The Effects of Mitogens, IL-2 and Anti-CD3 Antibody on the T-Cell Receptor Vβ Repertoire

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Phytohaemagglutinin (PHA), Concanavalin A (Con A), interleukin-2 (IL-2), and monoclonal antibodies to CD3 (CD3MoAbs) are used for the assessment of the T-cell receptor (TCR) BV gene family expression in autoimmune disorders and multiple sclerosis, and to produce clones for assessment of cytokine profiles in progressive human immunodeficiency virus infection. The authors examined the effects of these stimulants on the TCR Vβ repertoire of resting and blastic $CD4^+$ and $CD8^+$ normal human peripheral blood lymphocytes, using three-colour cytofluorometry and a panel of anti-TCR Vβ monoclonal antibodies. IL-2 was associated with an increased percentage of blastic $CD4^+$ cells expressing V_β5.1 (from median of 3.7% to 8.0%, $P = 0.0002$) and blastic $CD8^+$ cells expressing V_β5.3 (1.0 to 1.5%, $P = 0.0039$). CD3MoAb caused a slight increase in V_β6.7 + blastic $CD4^+$ cells (4.5 to 6.9%, $P = 0.0078$). PHA did not alter the Vβ repertoire of blastic cells. Con A caused skewing in $CD8^+$ blastic cells, toward expression of V_β5.2/5.3 (3.1 to 8.1%) and V_β5.3 (0.8 to 4.8%) ($P = 0.0020$). Thus, IL-2 stimulation causes slight alterations in the Vβ repertoire that should be taken into account in certain research settings. Con A produced skewing in $CD8^+$ blastic cells suggesting that, in the presence of CD8, either Con A binds selectively to certain Vβ or the three-dimensional complex created by Con A's binding to other T-cell surface molecules induces preferential V_β5 stimulation.

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INTRODUCTION

Numerous agents have been found to stimulate lymphocyte proliferation; many of these agents interact with the T-cell antigen receptor (TCR) [1]. Some appear to bind one or another section of the antigen-specific portion of the TCR, termed $T_{idiotypic}$ (Ti), composed of disulfide-linked α and β chains or γ and δ chains. Ti-binding substances include certain lectins, i.e. carbohydrate binding proteins that may also agglutinate erythrocytes, and superantigens (SAGs), substances binding the variable region of the Ti β chain. Other substances bind to the non-antigen-related portion of the TCR, a complex of five invariant polypeptide chains termed CD3. The most commonly used stimulatory CD3-binding agents are monoclonal antibodies to CD3 (CD3MoAb).

The best-studied SAGs include those expressed endogenously in inbred mice, genetically encoded by murine retroviruses and exogenous bacterial SAGs, including the enterotoxins of staphylococci and streptococci [2, 3]. These enterotoxins bind to the variable portion of the β chain of the TCR (Vβ), outside the antigen-binding cleft. Vβ-specificity varies among SAGs, but any given SAG stimulates a consistent pattern of Vβ families. Lectins have been isolated from viruses, fungi, bacteria, plants and animals. Two widely studied lectins, mitogenic for T lymphocytes, are phytohaemagglutinin (PHA), derived from *Phaseolus vulgarus*, and Concanavalin A (Con A), derived from *Canavalia ensiformis*. These substances cause massive T lymphocyte division and cytokine production. Both appear to interact with Ti [4–8]. Con A also binds to CD3, albeit less strongly than to Ti [4, 6, 8], but there is some debate as to whether PHA also binds to CD3 [4–7]. Con A precipitates more cell surface glycopeptides than does PHA [6], but both Con A and PHA bind CD2, another T lymphocyte surface glycoprotein molecule. Con A does so more strongly than PHA [7, 8].

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The mitogenic action of some lectins and CD3MoAbs has been extensively employed for T lymphocyte stimulation or propagation in short- and long-term culture, cell lines, or cloning. Interleukin-2 (IL-2) is usually added to the culture media to maximize T lymphocyte stimulation. It has been tacitly or explicitly assumed that with this combination of stimulants all T lymphocytes proliferate comparably, rather than subgroups being preferentially induced to replicate, as is the case with SAGs [9–14]. Early cloning data support this assumption [9], whilst later studies either accept the assumption [10–14] or provide/reference minimal data to support it [15–18].

Recent data suggest that this assumption may not always be valid. Certain stimulants may favour $CD4^+$ T lymphocyte proliferation over $CD8^+$ T lymphocyte proliferation or vice versa [11, 13]. Since the distribution of Vβ families differs between $CD4^+$ and $CD8^+$ lymphocytes [19, 20] this could be associated with biased Vβ stimulation. Similarly, it has been suggested that γδ T lymphocytes respond equally well to PHA, but less to IL-2 than do αβ T cells [14], again suggesting that biased stimulation is possible. In human immunodeficiency virus (HIV)-infected people, certain subsets of $CD4^+$ T lymphocytes respond more poorly than others to CD3MoAb [21], again suggesting variable responsiveness to these stimuli. Most directly, one study using a semi-quantitative polymerase chain reaction (PCR) technique found that PHA-stimulated T lymphocytes from healthy donors appeared to have a significantly increased expression of the BV6, 7S2, and 10S1 families compared with unstimulated cells [22]. Another study found an oligoclonal expansion of V_β5.3+ cells in one of 15 cultures surviving in IL-2 for 4 weeks, as assessed with single-colour cytofluorometry and monoclonal antibodies to seven AV or BV gene products [23].

Vβ-related skewing of cell distributions may be of little concern in the production of antigen-specific T lymphocyte clones. However, altered levels of cells expressing specific BV or AV gene products have recently been cited as evidence for SAG-related activity in autoimmune disorders and multiple sclerosis. In many of these studies mitogens, CD3MoAb and IL-2 have been used to stimulate cell proliferation before measurements of the cellular distributions [10, 12, 14–16]. Similarly, in studies of changes in cytokine secretion patterns with progressive HIV infection, T lymphocyte clones have been produced using PHA supplemented with IL-2 [24, 25]. Subsequent stimulation included CD3MoAb [24]. Activity in these research areas is intensive, making it important to validate the assumption that mitogens, CD3MoAb and IL-2 stimulate the TCR repertoire in an unbiased fashion. We therefore assessed the effects of PHA, Con A, CD3MoAb and IL-2 on the TCR Vβ distributions of resting and blastic $CD4^+$ and $CD8^+$ human peripheral blood lymphocytes of volunteer donors.

