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In vitro and in vivo T cell oligoclonality following chronic stimulation with staphylococcal superantigens

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Abstract

Microbial superantigens (SAg), including SEB and TSST-1, *polyclonally* activate T cells belonging to specific TCR BV families. A pathogenic role for SAg in various human diseases has been suggested, but enthusiasm for this view has been tempered by the T cell *oligoclonality* in these disorders. To assess whether T cell oligoclonality can emerge following protracted SAg stimulation, human PBMC were stimulated with SEB, TSST-1, or anti-CD3 mAb and maintained in culture with exogenous IL-2. Oligoclonality was appreciated by day 14 among CD4⁺ and CD8⁺ T cells. In addition, mice transgenic for human DR2 and DQ8 were injected weekly with SEB, and splenic CD4⁺ and CD8⁺ T cells were analyzed for oligoclonality. In mice that received one or three such injections, little-to-no oligoclonality was detected. In contrast, considerable oligoclonality was detected in mice that received eight weekly SEB injections. Many of these T cell oligoclonalities were identical to “spontaneously” arising oligoclonalities detected in SEB-naïve mice. Thus, T cell oligoclonality can emerge following chronic SAg stimulation. In hosts who have lost tolerance to self Ag, chronic exposure to SAg may preferentially promote expansion of autoreactive T cells and facilitate development of clinical disease.

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Introduction

Microbial superantigens (SAg), by virtue of their “bridging” MHC class II (MHCII) molecules on APC to specific TCR V_β elements on T cells [1–4], trigger polyclonal activation and expansion of T cells belonging to the specific TCR BV families [5,6]. Included among microbial SAg with potent stimulatory properties are staphylococcal enterotoxin B (SEB) and toxic-shock-syndrome toxin-1 (TSST-1) which preferentially stimulate human BV14 and BV2 T cells respectively [7].

Given the large number of SAg-producing microbial agents in the environment, it is highly likely that microbial SAg interact with and affect the host immune system in a (patho)physiologically significant manner. In rodents, SAg

can aggravate and/or reactivate EAE [8,9] and bacterial cell-wall-induced arthritis [10]. In humans, a pathogenic role has been implicated for SAg in organ-specific and systemic autoimmune diseases [11–15]. However, SAg pathogenicity in human disease has never been directly proven due to ethical proscriptions against performing the definitive *in vivo* experiments in humans.

Thus, although the collective circumstantial evidence in humans is compelling, the true contribution of SAg to disease pathogenesis can still be questioned. For example, based on the selective expansion of T cells belonging to specific BV families, Kawasaki disease has been proposed to be a SAg-driven disorder [14]. However, more recent TCR sequencing studies in Kawasaki disease have demonstrated an *oligoclonality* of T cells within a specific TCR BV family rather than the *polyclonality* of T cells expected from SAg stimulation [16]. Oligoclonality of peripheral blood T cells has also been observed in systemic immune-based rheumatic diseases, including mixed connective tissue disease, SLE, and polymyositis/dermatomyositis

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[17,18]. These findings point to a process driven by specific Ag and ostensibly raise doubts with regard to a fundamental role for SAg in disease development.

Although the initial host response to *acute* stimulation by SAg results in a broad polyclonal T cell response, the nature of the response under proinflammatory conditions or to *chronic* stimulation by SAg may be different. Following initial SAg-induced expansion of T cells belonging to the specific BV families, those T cells undergo rapid deletion due to apoptosis [19–22]. However, this deletion can be overcome by a proinflammatory stimulus such as LPS [23], and in vivo restimulation of SEB-primed mice and SEB leads to preservation and activation of both CD4⁺ T cells and CD8⁺ T cells belonging to the BV8 family [24].

What is not yet known is whether the extant T cells, following SAg stimulation under proinflammatory conditions or following repeated SAg stimulation, can show an *oligoclonal* bias. That is, among T cells belonging either to a BV family “highly reactive” to a given SAg or “poorly reactive” to the given SAg, will discrete oligoclones emerge as dominant following chronic stimulation with that given SAg? To address this question, we assessed T cell clonality at the single-cell level following in vitro or in vivo stimulation with SAg. We demonstrate that T cell oligoclonality is common within SAg-highly reactive and SAg-poorly reactive BV families after chronic SAg stimulation.

