer design

Primers specific to the Cβ and 23 different Vβ groups were designed using Primer Express version 2.0 (Applied Biosystems). Some Vβ groups have multiple Vβ subgroups genes showing high sequence similarity. Therefore, some Vβ primers are expected to amplify multiple Vβ subgroup genes within the corresponding Vβ group. Sequencing analysis of PCR products generated using these primers showed that 36 out of 49 functional Vβ genes were amplified. The primer sequences and amplified Vβ subgroup genes are summarized in Table 1.

[3]. Normally, we do not experience DNA contamination in the RNA preparation after the DNase I treatment. A contamination of DNA in RNA preparation could be verified by PCR reactions with primers specific to GAPDH (forward: 5’-GCAAATTCCATGGCACCGT-3’; reverse: 5’-TCGCCCCACTTGATTTTGG-3’).

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3.5 Quantitative real time PCR

3.5.1 Standard curve—For absolute quantification of the Cβ and Vβ genes, the standard curves for primers to Cβ and Vβ genes were generated.

1. The Cβ and Vβ genes were amplified by PCR using primers listed in Table 1. Each PCR product was purified using a PCR purification kit (Qiagen) and then cloned into pCR2.1 plasmid vector (Life Technologies). Cloned pCR2.1 plasmid vectors were purified using a plasmid MiniPrep kit (Qiagen) and the concentration of plasmid was determined by measuring the absorbance at 260 nm using a Nanodrop (Thermo Scientific). The plasmid copy number was determined by a following formula [13,14]:

$$Number\ of\ plasmid\ copies\ (molecules) = \frac{\mathrm{X\ ng} \times 6.0221 \times 10^{23}\ \mathrm{molecules/mole}}{(\mathrm{N} \times 660\ \mathrm{g/mole}) \times 1 \times 10^{9}\mathrm{ng/g}}$$

Where:

X = the amount of plasmid (ng)

N = the size (base pair) of plasmid

2. Set up triplicate qRT-PCR reactions (25 μl each) consisting of:

- 12.5 μl Power SYBR Green PCR Master mix
- 5 μl (2 pmoles) forward and reverse primers (2.5 μl each)
- 5 μl 10-fold serially diluted plasmid template (2.5 – 2.5 × 10^5 copies)
- 2.5 μl DNase-free water

3. Thermocycler conditions consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles of a denaturation at 95°C for 15s and an extension at 60°C for 1 min. Fluorescent data is measured during an extension period. After 40 cycles, a melting curve analysis is performed. The base line and threshold cycle (C_T) is determined using the Sequence Detector Systems version 1.2.2 (Applied Biosystems).

4. The standard curve is generated by plotting the C_T vs. the $\log_{10}$ copies of serially diluted plasmid harboring Cβ and Vβ genes (2.5 – 2.5 × 10^5 copies). The slope, intercept, and correlation coefficiency (R^2) are determined using Microcal OriginPro Vesion 7.5 (OriginLab) (see Note 5).

Representative standard curve for the Cβ gene is shown in Figure 1. Standard curves for the Cβ and Vβ genes are summarized in Table 2.

3.5.2. Quantification of Vβ gene—1. Set up triplicate qRT-PCR reactions (25 μl) consisted of:

[5]. Standard curves generated for Cβ and Vβ genes using other real time PCR equipment such as iCycler (Bio-rad) was not significantly different from those generated by ABI 7500 real-time PCR systems.

- 12.5 µl Power SYBR Green PCR Master mix
- 5 µl 2 pmoles forward and reverse primers (2.5 µl each) specific to the Cβ or Vβ gene
- 5 µl cDNA prepared in 3.3
- 2.5 µl DNase-free water

2. Perform and analyze the qRT-PCR as described in 3.5.1 (see Note 4).

3. For absolute quantification, the absolute copy numbers of the Cβ or Vβ gene is calculated by extrapolating the C_T to the standard curve listed in Table 2 (see Note 6).

4. The percentage of each Vβ (%Vβ) is calculated by the following equation:

$$\%V\beta_n = \left(\frac{V\beta_n}{C\beta}\right) \times 100$$

Where:

%Vβn= the percentage of indicated (n) Vβ group

Vβn= the copy number of indicated (n) Vβ group

Cβ= the copy number of Cβ gene

5. Selective expansion of Vβ by SAg is determined when the %Vβn from the culture stimulated with SAg is significantly higher than the corresponding %Vβn from the unstimulated culture (without stimuli) by paired *t*-test ($p<0.01$).