MATERIALS AND METHODS

Cells. Peripheral mononuclear cells were obtained and isolated from the blood of 36 normal blood donors and analysed before and after peak stimulation with $\geqslant 1$ of the following*: PHA (Difco, Detroit, MI, USA) 0.005 μg/ml (stimulated 1–4 days, median 4 days), Con A (Pharmacia AB, Uppsala, Sweden) 4 μg/ml (stimulated 4–7 days, median 4 days), purified IL-2 (Advanced Biotechnologies, Columbia, MD, USA) 64 units/ml (stimulated 1–5 days, median 3 days), CD3MoAb clone SK7 (Beckton Dickinson Immunochemistry Systems [BD], San Jose, CA, USA) 0.050 μg/ml (stimulated 1–5 days, median 4 days), and staphylococcal enterotoxin D (SED) (Serva Biochemicals, Westbury, NY, USA) 0.001 μg/ml (stimulated 3–7 days, median 6 days) (Table 1). All cells were cultured in RPMI 1640 medium containing 10% fetal calf serum and 0.29 mg/ml L-glutamine at a density of 2×10^6 cells/mm^3 at 37°C in humidified 5% CO_2.

Reagents. Fluorescein isothiocyanate-conjugated (FITC) monoclonal antibodies against the human TCR were purchased from three sources, with reactivities specified to date as follows: Immunotech (Westbrook, ME; BV2S1, clone E22E7.2; BV3S1, clone LE.89; BV5S2, clone 36213; BV8S1/8S2, clone 56C5.2; BV13S6, clone JU74.3; BV17S1, clone E17.5F3.15.13; BV21S3, clone IG.125; BV22S1, clone IMMU546), T Cell Diagnostics (Woburn MA, USA; AV2, clone F1; BV3S1, clone 8F10 (referred to by the manufacturer as '3A'); BV5S1, clone LC4; BV5S2/5S3, clone 1C1; BV5S3, clone W112; BV6S7, clone OT145; BV8, clone 16G8 (referred to by the manufacturer as '8A'); BV8, clone MX6 (referred to by the manufacturer as '8B'); BV12S1, clone S511; BV13S1/13S3, clone BAM13), and BD (A,B TCR). Phycoerythrin-conjugated (PE) monoclonal anti-CD8 and PerCP-conjugated (PerCP) monoclonal anti-CD4 and anti-CD8 were obtained from BD.

Flow cytofluorometry. Three-colour cytofluorometry was done using a FACScan or FACSort (BD) and Lysis II software. Negative control aliquots were stained with FITC mouse IgG1, PE-mouse IgG2a, and PerCP-mouse IgG1. Generally, 30,000 ungated events were collected from each sample and an additional 3000 events were collected from each sample using the following gating: (i) forward and side scattering of $TCR_{\alpha,\beta}$+ cells indicative of large, less dense (blastic) cells, as opposed to small, dense (resting) lymphocytes (Fig. 1a); and (ii) positivity for CD4 or CD8, but not both (Fig. 1b). This permitted the separate evaluation of blastic T lymphocytes for each analysis. Analyses are reported for the following populations: resting $CD4^+$ cells; resting $CD8^+$ cells; blastic $CD4^+$ cells; and blastic $CD8^+$ cells.

Analytical and statistical techniques. The percentage of resting and blastic $CD4^+$ and $CD8^+$ lymphocytes expressing a given BV gene product at the end of culture was compared to the percentage of resting $CD4^+$ or $CD8^+$ lymphocytes expressing that BV product at the beginning of culture. The same reagent panel and reagent lots was used at the beginning and end of each culture, but the monoclonal antibody panel used was enlarged as additional reagents became available (Table 1). Readings at both time points were carried out on the same machine, with the same compensation, and by the same operator. All analyses were done by a single individual; gates were consistent between the two time points.

A Wilcoxon signed rank test was used to compare the percentage of cells expressing gene product V_β'X' at the end of culture to the percentage of cells expressing V_β'X' at the beginning of culture (day 0). This test provides a nonparametric matched (pre- to post-) analysis of all samples stimulated with a given reagent. It takes into consideration the direction of change (negative/positive). Data herein will be expressed as the median subtractive differences

* Use of any and all trade names and commercial sources is for identification only and does not imply endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

Table 1. Numbers of donors evaluated, by antigen, BV reagent and lymphocyte population being compared to day 0 resting lymphocytes