Materials and methods

Human subjects

A healthy non-pregnant middle-aged female of Hispanic descent (donor I) and a healthy young-adult male of Chinese descent (donor II) were recruited from University of Southern California (USC) personnel.

Mice

A β 0-DR2/DQ8 mice have a C57B1/10 background, are genetically deficient for endogenous (murine) MHCII, and bear transgenes for human DR2 and DQ8 [25,26]. These mice were originally obtained from Chella David (Mayo Foundation, Rochester, MN, USA) and have been propagated and maintained at USC. Mice of either sex were used and were 9–17 weeks of age at the onset of any given experiment.

In vitro T cell stimulation

Human PBMC were isolated from venous blood by Ficoll density gradient centrifugation [27]. The PBMC were divided into aliquots of 10⁷ cells each. One aliquot was processed for single-cell TCR analysis (see below) without in vitro culture. The remaining aliquots (10⁷ cells/10 ml) were stimulated with 100 ng/ml of SEB (Sigma, St. Louis,

Table 1
PCR primers

Species	Primer name	Primer sequence
Human	V β 14	5'-GTCTCTCGAAAAGAGAAGAGGAAT-3'
	V β 2	5'-TCATCAACCATGCAAGCCTGAC-3'
	V β 5.1	5'-ATACTTCAGTGAGACACAGAGAAA-3'
	C β	5'-TTCTGATGGCTCAAACACAGCG-3'
Mouse	V β 8.2	5'-CATATGGTGTGTCGGCAGCACT-3'
	V β 15	5'-CGCCTCAAAAGGCATTGAA-3'
	C β	5'-GGCTCAAACAAGGAGACCTTG-3'

The primer sequences were designed to yield PCR products of 230–290 bp.

MO, USA), TSST-1 (Toxin Technology, Sarasota, FL, USA), or anti-CD3 mAb 454 [27] in RPMI 1640 medium supplemented with 10% FCS, glutamine, antibiotics, and rIL-2 (100 U/ml). On day 4 and on every subsequent third or fourth day, the cells were washed and recultured in fresh medium containing rIL-2 (100 U/ml) without additional SAg or anti-CD3 mAb. Replicate cultures were harvested on days 7 and 14.

In vivo T cell stimulation

Mice were injected i.p. weekly (up to 8 injections) with SEB (20 μ g in 0.2 ml) and were sacrificed 3 days after the last SEB injection. Spleen mononuclear cells were recovered by mechanical teasing followed by Ficoll density gradient centrifugation.

Single-cell TCR analysis

Fresh or cultured human PBMC or murine spleen mononuclear cells were enriched for T cells by passage through nylon wool columns. The T cell-enriched population was stained with the appropriate PE-conjugated anti-CD4 mAb + FITC-conjugated anti-CD8 mAb (BD PharMingen, San Diego, CA, USA), and $\geq 10^5$ CD4⁺ and $\geq 10^5$ CD8⁺ cells were isolated by positive-selection cell sorting (FACSDiVa, Becton-Dickinson, San Jose, CA, USA). Purity of the recovered populations was routinely >97%.

Total RNA was extracted from the recovered cells with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and was stored at –70°C. After all the RNA samples from a given individual had been collected, they were converted at the same time to cDNA by AMV reverse transcriptase using a random-primer mix (Promega, Madison, WI, USA). The PCR reaction mixture contained 0.2–0.4 μ g cDNA, 200 μ M dNTPs, 2 mM MgCl₂, 0.5 U Taq polymerase, and 50 pmol of the C β and individual respective V β primers listed in Table 1. PCR was performed for 40 cycles (each cycle running at 94°C for 60 sec, 62°C for 60 sec, and 72°C for 60 sec), and the PCR products were electrophoresed on a 1.5% agarose gel. The desired band was gel-purified by a commercial gel extrac-

Table 2
Oligoclonal T cell expansion in “highly reactive” BV families following in vitro stimulation with SAg

