

# Age-related CD8 T Cell Clonal Expansions Constrict CD8 T Cell Repertoire and Have the Potential to Impair Immune Defense

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## Abstract

Peripheral T cell diversity is virtually constant in the young, but is invariably reduced in aged mice and humans. CD8<sup>+</sup> T cell clonal expansions (TCE) are the most drastic manifestation of, and possible contributors to, this reduced diversity. We show that the presence of TCE results in reduced CD8<sup>+</sup>, but not CD4<sup>+</sup>, T cell diversity, and in functional inability to mobilize parts of the CD8<sup>+</sup> T cell repertoire affected by TCE. In the model of herpes simplex virus (HSV)-1 infection of B6 mice, >90% of the responding CD8<sup>+</sup> T cells use V $\beta$ 10 or V $\beta$ 8 and are directed against a single glycoprotein B (gB<sub>498-505</sub>) epitope, gB-8p. We found that old animals bearing CD8<sup>+</sup> TCE within V $\beta$ 10 or V $\beta$ 8 families failed to mount an effective immune response against HSV-1, as judged by reduced numbers of peptide-major histocompatibility complex tetramer<sup>+</sup> CD8 T cells and an absence of antiviral lytic function. Furthermore, V $\beta$ 8 TCE experimentally introduced into young mice resulted in lower resistance to viral challenge, whereas V $\beta$ 5<sup>+</sup> TCE induced in a similar fashion did not impact viral resistance. These results demonstrate that age-related TCE functionally impair the efficacy of antiviral CD8<sup>+</sup> T cell immunity in an antigen-specific manner, strongly suggesting that TCE are not the mere manifestation of, but are also a contributing factor to, the immunodeficiency of senescence.

Key words: aging • TCR • T cell clonal expansions • repertoire diversity • antiviral immunity

## Introduction

Aging of the immune system leads to the common but poorly understood state of immunodeficiency. Unlike the severe congenital or acquired immunodeficiencies, aging is not associated with a decrease in lymphocyte numbers, but rather with gradual shifts in lymphocyte responsiveness, population ratios, and repertoire (1–4). Of these three, the least well understood is the impact of progressive decrease in lymphocyte antigen receptor diversity (5–9). Within the peripheral T cell pool, TCR diversity remains remarkably constant between birth and adulthood, with nearly invariant representation of peripheral T cells expressing different TCRV $\beta$  segments and

highly diverse molecular distribution of TCR CDR3 lengths (10, 11). In senescence, representation of T cells bearing different TCRV $\beta$  becomes randomly uneven, with concomitant reduction of CDR3 length diversity. A frequent and drastic hallmark of age-related TCR diversity reduction is the appearance of CD8 T cell clonal expansions (TCE), which in some instances can comprise >80% of the total CD8 compartment (6, 7, 9, 12). There are numerous outstanding questions surrounding the appearance, maintenance, and the physiological role of TCE: are they a consequence of repeated antigenic challenges (12, 13), a consequence of cytokine dysregulation (14–17), or semi-transformed cells randomly selected from memory cells? Is their status linked to their replicative history (and how)? At the present, these questions are under intense scrutiny.

Perhaps an even more critical question relates to their impact upon the immune responsiveness of the organism.

*Abbreviations used in this paper:* ATX, adult thymectomy; CTLp, TEL precursors; FCM, flow cytometry; pMHC, peptide-MHC; TCE, T cell clonal expansion(s).

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TCE are often stable, persisting in the absence of overt pathogenic challenge. Individuals bearing TCE, therefore, exhibit natural loss of T cell repertoire diversity, but the extent of this loss and the relationship between the presence of TCE and the immunodeficiency of aging is presently unclear. To answer this question, we evaluated the impact of TCE upon the ability of old mice to mount a de novo immune response and found that TCE are likely to be one of the contributing factors to the poor immune responsiveness in the elderly.