	$CD4^+$ lymphocytes									
	SED	IL-2	∝CD3	PHA	CON A	SED	IL-2	∝CD3	PHA	CON A
	Resting					Blastic				
BV										
(A2)	10	11	9	8	8	10	11	9	8	8
2S1	14	13	11	15	11	14	13	10	15	11
3S1	7	14	11	6	11	7	14	11	6	11
3[8F10]	11	12	11	14	11	11	11	11	14	11
5S1	10	13	11	10	11	10	13	11	10	11
5S2	1	6	5	6	7	1	6	5	6	7
5S2/S3	16	14	11	15	11	17	14	11	15	11
5S3	9	13	11	8	11	9	14	11	8	11
6S7	11	13	11	10	11	11	13	11	10	11
8S1/S3	13	13	11	13	11	13	13	11	13	11
8[16G8]	14	14	11	14	11	14	14	11	14	11
8[MX6]	4	12	11	6	11	4	12	11	6	11
12S1	17	13	10	15	10	17	12	10	15	10
13S1/S3	8	12	11	10	11	8	12	11	10	11
13S6	5	8	5	6	7	4	8	5	6	7
17S1	16	13	11	15	11	16	12	11	15	11
21S3	10	11	10	14	10	10	11	10	14	10
22S1	10	12	11	14	11	10	12	11	14	11
	$CD8^+$ lymphocytes									
	Resting					Blastic				
(A2)	7	8	7	7	6	7	8	8	7	7
2S1	8	10	9	13	9	9	10	10	13	10
3S1	2	9	10	5	10	3	10	10	5	10
3[8F10]	9	10	10	13	9	10	9	10	13	10
5S1	7	10	10	9	10	7	10	10	9	10
5S2	1	5	4	5	6	1	5	4	5	6
5S2/S3	15	10	10	13	10	10	10	10	13	10
5S3	5	10	10	7	10	5	10	10	7	10
6S7	8	10	10	9	10	8	10	10	9	10
8S1/S2	6	10	10	11	10	7	10	10	11	10
8[16G8]	9	10	10	13	10	10	10	10	12	10
8[MX6]	3	10	10	5	10	3	10	10	5	10
12S1	9	9	9	13	9	10	8	9	13	9
13S1/S3	7	10	10	9	10	7	10	10	9	10
13S6	2	5	4	5	6	2	5	4	5	6
17S1	9	10	10	13	10	10	9	10	13	10
21S3	8	9	8	12	9	9	9	9	13	9
22S1	8	10	10	12	10	9	10	10	13	10

between the percentage of cells expressing a given Vβ at the end of culture and the percentage of resting $CD4^+$ or $CD8^+$ cells expressing that Vβ product on day 0, for all cultures done with that antigen.

Results for all BV gene families listed above are available from the authors but will be provided herein, in text or figures, only if a blastic expansion was found at at least one stimulant and the two-sided *P* value associated with this expansion was less than 0.01. This statistical testing provides one means to objectively estimate the magnitude and consistency of any given finding. In addition, for any blastic Vβ findings with a *P* value <0.01, we provide data concerning the number of donors with a positive subtractive difference (i.e. value at end of culture minus value at beginning of culture >0). This permits an additional means of assessing interdonor consistency of findings.

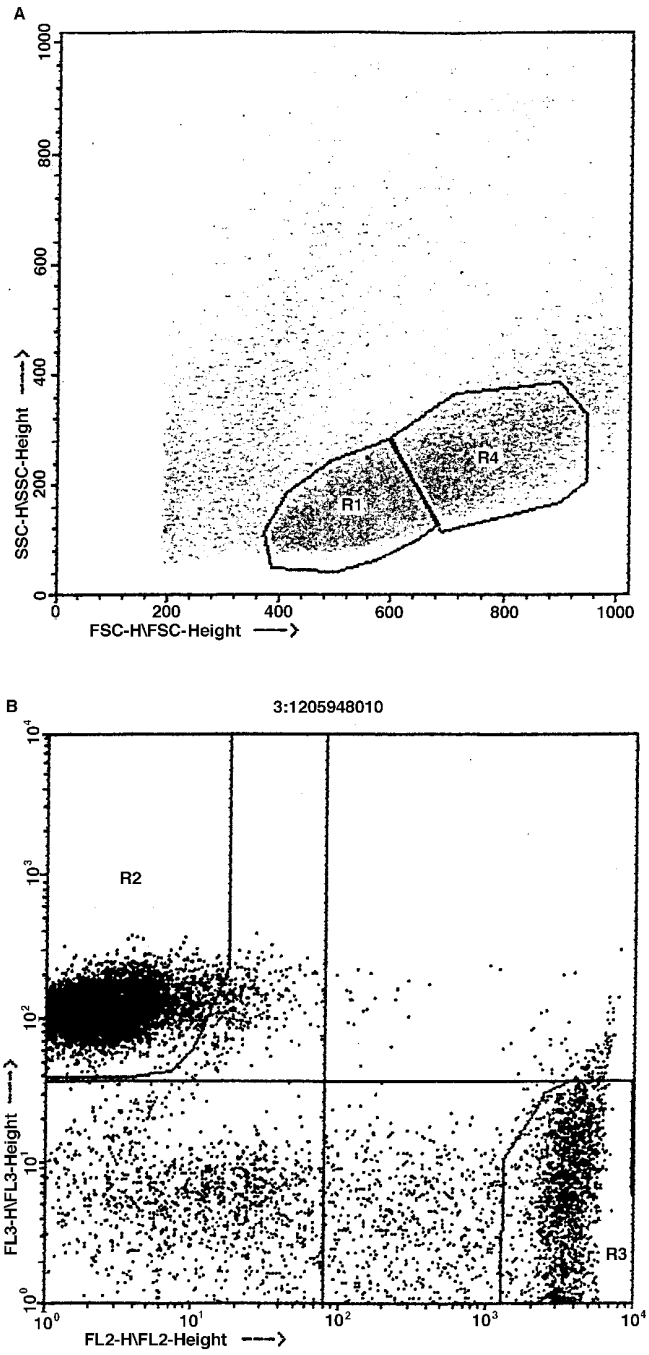

Fig. 1. (A) Example of scatter gates used to define resting lymphocytes (R1) and blastic lymphocytes (R4). (B) Example of gates used to define $CD8^+$ cells ($Fl2^+/Fl3^-$ = R3) and $CD4^+$ cells ($Fl3^+/Fl2^-$ = R2).