SAg	T cells	Donor	Day	CDR3 sequence			Clones		CDR3 sequence			Clones	
				BV14	nDn	BJ	Total	Oligo	BV2	nDn	BJ	Total	Oligo
SEB	CD4+	I	0	CASS	YSG	SDTQY(2.3)	35	10					
			14	CAS	GR	DTQYF(2.3)	30	10					
				CASS	P	RTDTQ(2.3)	30	6					
		II	0				26	0					
			14				24	0					
							23	0					
	CD8+	I	0	CASS	STSAG	EQFFG(2.1)	39	9					
			14				23	0					
		II	0				31	0					
			14				29	0					
TSST-1	CD4+	I	0									27	0
			14									35	0
		II	0									29	0
			14									27	0
	CD8+	I	0						CSAR	GETSNRGII	NEQFF(2.1)	30	25
			14									28	0
		II	0									43	0
			14									29	0

PBMC from each of two normal donors (I and II) were stimulated with SEB or TSST-1. On day 0 (prior to culture) and day 14, CD4⁺ and CD8⁺ cells were isolated and processed for single-cell TCR analysis as described in Materials and methods. The CDR3 sequences of the T cell clones oligoclonally expanded ($\geq 20\%$ of the total) are listed. The numbers in parentheses in the BJ column indicate the BJ segment utilized by the individual clone. The total number of individual sequenced clones for each BV family is listed under “total”, and the number of individual clones with the given sequence is listed under “oligo”. The listing of more than one CDR3 sequence in any single entry indicates that more than one T cell clone was oligoclonally expanded in that BV family. The absence of any CDR3 sequences in any single entry indicates that there was no observed T cell oligoclonal expansion in that BV family.

tion kit (QIAGEN, Valencia, CA, USA), cloned into pGEM T-easy vector (Promega), and used to transform *E. coli* DH5 α (Invitrogen). The colonies containing the recombinant plasmid were selected for ampicillin resistance and white color and were expanded in LB medium. The recombinant plasmids were purified with the QIAGEN miniprep system and sequenced with the 377A DNA sequencing system (Perkin Elmer/Applied Biosystem, Foster, CA, USA). The nomenclature of human and mouse TCR CDR3 is according to Arden et al. [28,29]. For clarity and simplicity of presentation, a T cell clone was arbitrarily listed as oligoclonally expanded when $\geq 20\%$ of the sequenced clones shared the same unique CDR3 sequence.

Results

T cell oligoclonality following in vitro stimulation with SAg or anti-CD3 mAb

SEB preferentially stimulates human BV14 (but not BV2 or BV5.1) T cells, whereas TSST-1 preferentially stimulates human BV2 (but not BV14 or BV5.1) T cells [7]. We anticipated that stimulation of human PBMC with SEB would result in polyclonal expansion of BV14 T cells with no appreciable T cell oligoclonality and that stimulation with TSST-1 would result in polyclonal expansion of BV2 T cells with no appreciable T cell oligoclonality. We supplemented cultures with rIL-2 to mimic a proinflammatory

environment which would promote and facilitate T cell survival and expansion.

Although no oligoclonality was observed among freshly-isolated (day 0) CD4⁺ T cells or CD8⁺ T cells and cells from donor II, some oligoclonality was observed among freshly-isolated CD4⁺ T cells and CD8⁺ T cells from donor I (Table 2). This likely reflected an ongoing in vivo response to some environmental immunogen(s) and is consistent with the limited oligoclonality appreciated by others among circulating T cells in normal donors that had not been intentionally immunized [17]. In this case (as in every case of T cell oligoclonality described in this report), not only were the expanded T cell clones identical in terms of amino acid sequence, but they were also identical in their primary DNA sequences (data not shown), indicating true oligoclonality.

Following stimulation of PBMC from either donor with SEB or TSST-1 and maintenance in rIL-2-containing medium, no oligoclonality of either CD4⁺ T cells or CD8⁺ T cells was appreciated among BV14 (SEB-highly reactive) or BV2 (TSST-1-highly reactive) T cells at day 7 (data not shown). In addition, no T cell oligoclonality was observed when SEB-stimulated or TSST-1-stimulated cultures from donor II or TSST-1-stimulated cultures from donor I were extended to 14 days. However, SEB-stimulated PBMC cultures from donor I *did* lead to oligoclonality of two distinct BV14 CD4⁺ T cell clones by day 14 (Table 2). Of considerable note, these T cell oligoclones were *different* from the T cell oligoclones detected in freshly-isolated PBMC.