## Materials and Methods

**Mice.** 21-mo-old female C57/BL6 (B6) mice were purchased from the National Institute of Aging (via Charles River Laboratories). Animals were housed under specific pathogen-free conditions at the Oregon Health and Science University or at the Memorial Sloan-Kettering Cancer Center, and were used at 22–26 mo of age. All animal studies were approved by the respective Institutional Animal Care and Use Committees, in accordance with all applicable federal, state, and local regulations. Young (2–4-mo-old) control female B6 mice were purchased from the same source. At the conclusion of the experiment, thorough clinical examination and necropsy were performed on all animals and those exhibiting signs of poor health or tumors were excluded from the analysis. There was essentially no difference in absolute numbers of CD8 T cells between old and young animals, regardless of the TCE status. Thus, old mice had larger spleens than the young ( $86.9 \times 10^6 \pm 10.3 \times 10^6$  cells vs.  $60.2 \pm 8.4 \times 10^6$ ), but with lower relative CD8 representation ( $10.6 \pm 2.9\%$  vs.  $15.4 \pm 3.7\%$ ), yielding average absolute numbers of  $9.2 \pm 2.5$  and  $9.3 \pm 2.2 \times 10^6$  cells for old and young animals, respectively. As reported previously (1, 3, 18), numbers of CD4 T cells, and thus CD8/CD4

ratios, were decreased in old animals, regardless of the presence of TCE (see Table I). For viral-resistance studies, C57BL/6 mice underwent adult thymectomy (ATX) and antibody-mediated depletion at 4 wk of age by vacuum suction, and were rested for 4 mo before viral challenge. In select experiments, these animals were reconstituted with cells from OT-1 and 2C mice (references 19 and 20, respectively). The effectiveness of thymectomy was verified at necropsy and only fully thymectomized animals were included in this study.

**Virus, Peptides, Peptide-MHC (pMHC) Tetramers, and Flow Cytofluorometric Analysis (21).** HSV-1 (strain 17; provided by S. Silverstein, Columbia University, New York, NY, or by J. McGeoch, University of Glasgow, Glasgow, Scotland) was used throughout the experiments. Peptides gB-8p (HSV-1 glycoprotein B<sub>498-505</sub>, SSIEFARL) and OVA-8p (OVA<sub>257-264</sub>, SIINFEKL) were purchased from Research Genetics. H-2K<sup>b</sup> tetramers loaded with OVA-8p or gB-8p were prepared as described previously (21). To ensure the comparability of the presented studies, all experiments were performed using the same batch of tetramer. K<sup>b</sup>/OVA-8p tetramer was used as a negative control and revealed <0.2% nonspecific staining.

For flow cytometry (FCM),  $1-2 \times 10^6$  splenocytes were blocked with Fc block (anti-mouse Fc $\gamma$ RI/III; BD Biosciences) and incubated for 30 min on ice in the presence of a saturating dose of K<sup>b</sup>/HSV-8 tetramer-PE and/or the indicated FITC-conjugated V $\beta$ -specific mAb and anti-CD8 $\beta$ -CyChrome (BD Biosciences). After washing, the samples were analyzed using a FACScan or FACSCalibur instrument/CELLQuest 3.1 software (Becton Dickinson).  $1-4 \times 10^4$  cells/sample were analyzed, with dead cells excluded by selective gating. Markers were set so as to delimit the discrete population of cells staining positively for a given V $\beta$ . This was possible because of the presence of two distinguishable populations of positive and negative cells in each case. Due to the variability in staining intensity by different

**Table I.** Depending on the Degree of Expansion, CD8<sup>+</sup> TCE Numerically Constrict Other CD8<sup>+</sup>, but Rarely, If Ever, CD4<sup>+</sup> T Cell Populations