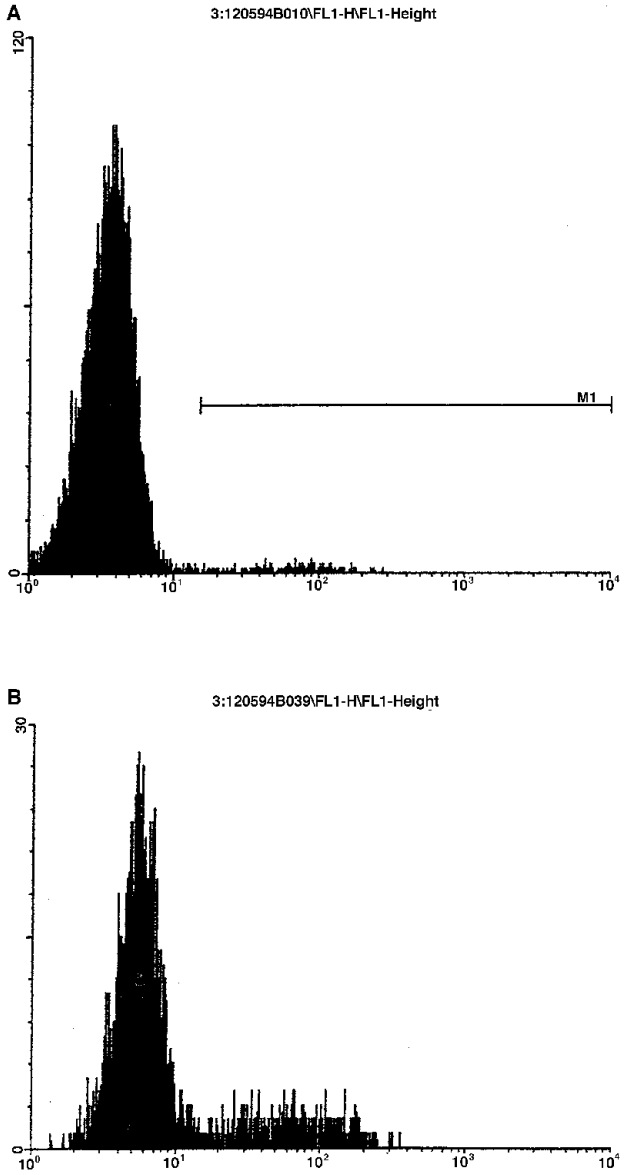

Fig. 2. (A) Histogram of distribution of $V_\beta 8$ (clone 16G8) reactivity of resting $CD4^+$ lymphocytes, following stimulation with staphylococcal enterotoxin D. Gating shown in Figs 1A (R1) and 1B (R2). (B) Histogram of distribution of $V_\beta 8$ (clone 16G8) reactivity of blastic $CD4^+$ lymphocytes, following stimulation with staphylococcal enterotoxin D. Gating shown in Figs 1A (R4) and 1B (R2).

RESULTS

Stimulation with SED, a known SAG, produced extensive blastogenesis (data not shown) and an increased percentage of cells expressing BV5S2/5S3 subfamily gene products in both $CD4^+$ and $CD8^+$ blastic ('Bl') cells at the end of culture, compared with resting cells placed into culture ('D0') ($CD4^+$ $V_\beta 5.2/5.3$: medians: D0 2.4%, Bl 9.4%; $CD8^+$: medians: D0 2.7%, Bl 12.7%) ($CD4^+$ $V_\beta 5.3$: medians: D0 1.0%, Bl 5.7%; $CD8^+$: medians: D0 0.4%, Bl 2.6%*) (Table 2, Fig. 3). In 15 of the 17 donors, SED also produced an increase in the percentage of blastic $CD4^+$ cells expressing $V_\beta 8$, based on results with $\geqslant 1$ $V_\beta 8$-specific reagent ($CD4^+$ $V_\beta 8.1/.2$: D0 4.5%, Bl 15.0%) ($CD4^+$ $V_\beta 8$ [clone 16G8]: D0 4.9%, Bl 14.8%) (Figs 2A & B) ($CD4^+$ $V_\beta 8$ [clone MX6]: four out of four cultures with increases, medians: D0 4.5%, Bl 13.5%, $n = 4$, NS) (Table 2, Fig. 3). These alterations are consistent with activation and