The established method was applied to determine the Vβ specificity of SEA, SEB, SEC1, SED, SEE, SEG, SEI, SElM, SElN, SElO, and TSST-1. Results are summarized in Figure 2 and Table 3 and showed accurate, reproducible, and comparable results observed in previous studies [15–17]

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[6] To synchronize real-time PCR data analysis, the base line signal and threshold was set to be determined automatically by the Sequence Detector System version 1.2.2.

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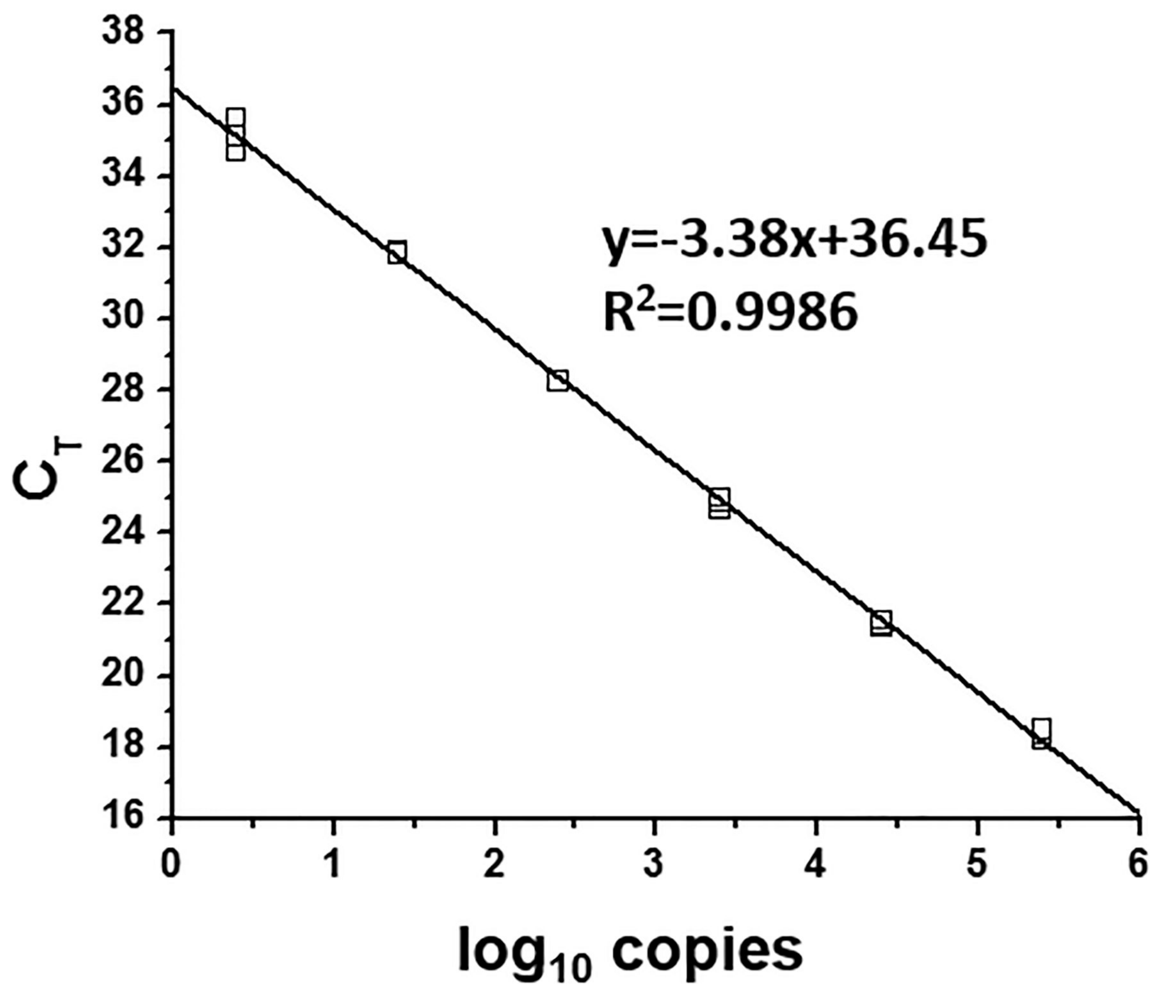

Figure 1.

A representative standard curve generated for the Cβ gene, reproduced from [12] with permission from JTM. Quantitative real-time PCR was performed using 10-fold serially diluted templates ($2.5 - 2.5 \times 10^5$ copies). The C_T was determined and plotted over the $\log_{10}$ copies to calculate the slope, Y axis intercept, and correlation coefficiency (R^2).