Mouse no. (TCE, %) <sup>a</sup>	no. CD8 <sup>+</sup> ( $\times 10^6$ )		no. CD4 <sup>+</sup> ( $\times 10^6$ )		%V $\beta$ 2 <sup>+</sup> CD8 CD4		%V $\beta$ 9 <sup>+</sup> CD8 CD4		%V $\beta$ 5 <sup>+</sup> CD8 CD4		%V $\beta$ 8 <sup>+</sup> CD8 CD4		%V $\beta$ 10 <sup>+</sup> CD8 CD4		%V $\beta$ 11 CD8 CD4	
	Old control ( <i>n</i> = 7)	10.1 $\pm$ 2.7	13.8 $\pm$ 3.7	5.2 $\pm$ 1.1	4.8 $\pm$ 1.1	3.3 $\pm$ 1.2	2.5 $\pm$ 0.9	14.2 $\pm$ 2.6	2.6 $\pm$ 0.8	20.6 $\pm$ 3.3	20.0 $\pm$ 2.9	5.9 $\pm$ 1.6	5.6 $\pm$ 1.2	6.6 $\pm$ 1.5	4.7 $\pm$ 0.6	
6050 (V $\beta$ 10, 41.1%)	11.9	12.2	ND	ND	ND	ND	<b>7.4<sup>b</sup></b>	2.5	<b>12.0</b>	17.4	<b>TCE<sup>c</sup></b>	4.7				
4696 (V $\beta$ 9, 17.3%)	7.8	12.9	ND	ND	<b>TCE<sup>c</sup></b>	2.8	<b>8.8</b>	2.2	<b>13.8</b>	16.9	ND	ND				
4824 (V $\beta$ 5, 26.4%)	8.4	9.0	<b>3.4</b>	5.4	ND	ND	<b>TCE<sup>c</sup></b>	1.9	<b>15.3</b>	20.0	4.4	5.5				
6040 (V $\beta$ 5, 86.8%)	9.5	12.8	ND	ND	<b>0.6</b>	3.9	<b>TCE<sup>c</sup></b>	2.7	<b>10.2</b>	17.3	<b>0.4</b>	5.4				
6560 (V $\beta$ 11, 23.8%)	9.9	13.7	ND	ND	<b>TCE<sup>c</sup></b>	5.1	<b>10.4</b>	2.0	<b>16.3</b>	17.4	4.5	5.1				

Spleen T cells from individual old animals were analyzed by viable cell counting and multiparameter FCM for the expression of indicated TCR segments, CD4 and CD8. In parallel, the clonal nature (>97% of the family belonged to a single clone) of their expanded T cells was verified by CDR3 length analysis. Results are representative of 17 control and 19 animals with TCE analyzed in this manner.

<sup>a</sup>Calculated from percentages obtained by FCM and total spleen cell numbers (trypan blue exclusion counting).

<sup>b</sup>Families exhibiting a decrease in representation >1 SD are outlined in bold.

<sup>c</sup>Indicates the TCRV $\beta$  makeup of the TCE (percent of total CD8<sup>+</sup> cells belonging to the TCE is indicated in brackets underneath the animal number).