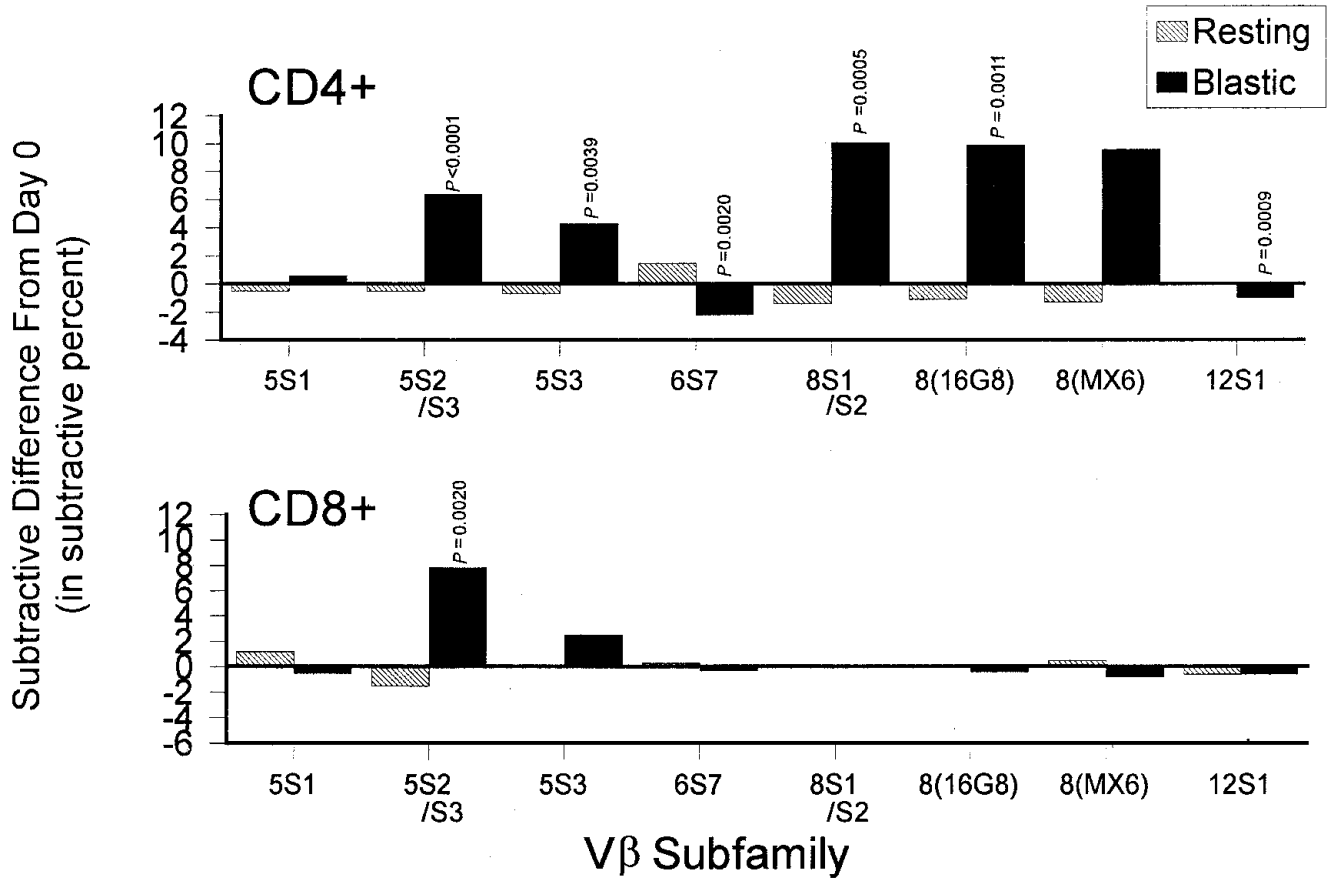

Fig. 3. Change in median percentage of $CD4^+$ and $CD8^+$ lymphocytes expressing TCR BV subfamily post SED stimulation, by cell state. Note that the scale for Fig. 3 differs from the scale for Figs 4–7.

expansion of $CD4^+$ and $CD8^+$ $V_\beta 5.2/5.3+$ cells and $CD4^+$ $V_\beta 8+$ cells.

Stimulation with IL-2 produced minimal blastogenesis and increased percentages of blastic $CD4^+$ cells expressing $V_\beta 5.1$ (medians: D0 3.7%, Bl 8.0%), $V_\beta 5.3$ (medians: D0 1.0%, Bl 1.5%), $V_\beta 8$ [clone 16G8] (medians: D0 4.5%, Bl 6.8%), and $V_\beta 12.1$ (medians: D0 1.7%, Bl 2.5%) (Table 2, Fig. 4). Increases were seen with the other two reagents to $V_\beta 8$, but these changes did not reach significance ($V_\beta 8.2/.3$: 9 of 13 with increases; clone MX6: nine of 12 with increases) (Fig. 4). Very small but statistically significant increases in the percentage of blastic $CD8^+$ cells expressing BV5S3 gene products were also seen (medians: D0 0.9%, Bl 1.4%) (Table 2, Fig. 4). For 10 of 10 donors, IL-2 produced an increase in the percentage of resting $CD8^+$ cells expressing $V_\beta 17.1$ (D0 4.1%, Resting [R] 4.8%, $P = 0.0020$).

The only consistent blastic increase found with CD3MoAb was in the percentage of blastic $CD4^+$ cells expressing BV6S7 products (medians: D0 4.5%, Bl 6.9%) (Table 2, Fig. 5). PHA produced vigorous mitogenesis, but no significant expansions in the blastic cell (Fig. 6).

*Latter difference not significant (NS).

Table 2. Number of cultures with increases in $CD4^+$ or $CD8^+$ blastic cell populations by stimulant

Stimulant	Vβ family	$CD4^+$ blastic cells	$CD8^+$ blastic cells
SED	5.2/5.3	17/17 (100%)	10/10 (100%)
SED	5.3	9/9 (100%)	5/5 (100%)
SED	8.2/8.3	12/13 (92%)	—
SED	8 (16G8)	12/14 (86%)	—
IL-2	5.1	12/13 (92%)	—
IL-2	5.3	13/14 (93%)	9/10 (90%)
IL-2	8 (16G8)	13/14 (93%)	—
IL-2	12.1	9/12 (75%)	—
CD3MoAb	6.7	9/11 (82%)	—
PHA	—	—	—
Con A	5.2/5.3	—	10/10 (100%)
Con A	5.3	—	10/10 (100%)