This unexpected T cell oligoclonality suggested that

Table 3
Oligoclonal T cell expansion in “poorly reactive” BV families following in vitro stimulation with SAg

Donor	T cells	SAg	Day	CDR3 sequence			Clones		CDR3 sequence			Clones		CDR3 sequence			Clones	
				BV14	nDn	BJ	Total	Oligo	BV5.1	nDn	BJ	Total	Oligo	BV2	nDn	BJ	Total	Oligo
I	CD4+	—	0	CASS	YSG	SDTQ(2.3)	35	10				28	0				27	0
		SEB	14						CASS	AEGYRGL	DEQF(2.1)	29	8	CSAR	GWQGA	TQYF(2.5)	32	26
									CASS	PSPGQGMG	DNEQ(2.1)	29	7					
	TSST-1	14				39	0	CASR	PGV	DNEQ(2.1)	32	9						
									CASS	PR	YEQY(2.7)	32	7					
CD8+	—	0	CASS	STSAG	EQFF(2.1)	39	9				32	0	CSAR	GETSNRG II	NEQF(2.1)	30	25	
	SEB	14						CASR	PGV	DNEQ(2.1)	35	29				21	0	
	TSST-1	14	CASS	YSG	SDTQ(2.3)	25	7	CASS	PREG	HNEQ(2.1)	32	12						
II	CD4+	—	0				26	0				32	0				29	0
		SEB	14								28	0				26	0	
		TSST-1	14	CAS	GR	DTQY(2.3)	31	13				43	0					
	CD8+	—	0				31	0	CASSL	GLAGPR	TQYF(2.5)	29	12				43	0
		SEB	14						CASSL	TSGT	QETQ(2.5)	31	9				24	0
		TSST-1	14	CAS	GR	DTQY(2.3)	29	7				30	0					

TCR analysis was performed as in Table 2, except that “poorly reactive” BV families were analyzed rather than “highly reactive” BV families.

some T cell clones (presumably reacting with undefined Ag present in the cultures) could preferentially expand and/or survive in a SAg-primed environment. To assess whether oligoclonal T cell expansion/survival could extend beyond SAg-highly reactive BV families, oligoclonality among SAg-poorly reactive BV families was also determined. Indeed, cultures from each donor displayed oligoclonality among CD4⁺ T cells and CD8⁺ T cells belonging to SAg-poorly reactive families (Table 3). As the case with the SAg-highly reactive oligoclones detected at day 14, the SAg-poorly reactive oligoclones detected at day 14 of culture were different from the oligoclones detected at day 0.

To demonstrate that this in vitro SAg-driven T cell oligoclonality was not an Ag-specific response to some immunogenic epitope derived from SEB or TSST-1, parallel PBMC cultures from each donor were stimulated with anti-CD3 mAb rather than with SAg. By day 14 of cultures, CD4⁺ and CD8⁺ T cell oligoclonality was often appreciated, and the T cell oligoclones were again different from those detected on day 0 (Table 4). The fact that identical oligoclones occasionally emerged among both CD4⁺ and CD8⁺ T cells from a single donor (e.g., BV14 clone CAS-GR-DTQY in Table 3) or from different donors (e.g., BV5.1

clone CASSL-SVAGGFVG-EQFF in Table 4) strongly suggests an Ag-driven response to some unidentified potent Ag in the culture milieu in the setting of SAg- or anti-CD3-driven polyclonal stimulation.

T cell oligoclonality following repeated in vivo stimulation with SEB

Due to ethical proscriptions against injecting potentially toxic material into humans without anticipation of clinical benefit, we could not assess whether the in vitro SAg-driven oligoclonality of human T cells would be replicated in vivo. Accordingly, we turned to mice for in vivo experimentation. However, the ability of staphylococcal SAg to trigger proliferation of murine T cells is orders of magnitude weaker than that to trigger proliferation of human T cells [30]. This striking difference is not inherent to the T cells themselves but rather is due to the greater ability of human MHCII than that of murine MHCII to present staphylococcal SAg to T cells. Indeed, T cells from mice transgenic for human MHCII proliferate vigorously in response to SEB in the presence of APC that also express human MHCII [31]. Accordingly, we utilized A_β0-DR2/DQ8 mice which are

Table 4
Oligoclonal T cell expansion following in vitro stimulation with anti-CD3 mAb