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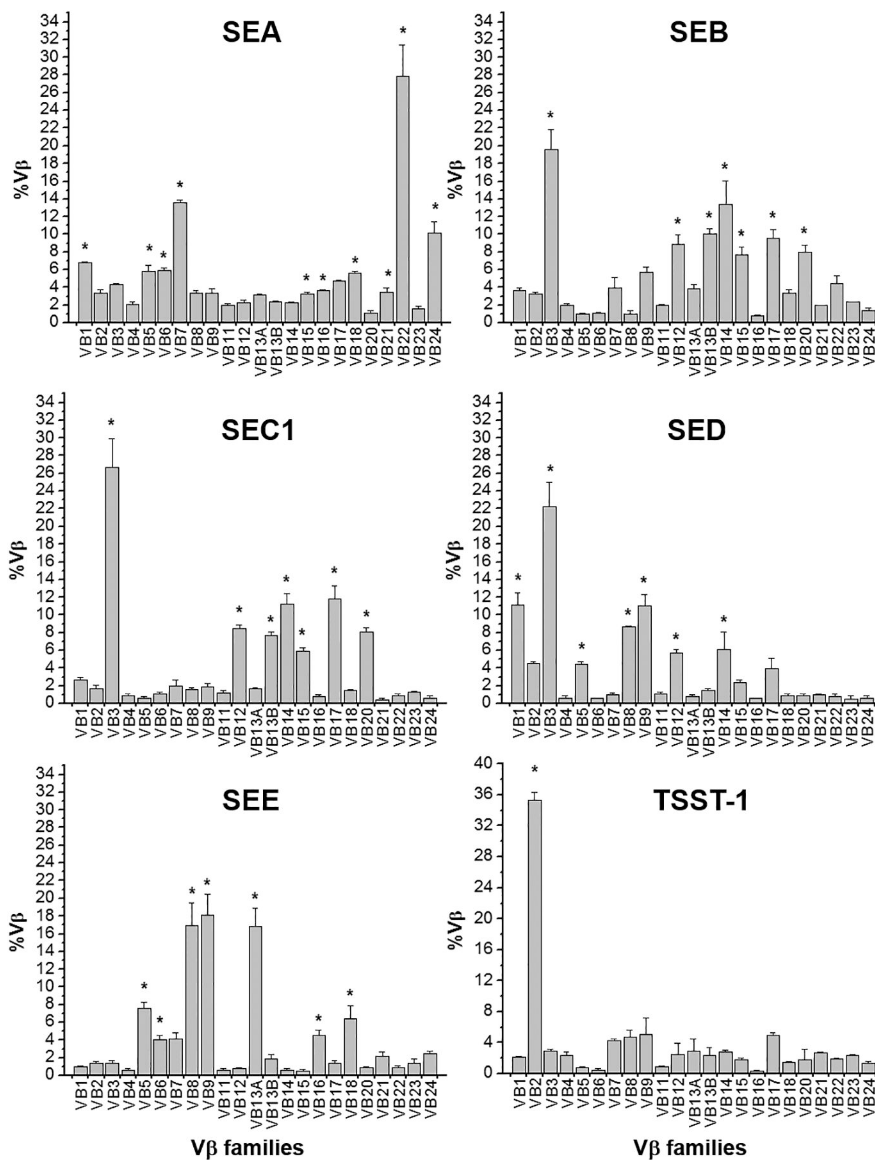