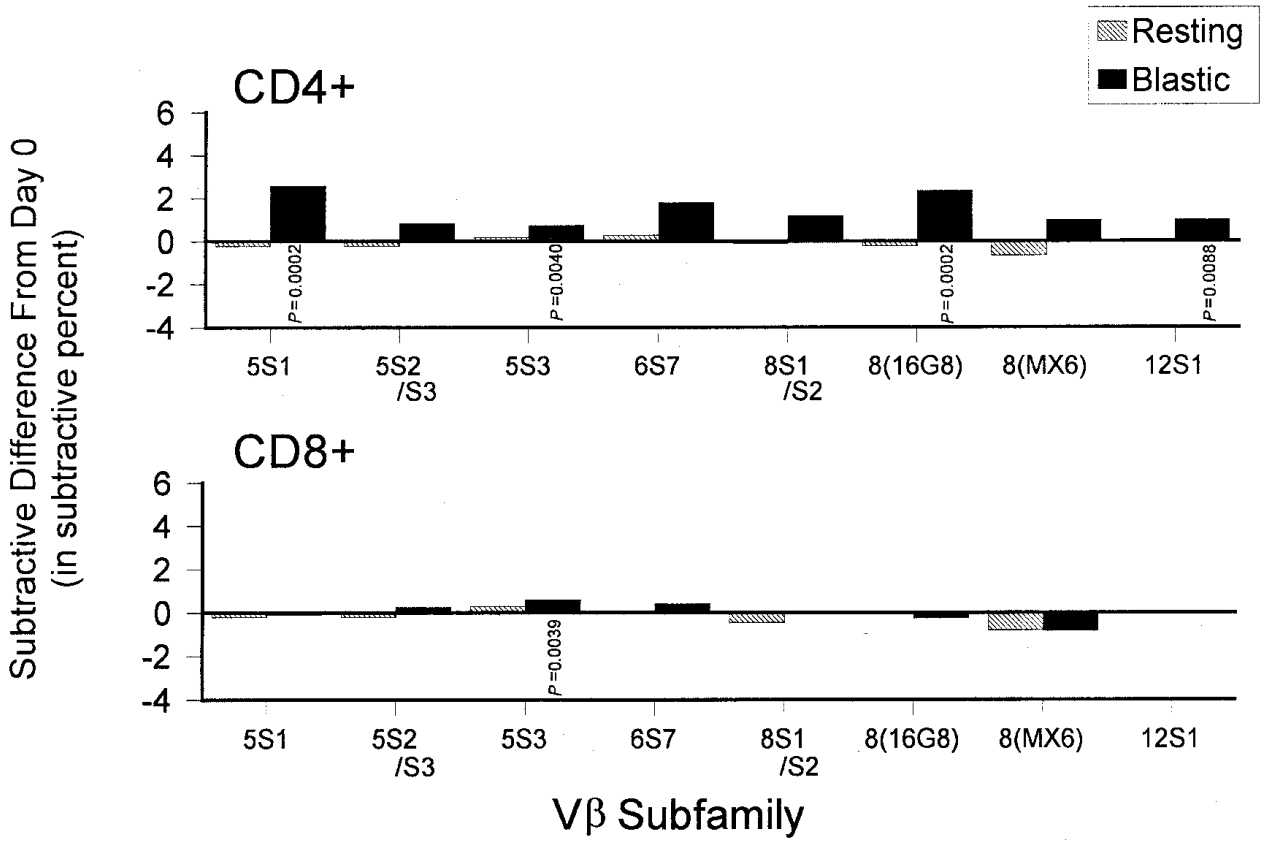

Fig. 4. Change in median percentage of $CD4^+$ and $CD8^+$ lymphocytes expressing TCR BV subfamily post IL-2 stimulation, by cell state.

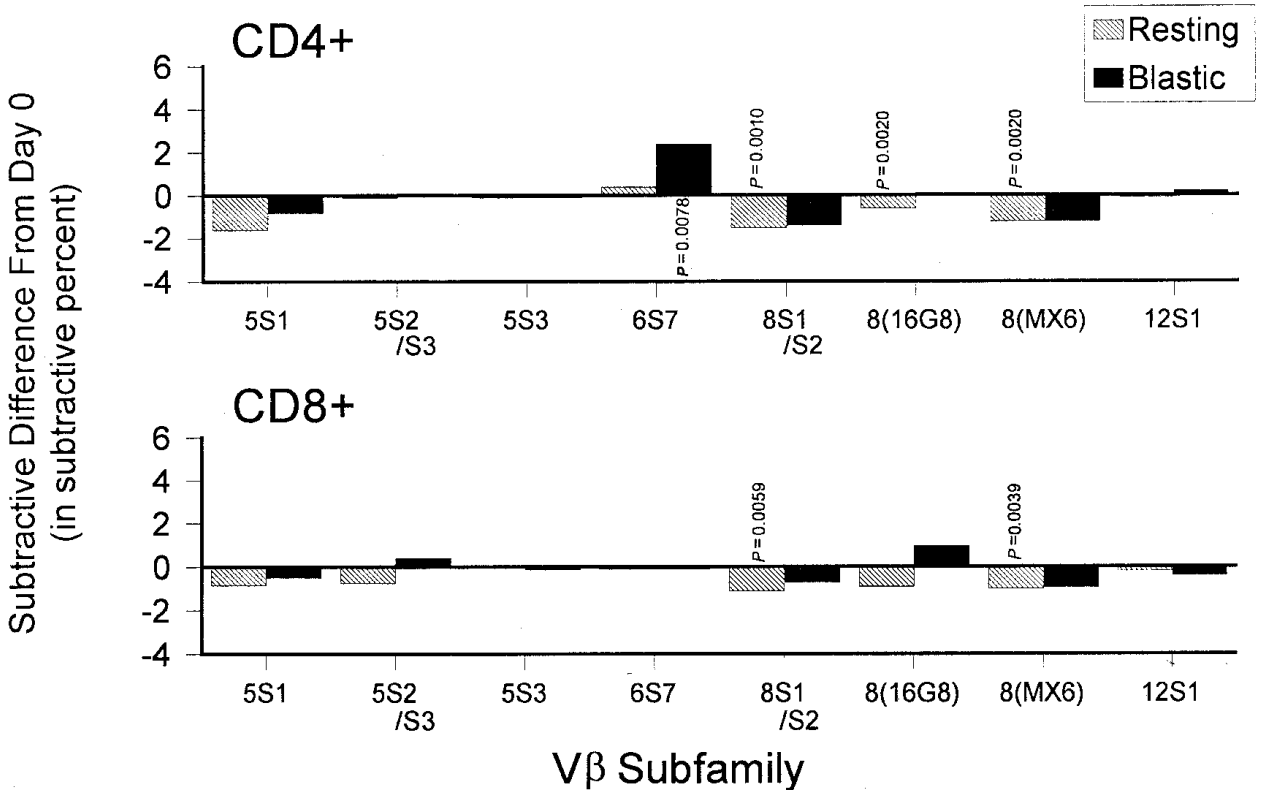

Fig. 5. Change in median percentage of $CD4^+$ and $CD8^+$ lymphocytes expressing TCR BV subfamily post CD3MoAb stimulation, by cell state.