T cells	Donor	Day	CDR3 sequence			Clones		CDR3 sequence			Clones		CDR3 sequence			Clones	
			BV14	nDn	BJ	Total	Oligo	BV5.1	nDn	BJ	Total	Oligo	BV2	nDn	BJ	Total	Oligo
CD4+	I	0	CASS	YSG	SDTQ(2.3)	35	10				28	0				27	0
		14	CAS	SEGWDPRN	QFFG(2.1)	35	7	CASSL	SVAGGFVG	EQFF(2.1)	46	26				26	0
	II	0				26	0				31	0				29	0
		14				30	0	CASSL	GTGLP	EAFF(1.1)	32	21				27	0
CD8+	I	0	CASS	STSAG	EQFF(2.1)	39	9				32	0	CSAR	GETSNRGII	NEQF(2.1)	30	25
			CAS	GR	DTQY(2.3)	30	6	CASS	PREG	HNEQ(2.1)	34	9	CSAR	GWQGA	TQYF(2.5)	24	12
		14	CASS	SGRS	TDTQ(2.3)	30	9										
			CASS	YSG	SDTQ(2.3)	30	7										
	II	0				31	0	CASSL	GLAGPR	TQYF(2.5)	29	12				43	0
		14	CASS	YSGS	NEQF(2.1)	36	14	CASSL	SVAGGFVG	EQFF(2.1)	46	15				23	0
			CAS	GR	DTQY(2.3)	36	18										

TCR analysis was performed as in Tables 2 and 3.