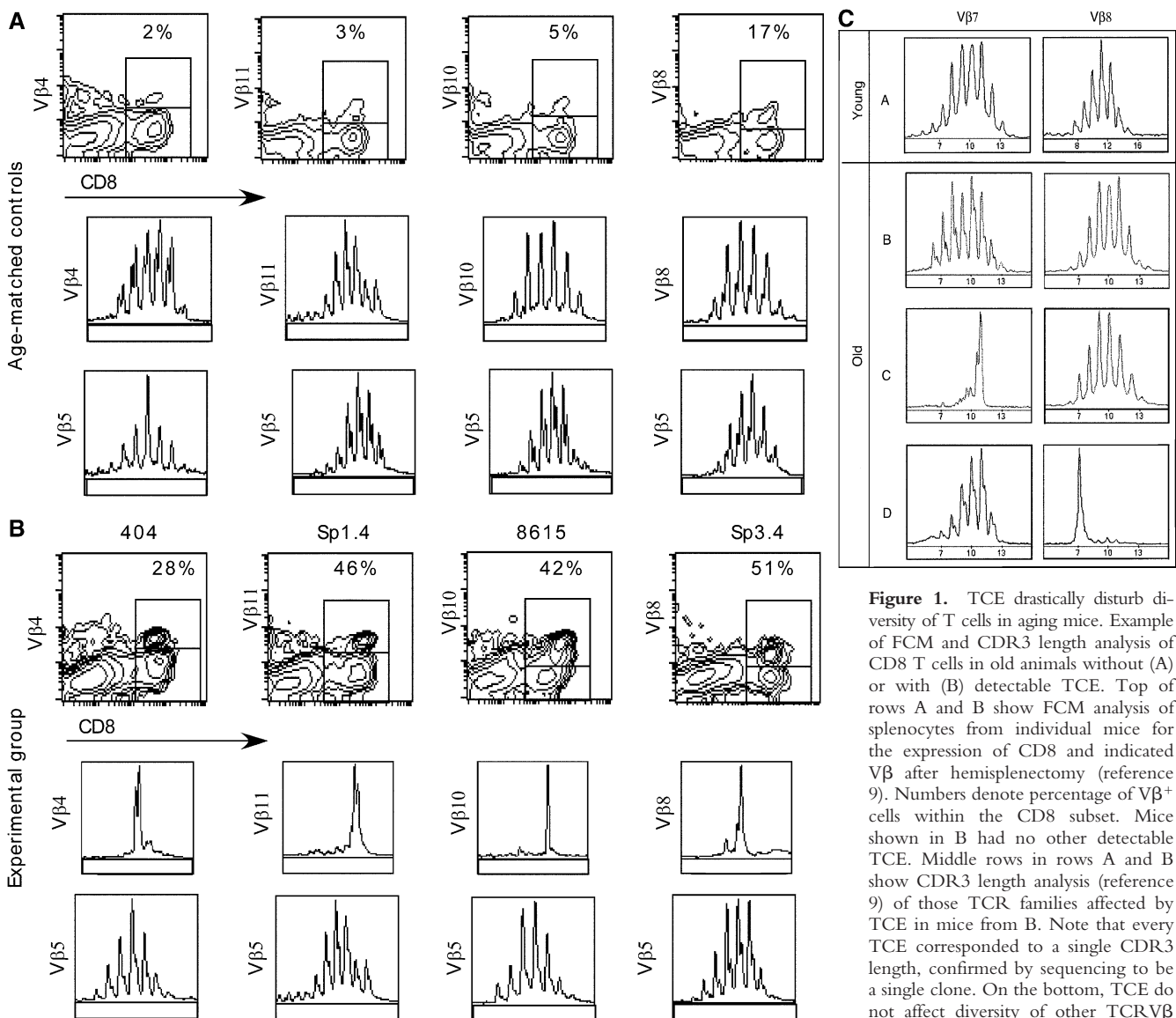
TCRV $\beta$ -specific mAbs, these markers were not identically positioned for all TCRV $\beta$  segments. However, they were identically positioned for the comparative analysis of each individual TCRV $\beta$  segment in all animals (young, old with no TCE, old with "irrelevant" TCE, and old with V $\beta$ 8 or V $\beta$ 10 TCE).

**Immunization and CTL Analysis.** Mice were immunized i.p. with  $10^6$  PFU of HSV-1 virus strain 17, or s.c. with 10  $\mu$ M gB-8p emulsified in adjuvant as described previously (22). 7 d after HSV-1 injection, splenocytes were used for direct FCM analysis by tetramer staining and for in vitro restimulation to generate CTLs. For CTL generation, splenocytes from primed mice were cocultured with irradiated (30 Gy), gB-8p-coated (1  $\mu$ g/ml) syngeneic spleen cells. Thereafter, CTL lines were maintained in vitro by weekly restimulations with syngeneic irradiated splenocytes coated with gB-8p in the absence of exogenous growth fac-

tors. CTL activity was determined on day 5 after each restimulation in a standard  $^{51}\text{Cr}$  assay using H-2 $^b$ -expressing EL-4 cells with or without gB-8p as targets.

**CDR3 Length Analysis.** The assay on CD8- or CD4-depleted spleen cells obtained by immunomagnetic sorting or antibody plus C'-mediated depletion as described previously (9, 22). CDR3 length polymorphism profiles of V $\beta$  families not containing TCE exhibited comparable diversity to those shown for the V $\beta$ 5 family (see Fig. 1, A and B). When single peaks, suggestive of TCE, were detected, the PCR products were sequenced exactly as detailed previously (9), revealing a single readable sequence in each case.