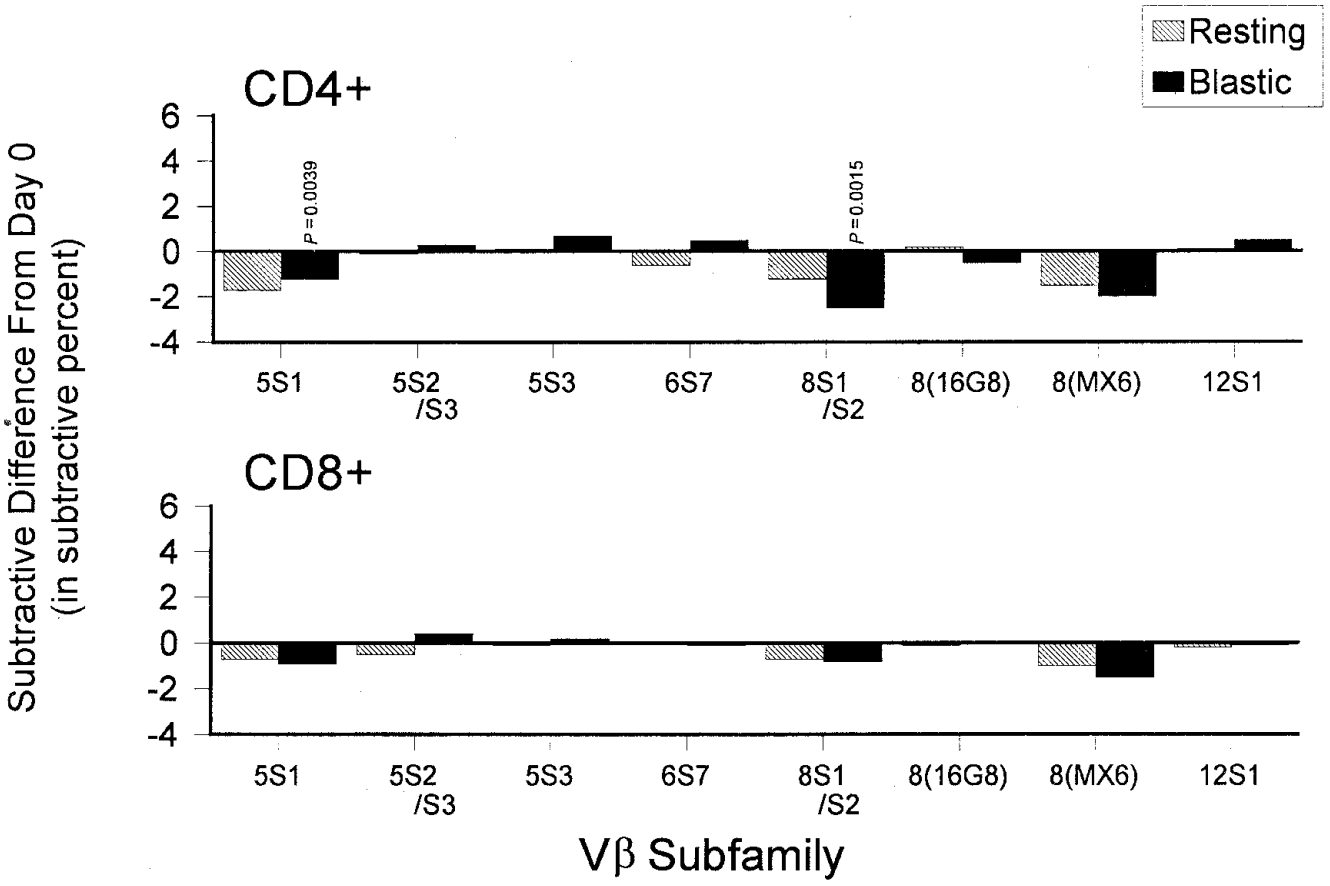

Fig. 6. Change in median percentage of $CD4^+$ and $CD8^+$ lymphocytes expressing TCR BV subfamily post PHA stimulation, by cell state.

Con A produced increases in the percentage of blastic $CD8^+$ cells expressing V_β5.2/5.3 (medians: D0 3.1%, Bl 8.1%) and V_β5.3 (medians: D0 0.8%, Bl 4.8%) (Table 2, Fig. 7).

DISCUSSION

We examined the proliferation phase of *in vitro* Vβ-specific responses to various stimulants, four of which are used extensively to evaluate T lymphocyte reactivity and/or to induce mitosis for T lymphocyte culture and cloning. These stimulants included IL-2, CD3MoAb, PHA and Con A. SED was also examined as a comparison stimulant known to produce skewing of the TCR BV gene product repertoire.

SED produced a skewed response pattern with expansions of cells expressing V_β5.2/5.3, to a degree consistent with Marrack & Kappler's early findings [2]. SED also produced an expansion of $CD4^+$ V_β8+ cells, based on results using two different reagents. We did not find an expansion of V_β12-expressing cells, as has been previously reported [2]; however, our reagent was restricted to subfamily 12S1.

Stimulation with IL-2 produced slight skewing of the Vβ repertoire. This finding is consistent with that of Morita *et al.* who found an expansion of V_β5.3+ cells in one of 15 cultures [23]. Of our cultures, 12 of 13 had slightly increased percentages of V_β5.1+ cells in the $CD4^+$ blastic population, 13 of 14 had increased percentages of $CD4^+$ V_β5.3+ blastic cells, and nine of 10 had increased percentages of $CD8^+$ V_β5.3+ blastic cells. In addition, we found that the majority of IL-2-stimulated cultures had increased proportions of $CD4^+$ V_β8+ and $CD4^+$ V_β12.1+ blastic cells. In general, one would doubt that this degree of skewing would have laboratory implications. However, in some research situations the slight alterations in the Vβ repertoire seen with IL-2 might lead to erroneous conclusions. For example, in studies concerning the effects of HIV infection on the Vβ repertoire, purportedly positive findings are well within the range of the differences reported herein and often involve the same Vβ families we found affected by IL-2 [26–31].