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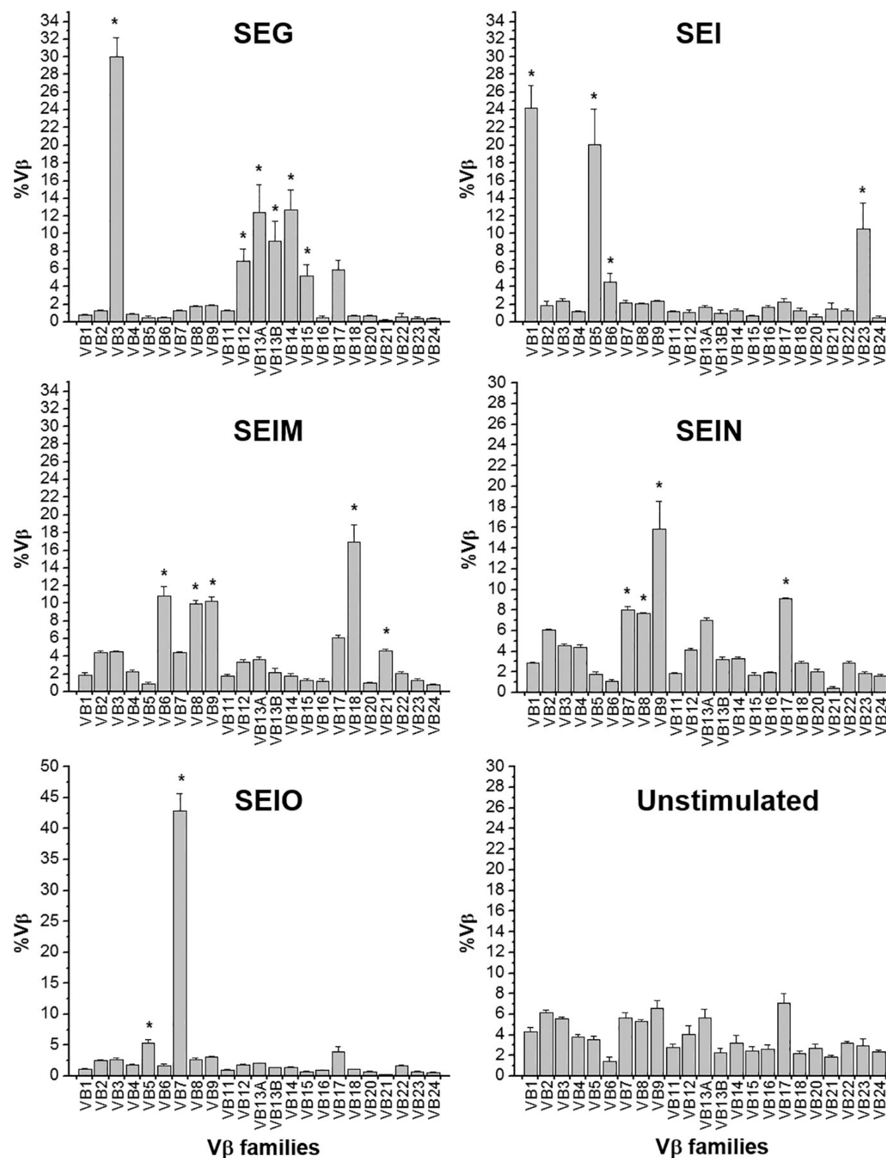

Figure 2.

Selective expansion of TCR Vβ by SAgs, reproduced from [12] with permission from JTM. Enriched human lymphocytes were stimulated with an indicated SAg (0.5 μg/ml) for 4 days. Quantitative real time PCR was performed and the %Vβ was calculated. Selective expansion of Vβ by SAg is determined when the %Vβn from the culture stimulated with SAg is significantly higher than the corresponding %Vβn from the unstimulated culture (without stimuli) by paired t-test. The asterisk indicates a statistically significance ($p<0.01$).

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Table 1.

List of primers[a] and amplified Vβ gene(s), reproduced from [12] with permission from JTM.

Primer name	GenBank access number	Forward primer ('5 to 3')	Reverse primer ('5 to 3')	Amplified Vβ gene(s)[b]
Cβ	L36092	tccagttctacgggctctcg	gacgatctgggtgacgggt	
VB1	L36092	ggagcaggcccagtggat	cgctgtccagttgctggtat	TCRVB1s1
VB2	M11955	gagtctcatgctgatggcaact	tctcgacgccttgctcgtat	TCRVB2s1
VB3	U08314	tcctctgtcgtgtggccttt	tctcgagctctgggttactttca	TCRVB3s1
VB4	L36092	ggctctgaggccacatatgag	ttaggtttgggcggctgat	TCRVB4s1
VB5	L36092	gctccaggctgctctgttg	tttgagtgactccagcctttactg	TCRVB5s1, 5s3
VB6	X61440	ggcagggcccagagtttc	gggcagccctgagtcatct	TCRVB6s1, 6s2, 6s3, 6s4, 6s5, 6s6
VB7	U07977	aagtgtgccaagtcgcttctc	tgcagggcgtgtaggtgaa	TCRVB7s1, 7s2, 7s3
VB8	X07192	tgcccgaggatcgattctc	tctgagggctggatcttcaga	TCRVB8s1, 8s2, 8s3
VB9	U07977	tgcccgaggatcgattctc	tctgagggctggatcttcaga	TCRVB9s1
VB11	L36092	catctaccagaccccaagatacct	atggcccatggtttgagaac	TCRVB11s1
VB12	U03115	gttcttctatgtggccctttgtct	tcttgggctctgggtgattc	TCRVB12s1, 12s3
VB13[c]	L36092	tggtgctggtatcactgaccaa	ggaaatcctctgtggttgatctg	TCRVB13s1, 13s6
VB13[d]	X61445	tgtgggcaggtccagtga	tgtcttcaggacccggaatt	TCRVB13s2, 13s9
VB14	L36092	gctccttggctatgtggtcc	ttgggttctgggtcacttgg	TCRVB14s1
VB15	M11951	tgttacccagaccccaagga	tgacccttagtctgagaacattcca	TCRVB15s1
VB16	X06154	cggtatgcccaacaatcgat	caggctgcaccttcagagtaga	TCRVB16s1
VB17	U48260	caaccaggtgctctgctgtgt	gactgagtgattccaccatcca	TCRVB17s1
VB18	L36092	ggaatgccaaaggaacgattt	tgctggatcctcaggatgct	TCRVB18s1
VB20	L36092	aggtgccccagaatctctca	ggagcttcttagaactcaggatgaa	TCRVB20s1
VB21	M33233	gctgtggctttttggtgtga	caggatctgccggtaccagta	TCRVB21s1
VB22	L36092	tgaaagcaggactcacagaacct	tcacttcctgtcccatctgtgt	TCRVB22s1
VB23	U03115	ttcagtggctgctggagtca	cagagtggctgtttccctcttt	TCRVB23s1
VB24	U03115	acccctgataacttccaatcca	cctggtgagcggatgtcaa	TCRVB24s1