Table 5
T cell oligoclonality following in vivo stimulation with SEB

T cells	# of injections	Mouse	CDR3 sequence			clones		CDR3 sequence			Clones	
			BV8.2	nDn	BJ	Total	Oligo	BV15	nDn	BJ	Total	Oligo
CD4+	0	A	CASG	DGTG	NTEVF(1.1)	63	27	CGA	GLGF	QNTLY(2.4)	47	22
			CASG	DGTG	NTEVF(1.1)	72	21	CGA	GF	NQDTQ(2.5)	47	10
			CASG	RQG	NSDYT(1.2)	69	65	CGAR	DRAG	NYAEQ(2.1)	36	11
	1	D	CASG	DGTG	NTEVF(1.1)	40	0	CGA	GLGF	QNTLY(2.4)	36	9
			CASG	DGTG	NTEVF(1.1)	43	0	CGA	GLGF	QNTLY(2.4)	49	0
			CASG	DGTG	NTEVF(1.1)	56	0	CGA	GLGF	QNTLY(2.4)	44	0
	3	E	CASG	DGTG	NTEVF(1.1)	40	0	CGA	GLGF	QNTLY(2.4)	56	0
			CASG	DGTG	NTEVF(1.1)	40	0	CGA	GLGF	QNTLY(2.4)	59	0
			CASG	DGTG	NTEVF(1.1)	40	0	CGA	GLGF	QNTLY(2.4)	59	0
	8	F	CASG	DGTG	NTEVF(1.1)	40	0	CGA	GLGF	QNTLY(2.4)	59	0
			CASG	DGTG	NTEVF(1.1)	40	0	CGA	GLGF	QNTLY(2.4)	59	0
			CASG	DGTG	NTEVF(1.1)	40	0	CGA	GLGF	QNTLY(2.4)	59	0
	8	G	CASG	DGTG	NTEVF(1.1)	40	0	CGA	GLGF	QNTLY(2.4)	59	0
			CASG	DGTG	NTEVF(1.1)	40	0	CGA	GLGF	QNTLY(2.4)	59	0
			CASG	DGTG	NTEVF(1.1)	40	0	CGA	GLGF	QNTLY(2.4)	59	0
CD8+	0	A	CASG	DGTG	NTEVF(1.1)	60	47	CGAS	RGQG	SDYTF(1.2)	51	20
			CASG	DGTG	NTEVF(1.1)	60	47	CGA	LGQGA	SNTGQ(2.2)	51	12
			CASG	DGTG	NTEVF(1.1)	60	47	CGA	LGQGA	SNTGQ(2.2)	51	12
	1	B	CASG	DGTG	NTEVF(1.1)	68	24	CGAR	DRAG	NYAEQ(2.1)	55	11
			CASG	DGTG	NTEVF(1.1)	68	24	CGA	GLGF	QNTLY(2.4)	55	15
			CASG	DGTG	NTEVF(1.1)	68	24	CGA	GLGF	QNTLY(2.4)	55	15
	3	C	CASG	DGTG	NTEVF(1.1)	72	70	CGAR	RQ	DTGQL(2.2)	52	19
			CASG	DGTG	NTEVF(1.1)	72	70	CGAR	RQ	DTGQL(2.2)	52	19
			CASG	DGTG	NTEVF(1.1)	72	70	CGAR	RQ	DTGQL(2.2)	52	19
	8	D	CASG	DGTG	NTEVF(1.1)	67	28	CGA	GLGF	QNTLY(2.4)	46	16
			CASG	DGTG	NTEVF(1.1)	67	28	CGA	GLGF	QNTLY(2.4)	46	16
			CASG	DGTG	NTEVF(1.1)	67	28	CGA	GLGF	QNTLY(2.4)	46	16
	1	E	CASG	DGTG	NTEVF(1.1)	61	0	CGA	GLGF	QNTLY(2.4)	46	13
			CASG	DGTG	NTEVF(1.1)	61	0	CGA	GLGF	QNTLY(2.4)	46	13
			CASG	DGTG	NTEVF(1.1)	61	0	CGA	GLGF	QNTLY(2.4)	46	13
	3	F	CASG	DGTG	NTEVF(1.1)	71	67	CGA	GLGF	QNTLY(2.4)	50	14
			CASG	DGTG	NTEVF(1.1)	71	67	CGA	GLGF	QNTLY(2.4)	50	14
			CASG	DGTG	NTEVF(1.1)	71	67	CGA	GLGF	QNTLY(2.4)	50	14
	8	G	CASG	DGTG	NTEVF(1.1)	43	0	CGA	GLGF	QNTLY(2.4)	37	0
			CASG	DGTG	NTEVF(1.1)	51	0	CGAR	DQG	YFGPG(2.6)	36	8
			CASG	DGTG	NTEVF(1.1)	42	0	CGAR	DQG	YFGPG(2.6)	36	8
	3	H	CASG	DGTG	NTEVF(1.1)	46	0	CGAR	DQG	YFGPG(2.6)	46	13
			CASG	DGTG	NTEVF(1.1)	46	0	CGAR	DQG	YFGPG(2.6)	46	13
			CASG	DGTG	NTEVF(1.1)	46	0	CGAR	DQG	YFGPG(2.6)	46	13
	8	I	CASG	DGTG	NTEVF(1.1)	36	0	CGAR	DQG	YFGPG(2.6)	45	9
			CASG	DGTG	NTEVF(1.1)	36	0	CGAR	DQG	YFGPG(2.6)	45	9
			CASG	DGTG	NTEVF(1.1)	36	0	CGAR	DQG	YFGPG(2.6)	45	9
	8	J	CASG	DGTG	NTEVF(1.1)	46	0	CGAR	DQG	YFGPG(2.6)	46	15
			CASG	DGTG	NTEVF(1.1)	46	0	CGAR	DQG	YFGPG(2.6)	46	15
			CASG	DGTG	NTEVF(1.1)	46	0	CGAR	DQG	YFGPG(2.6)	46	15
	8	J	CASG	DGTG	NTEVF(1.1)	52	23	CGA	GLGF	QNTLY(2.4)	53	33
			CASG	DGTG	NTEVF(1.1)	52	23	CGA	GLGF	QNTLY(2.4)	53	33
			CASG	DGTG	NTEVF(1.1)	52	23	CGA	GLGF	QNTLY(2.4)	53	33
	8	J	CASG	DGTG	NTEVF(1.1)	74	64	CGAR	DQG	DTGQL(2.2)	30	10
			CASG	DGTG	NTEVF(1.1)	74	64	CGAR	DQG	DTGQL(2.2)	30	10
			CASG	DGTG	NTEVF(1.1)	74	64	CGAR	DQG	DTGQL(2.2)	30	10

Mice were injected weekly with SEB for the indicated number of times. Splenic CD4⁺ and CD8⁺ cells were isolated 3 days after the last injection and processed for single-cell TCR analysis. T cell oligoclonalities common to both SEB-injected and SEB-naïve mice are indicated by bold type, bold/italics type, and bold/underlined type.

genetically deficient in endogenous (murine) MHCII but are transgenic for (and express) human DR2 and DQ8 [25,26].