CD3MoAb produced slight skewing toward V_β6.7+ cell expansion in the $CD4^+$ blastic population. PHA produced no skewing of Vβ responses, as evidenced by a lack of Vβ-specific expansions in the blastic $CD4^+$ and $CD8^+$ lymphocyte population. This result differs from those of Wong *et al.* [22]. These researchers applied a semiquantitative PCR technique and found expansions in the levels of expression of BV6, 7S2, and 10S1 in eight of eight donors. Expansions were also found in BV1, 3, 5, 12, 13S1, 13S3, 14, 15, 17, and 18 in seven of eight donors. Although we did not have reagents to V_β7.2 or 10.1, we did have

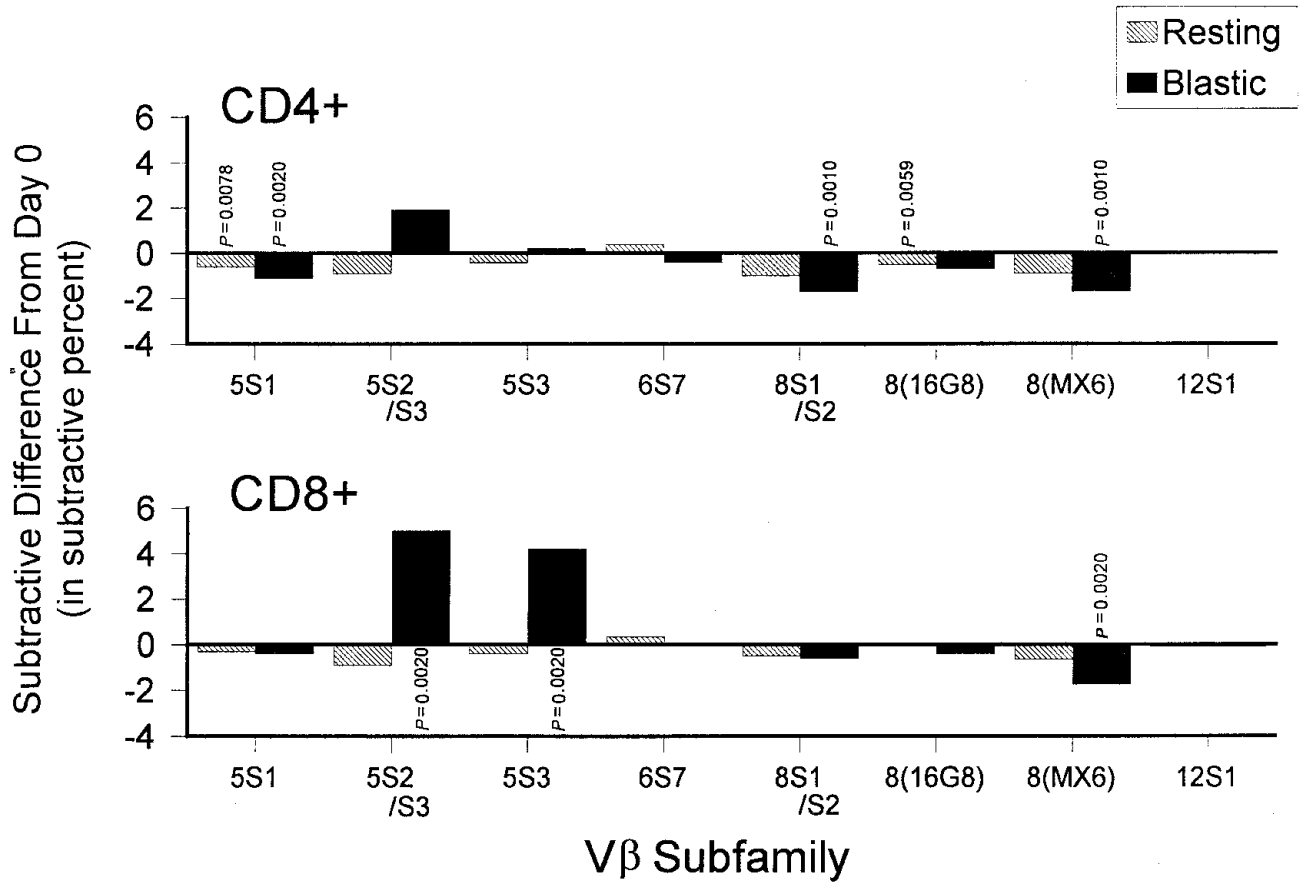

Fig. 7. Change in median percentage of $CD4^+$ and $CD8^+$ lymphocytes expressing TCR BV subfamily post Con A stimulation, by cell state.

reagents to a number of the other families cited in that publication and found no expansion.

The most likely source of the difference between our findings and those of Wong is differing analytical techniques. One group of investigators that published PCR-based BV results has subsequently suggested that PCR is insufficiently quantitative for examining the TCR repertoire in many settings [26]; similar concerns have been raised by others [32]. An additional problem with PCR may be the variation in amplification efficiency among Vβ families. Three studies suggesting good concordance between PCR-based and fluorescence-based TCR analyses compared results for only BV5S2/S3, 8, and 12 [17]; AV1 in a cell line [18]; or BV5 and 8 [33]. One study used a large panel of monoclonal antibodies to compare immunofluorescence and quantitative PCR determinations of the TCR repertoire of normal donors and found that PCR findings were not related to the percentage of cells expressing BV gene products in any predictable or even consistent fashion [34]. One murine-based study found that PCR consistently underestimated or overestimated the percentage of cells expressing certain BV families relative to fluorescence findings [35]. Until these issues are resolved, PCR-based results might best be viewed as potentially useful for assessment of levels of BV gene products but not necessarily for determining the distribution of cells expressing various BV gene products.

Our findings for PHA are limited in that we do not have reagents for all BV families and subfamilies. Of those we have assessed, PHA does not appear to have an effect on the Vβ repertoire. Thus, our findings support but cannot confirm that the skewed Vβ repertoire reported in investigations of multiple sclerosis [10, 14, 36], rheumatoid diseases [12], inflammatory myopathies [37], and autoimmunity [38] are not secondary to *in vitro* cell stimulation with PHA.

Our most intriguing finding was a relatively large Vβ-related skewing of $CD8^+$, but not $CD4^+$, blastic cells with Con A stimulation. To our knowledge, this has not been reported previously. The magnitude is close to that found with the SED, a SAG also assessed here. Previous studies have shown that Con A binds both the α and β chains of Ti, as well as CD3, CD2 and other surface glycoproteins [4, 6–8]. The site of its binding to the TCR β chain has not yet been elucidated. Our results suggest that either Con A selectively or preferentially binds to certain BV gene products in the presence of CD8, or Con A's binding to other surface molecules, in the presence of CD8, create a three-dimensional structure that secondarily induces preferential Vβ stimulation.

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