[a]The pseudogenes (Vβ10 and Vβ19) were not included.

[b]Vβ subgroup nomenclature followed the classification of Arden et al.

[c]VB13A corresponds to Vβ13.1 in previous studies.

[d]VB13B corresponds to Vβ13.2 in previous studies.

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Table 2.

Standard curve slopes, Y axis intercepts and correlation coefficients (R^2), reproduced from [12] with permission from JTM.

Primers	Slope	Y axis intercept	Correlation coefficient (R^2)
Cβ	–3.38	36.45	0.9986
VB1	–3.39	36.54	0.9977
VB2	–3.36	36.38	0.9982
VB3	–3.41	36.57	0.9987
VB4	–3.37	36.62	0.9984
VB5	–3.35	36.33	0.9976
VB6	–3.40	36.53	0.9978
VB7	–3.36	36.43	0.9983
VB8	–3.37	36.40	0.9986
VB9	–3.38	36.49	0.9985
VB11	–3.41	36.52	0.9986
VB12	–3.42	36.53	0.9972
VB13A	–3.34	36.34	0.9978
VB13B	–3.41	36.54	0.9974
VB14	–3.36	36.33	0.9981
VB15	–3.35	36.44	0.9976
VB16	–3.37	36.44	0.9984
VB17	–3.39	36.53	0.9982
VB18	–3.35	36.44	0.9986
VB20	–3.33	36.39	0.9973
VB21	–3.36	36.38	0.9986
VB22	–3.39	36.47	0.9981
VB23	–3.37	36.43	0.9980
VB24	–3.41	36.53	0.9984

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Table 3.

Summary of Vβ specificity observed with this methods and comparison with those in selected previous studies, reproduced from [12] with permission from JTM.

SAgs	Vβ specificity observed in this study	Vβ specificity observed in previous studies[a]	References
SEA	Vβ1, 5, 6, 7, 15, 16, 18, 21, 22, 24	Vβ1, 5, 6, 7, 9, 16, 18, 21	[15]
SEB	Vβ3, 12, 13B[b], 14, 15, 17, 20	Vβ1, 3, 6, 12, 13.2, 15, 17, 20	[16]
SEC1	Vβ3, 12, 13B, 14, 15, 17, 20	Vβ3, 12, 13.2, 14, 15, 17, 20	[9]
SED	Vβ1, 3, 5, 8, 9, 12, 14	Vβ1, 5, 6, 7, 8, 12	[18,7]
SEE	Vβ5, 6, 8, 9, 13A[c], 16, 18	Vβ5, 6, 8, 13.1, 18, 21	[15,16]
SEG	Vβ3, 12, 13A, 13B, 14, 15	Vβ3, 12, 13, 14	[17]
SEI	Vβ1, 5, 6, 23	Vβ1, 5, 6, 23	[17]
SElM	Vβ6, 8, 9, 18, 21	Vβ6, 8, 9, 18, 21	[17]
SElN	Vβ7, 8, 9, 17	Vβ9	[17]
SElO	Vβ5, 7	Vβ5, 7, 22	[17]
TSST-1	Vβ2	Vβ2	[16]

[a]Vβ specificities were results from previous studies using semi-quantitative PCR or FACS methods.

[b]Vβ13B corresponds to Vβ13.2 in previous studies.

[c]Vβ13A corresponds to Vβ13.1 in previous studies.