To assess whether T cell oligoclonality would ensue following SA_g stimulation in vivo, A_β0-DR2/DQ8 mice were injected weekly with SEB, and splenic CD4⁺ and CD8⁺ T cells were assessed for oligoclonality. In mice, SEB preferentially stimulates BV8 T cells but not BV15 T cells [7], so BV8.2 T cells were examined as representative of SEB-highly reactive T cells, and BV15 T cells were examined as representative of SEB-poorly reactive T cells.

In the three control mice not injected with SEB, oligoclonality among CD4⁺ T cells and CD8⁺ T cells within BV8.2 as well as BV15 families was common (Table 5), likely reflecting exposure to environmental immunogens (e.g., food Ag). Of note, two of the three control mice (A and B) shared a BV8.2 CD4⁺ T cell oligoclone (bolded italics), all three control mice (A, B, and C) shared a BV15 CD4⁺ T cell oligoclone (bolded italics), and all three control mice shared a BV15 CD8⁺ T cell oligoclone (bolded italics), suggesting that there are a few common environmental immunogens which elicit dominant T cell responses in A_β0-DR2/DQ8 mice.

In sharp contrast to the T cell oligoclonality detected in SEB-naïve mice, no T cell oligoclonality was appreciated among CD4⁺ or CD8⁺ T cells in the SEB-highly reactive

BV8.2 family in any of the mice given a single SEB injection (D, E, and F), consistent with SEB acutely promoting *polyclonal* activation of BV8.2 T cells. Similar absent oligoclonality in BV8.2 CD4⁺ and BV8.2 CD8⁺ T cells was observed in an additional mouse (G) given three weekly injections of SEB. Not only was no T cell oligoclonality appreciated in the SEB-highly reactive BV8.2 family, but no CD4⁺ T cell oligoclonalities were detected in the SEB-poorly reactive BV15 family as well. However, BV15 CD8⁺ T cell oligoclonalities were detected in three of these mice (E, F, and G), and these oligoclonalities differed from the oligoclonalities detected in any other SEB-injected or SEB-non-injected mouse, suggesting that the BV15 CD8⁺ T cell oligoclonalities were consequent to the SEB injection rather than simply being a vestige of pre-existing T cell oligoclonality.

Indeed, T cell oligoclonality was a striking feature of mice subjected to eight weekly SEB injections. Levels of CD4⁺ and CD8⁺ T cell oligoclonality in these mice were similar to those observed in control SEB-naïve mice. Each of the three mice that received eight weekly SEB injections (H, I, and J) manifested CD4⁺ T cell oligoclonality in both BV8.2 and BV15 families and CD8⁺ T cell oligoclonality in the BV15 family. In addition, two of the mice (I and J) also manifested CD8⁺ T cell oligoclonality in the BV8.2

family. Remarkably, seven of the 15 total oligoclones detected in these three mice were identical to oligoclones detected in SEB-naïve mice (compare all bolded oligoclones in mice H, I, and J with those in mice A, B, and C), suggesting that dominant T cell oligoclonalities which emerge in the absence of SAg stimulation can also emerge under conditions of chronic SAg stimulation.

Discussion

Investigators have long sought to implicate microbial SAg in human disease. Preferential expansion of specific BV families among circulating T cells in toxic-shock-syndrome [32] and preferential expansion of specific BV families among T cells in the joints of patients with rheumatoid arthritis [11,12] point to the presence of SAg and raise the possibility of SAg involvement in these disorders. Moreover, a compelling (albeit largely theoretical) argument has been offered in support of a pathogenic role for microbial SAg in T cell-dependent autoantibody-mediated disorders such as SLE [13].

However, the early excitement regarding a contributory role for SAg in human disease has been tempered by TCR sequencing studies which have demonstrated *oligo*clonality of, e.g., peripheral blood T cells in Kawasaki disease [16] and peripheral blood and kidney-infiltrating T cells in SLE [33,34]. This T cell oligoclonality points to an Ag-driven process and questions the actual role (if any) for SAg. Although our *in vivo* experiments do not address the issue of SAg pathogenicity, they clearly demonstrate that T cell oligoclonality cannot be taken as a marker for absence of ongoing SAg stimulation (Table 5). In spite of (or, perhaps, as a consequence of) chronic *in vivo* stimulation with SEB, unequivocal T cell oligoclonality does emerge.