**mAb-mediated Depletion of T Cell Subsets.** V $\beta$ 8 $^+$  and V $\beta$ 5 $^+$  T cells were depleted by i.p. injection of 100  $\mu$ g F23.1 and MR9.4 mAb, respectively. Control animals received an injection of 19E12



**Figure 1.** TCE drastically disturb diversity of T cells in aging mice. Example of FCM and CDR3 length analysis of CD8 T cells in old animals without (A) or with (B) detectable TCE. Top of rows A and B show FCM analysis of splenocytes from individual mice for the expression of CD8 and indicated V $\beta$  after hemisplenectomy (reference 9). Numbers denote percentage of V $\beta$  $^+$  cells within the CD8 subset. Mice shown in B had no other detectable TCE. Middle rows in rows A and B show CDR3 length analysis (reference 9) of those TCR families affected by TCE in mice from B. Note that every TCE corresponded to a single CDR3 length, confirmed by sequencing to be a single clone. On the bottom, TCE do not affect diversity of other TCRV $\beta$  families, as exemplified by V $\beta$ 5 profiles (not depicted). (C) Independent regulation of related T cell subsets: TCE affect non-TCE cells within the same TCRV $\beta$  family, but not the cells outside this family, even if they are related by primary sequence to TCE cells. The CDR3 profile of young (A) and old mice (B–D) bearing either no (B), V $\beta$ 7 (C), or V $\beta$ 8 (D) TCE are shown. The presence of a TCE in V $\beta$ 8 subset does not affect the CDR3 length polymorphism of V $\beta$ 7 subset or vice versa.

of all mice analyzed in this figure. The same pattern of polymorphic, gaussian-distributed peaks was seen in all other TCRV $\beta$  families (not depicted).

( $\alpha$ Thy-1.1, isotype control) mAb. All antibodies were obtained from the mAb Core Facility, Memorial Sloan-Kettering Cancer Center. To test the level of depletion, splenocytes from an independent group of depleted animals were used in a proliferation assay. Net corrected proliferation was as follows: control mice: anti-V $\beta$ 8: 26,349  $\pm$  3,425 cpm, anti-CD3: 80,133  $\pm$  6,299; mice depleted of T cells bearing V $\beta$ 8: anti-V $\beta$ 8: 152  $\pm$  88 cpm, anti-CD3: 76,842  $\pm$  5,246. Furthermore, the pattern of V $\beta$  use in the 5-d MLR using splenocytes from depleted or nondepleted animals responding against irradiated BALB/c cells revealed a complete lack of V $\beta$ 8 response in depleted mice, but an otherwise similar use of the other TCRV $\beta$  segments. Similar results were obtained after MR9.4-mediated V $\beta$ 5 depletion. Animals were challenged with the virus after depletion with or without prior transfer of TCR transgenic cells to simulate the TCE-like situation (see below).

**Experimental Induction of TCE in Young Animals.** V $\beta$ 8 or V $\beta$ 5 T cells were depleted in ATX B6.CD45.1 animals by intraperitoneal injection of 100  $\mu$ g F23.1 and MR9.4 mAb, respectively, 1 wk after ATX surgery. Control animals received an injection of mAb 19E12 (isotype control). Success of depletion (<96%) was verified by FCM at 7 and 14 d after injection as well as immediately before T cell transfer. OT-1 and 2C transgenic T cells were enriched by negative selection of B220<sup>+</sup> CD11B<sup>+</sup> cells using MACS separation (Miltenyi Biotec) in accordance with manufacturer's directions. Obtained cells were 65–75% CD8<sup>+</sup> as determined by FCM, with most of the remaining cells being CD4<sup>+</sup>.  $2 \times 10^7$  cells were injected i.v. and engraftment success was evaluated by FCM 24 h later using either IB2 (clonotypic antibody specific for 2C TCR) or anti-V $\beta$ 5 (clone MR9.4) and anti-CD45.2 antibodies. Animals were challenged 2 d after transfer with  $6 \times 10^7$  PFU HSV-1 strain 17, clone syn2<sup>+</sup>, cloned in our laboratory by three successive rounds of plaque picking. Survival was scored on a daily basis. Death occurred between days 6 and 10, and all animals surviving this period remained disease free for 100 d at which point the experiment was discontinued. Data is shown as percent survival at the termination of the experiment, with the statistical significance determined using Fisher's exact test.