How does T cell oligoclonality develop in the context of SAg-driven T cell stimulation? One possibility is that there is unequal SAg-triggered activation of individual clones within a BV family. The stimulatory capacities of individual SAg are largely consequent to the bridging of TCR V_{β} elements to MHCII on APC [1–6]. Although a major site of SAg binding to MHCII lies outside the Ag peptide-binding groove [3,4,35–37], the binding of certain Ag peptides to MHCII can affect binding of SAg to MHCII, with some Ag peptides leading to *enhanced* SAg binding to MHCII [37–41]. When T cells reactive with an “enhancing” Ag peptide specifically engage APC bearing such peptide/MHCII complexes, they concomitantly engage (and are stimulated by) SAg. T cells that do not recognize an “enhancing” Ag peptide also specifically engage APC, but these APC bear Ag peptides that do not enhance (and may inhibit) SAg binding to MHCII. Thus, SAg bind to MHCII less well on the latter APC, so the “enhancing” Ag-nonreactive T cells will not be stimulated as readily or as potently as will the “enhancing” Ag-reactive T cells.

Although we cannot formally exclude this possibility, we

believe it to be an inadequate explanation for the T cell oligoclonality, since T cell oligoclonality that arose following *in vitro* stimulation with anti-CD3 mAb was qualitatively similar to that which arose following *in vitro* stimulation with SAg (Tables 2–4). Thus, as formally demonstrated under *in vitro* conditions and as the case, by implication, for *in vivo* conditions, SAg are no more capable of promoting T cell oligoclonality than is anti-CD3 mAb. Repetitive *in vitro* stimulation of PBMC with PHA also leads to emergence of T cell oligoclonality as determined by spectratype analysis [42], so T cell oligoclonality ensues following chronic stimulation with one of two different agents (anti-CD3 mAb and PHA) that polyclonally activate T cells regardless of the BV family to which the T cells belong. For this same reason, we also believe it unlikely that *in vitro* or *in vivo* T cell oligoclonality represents an Ag-driven response to an immunogenic epitope of SEB or TSST-1.

The explanation we favor is that SAg and conventional Ag act in concert to enhance/amplify the Ag-driven response. In a host undergoing an ongoing Ag-specific response, initial contact of the host with SAg will result in polyclonal expansion of T cells that recognize the given Ag as well as T cells that do not recognize the given Ag. Unless the underlying Ag-specific response is a “strong” one, the SAg-induced polyclonal T cell response will “overwhelm” the ongoing “weak” Ag-specific response, rendering the Ag-restricted (oligoclonal) T cell response not readily detectable. Indeed, no T cell oligoclonality in the SEB-highly reactive BV8.2 family was appreciated in mice that received 1–3 weekly SEB injections (Table 5). Nevertheless, a subtle Ag-driven growth and/or survival advantage for the Ag-specific T cells would, with repeated rounds of encounters with SAg, select for the Ag-specific T cells and result in overt T cell oligoclonality. It already has been demonstrated that *in vivo* stimulation with specific Ag may protect the Ag-specific, SAg-reactive T cells from their apoptotic fate [43]. Thus, it should not be surprising that considerable T cell oligoclonality emerged after eight weekly injections of SEB (Table 5).

The nature of the expanded clones will likely differ among individuals expressing different MHCII. That is, T cells which oligoclonally expand in hosts expressing DR2/DQ8 might be very different from those which oligoclonally expand in hosts expressing other MHCII. This may be especially important for development of autoimmunity where the Ag in question is a self Ag to which tolerance has been broken. Potentially pathogenic T cell clones may emerge (likely influenced by MHCII-driven T cell selection), but endogenous regulatory mechanisms may prevent expansion of such cells to levels that would effect tissue damage and result in clinical disease. Infection with a microbial agent could induce a profoundly proinflammatory environment by triggering the innate immune response (e.g., via toll-like receptors). With superimposed SAg stimulation, these pathogenic T cell clones could expand in size

and quantitatively reach a “critical mass” needed to trigger and/or maintain disease.

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