**Calculation of TCR Repertoire Constriction by TCE.** The proportional reduction of T cells bearing other TCRV $\beta$  can be calculated in each mouse using the equation (i), (i)  $x = y/z$ , where  $x$  is the reduction factor of a T cell population bearing a given TCRV $\beta$ ,  $y$  is the percentage in control mice of a repertoire normally occupied by all other families except the one harboring a TCE, and  $z$  is the residual percentage of a repertoire not occupied by TCE in the TCE-bearing mouse. Thus, for the animal Sp3.4,  $x = 79.6\%: 29\% = 2.74$ . It follows that T cells bearing V $\beta$ 10 (usually 6.5% of all T cells) will make up 2.37% (6.5:2.74) of all T cells in mouse Sp3.4 with a V $\beta$ 8 TCE. The accuracy of this calculation can be verified for individual families using the equation (ii), (ii)  $p = q \times z/y$ , where  $p$  is the percentage of T cells bearing a given TCRV $\beta$  in a TCE-bearing mouse,  $q$  is the percentage of T cells bearing the same TCRV $\beta$  in control mice, and  $z$  and  $y$  are as defined for (i). These equations hold true regardless of the relative representation of T cell populations bearing different TCRV $\beta$ .

## Results

**Identification and Characterization of TCE.** TCE are diagnosed by a combination of FCM to detect expansion (Fig. 1, A and B, top rows) and TCR CDR3 length analysis (Fig. 1, A and B, middle and bottom rows), a PCR-based technique that determines molecular diversity (or clonality) of involved T cells. To be considered a TCE, an expansion should be overrepresented by >3 SDs for that family, and also have a CDR3 length profile showing a dominant peak. CDR3 regions are the most polymorphic parts of the TCR and their diversity is representative of T cell diversity within a given population. CDR3 length analysis determines the relative abundance of mRNA encoding TCR chains of individual CDR3 lengths, which usually range between 18–39 nucleotides, corresponding to 6–13 amino acids, in length (11), and thereby measures relative diversity of T cells within the population that ex-

**Table II.** No Cross-Regulation between V $\beta$ 7 and V $\beta$ 8 Family Members in the CD8 T Cell Compartment

Mouse	% F23.1 <sup>a</sup>	% V $\beta$ 8.2/8.3	% V $\beta$ 8.1	Fold reduction V $\beta$ 8.2/8.3	% V $\beta$ 7	Fold reduction V $\beta$ 7	% V $\beta$ 5	Fold reduction V $\beta$ 5
Control 1	23.57	12.23	11.31	NA	5.3	NA	10.1	NA
Control 2	19.6	6.7	12.9	NA	6.7	NA	13.3	NA
Control 3	24.2	7.1	17.13	NA	6.3	NA	14.3	NA
Av control	22.5	8.7	13.8		6.1		12.9	
SD control	2.5	3.1	3		0.7		2.4	
TCE 1	47.6	1.2	46.4	7.3	4.7	1.3	8	1.6
TCE 2	61.7	1.1	60.6	7.9	4.1	1.5	11.4	1.1
TCE 3	44.5	1.3	43.2	6.7	4.3	1.4	6.46	2
Av TCE	51.3	1.2	50.1	7.3	4.4	1.4	8.6	1.6
SD	9.2	0.1	9.3	0.6	0.4	0.1	2.5	0.4

Splenocytes of indicated animals were analyzed by multiparameter FCM for the expression of CD8 and of indicated TCRV $\beta$  segments. Results are expressed as percent of CD9 T cells.

NA, not applicable.

<sup>a</sup>F23.1 detects all V $\beta$ 8 family members.

presses the amplified mRNA. In young nonimmunized mice, CDR3 length analysis of CD8<sup>+</sup> T cells reveals within each TCRV $\beta$  family a diverse pattern of CDR3 length peaks spaced 3 bp (1 amino acid) apart in a Gaussian distribution around the most frequent length (27–30 nucleotides or 9–10 amino acids), representative of a diverse T cell repertoire (11). The same pattern is found in old mice not containing TCE (Fig. 1 A, V $\beta$ 4, 8, V $\beta$ 10, and V $\beta$ 11 shown in the middle, and Fig. 1, A and B, V $\beta$ 5 profiles on the bottom). Usually, antigenic challenge induces expansions of a handful of T cell clones, leading to relative or absolute dominance of one (monoclonal response) or a few (oligoclonal response) peaks in the profile (23, 24). Upon clearing the antigen, most of the expanded cells die by apoptosis, restoring the Gaussian CDR3 length profile. In aged mice and humans, the presence of TCE (defined as single dominant CDR3 length peaks) disturbs or completely distorts the polymorphic pattern of CDR3 length distributions in one or more TCRV $\beta$  families. Four mice shown in Fig. 1 B (middle) exhibit such dominant peaks,

each in a different V $\beta$  family. By densitometry and area integration calculation, TCE peaks in these and other mice used in this study (Tables I and III) occupied >97% of their respective TCRV $\beta$ <sup>+</sup> populations. Upon sequencing, each peak was shown to represent a single clone (refer to Materials and Methods), confirming our previously published (9) and unpublished data on >300 mice. However, although these TCE affected the percentages of T cells expressing other V $\beta$ s (Fig. 1 B and Tables I–III), they did not demonstrably affect their diversity (V $\beta$ 5 profiles in Fig. 1 B, bottom; other V $\beta$  profiles are not depicted).

FCM analysis demonstrated that the above TCE are expanded in absolute terms. The percentage of T cells bearing each TCRV $\beta$  is virtually constant in young mice, such that in C57BL/6 (B6) mice examined there was 3.2% V $\beta$ 4<sup>+</sup>, 20.4% V $\beta$ 8<sup>+</sup>, 6.5% V $\beta$ 10<sup>+</sup>, and 4.3% V $\beta$ 11<sup>+</sup> CD8 T cells (Tables I–III, reference 25, and not depicted). Old mice without detectable TCE exhibited similar V $\beta$  distribution amongst CD8 cells (Fig. 1 A, top row, and Table I). By contrast, FCM profiles of the old mice containing a single CDR3 peak in one of the above families demonstrate that TCE distort T cell homeostasis and can make up >80% of the total of CD8 T cells (Fig. 1 B, top row, and Tables I and III). As expected from previous data (1, 3), the total CD8/CD4 ratios increased in most aging animals (averages of CD8/CD4 ratios in TCE- and non-TCE-bearing old animals varied between 0.67 and 1.0; Tables I–III and not depicted) compared with young B6 animals (ratio: 0.49  $\pm$  0.05).

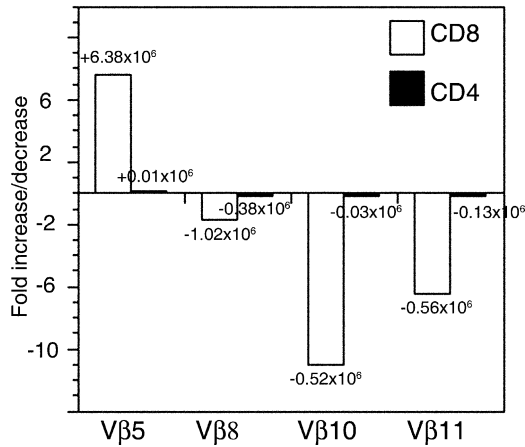
Next, we investigated the impact of TCE upon the diversity and numerical representation of other TCRV $\beta$  families of both CD8 and CD4 phenotypes. For example, certain TCRV $\beta$  families are closely related to each other and are more divergent from other families. Such is the case with the members of the V $\beta$ 8 family (V $\beta$ 8.1, V $\beta$ 8.2, and V $\beta$ 8.3) and their nearest cousin, V $\beta$ 7 (26). Thus, it was of interest to examine whether the CD8<sup>+</sup> TCE in these families influence each other more than they would influence other families. As seen in Fig. 1 C and Table II, we found no evidence of any cross-influence between V $\beta$ 7 and V $\beta$ 8 families, and a profound cross-influence between the members of the V $\beta$ 8 family. For example, a CD8<sup>+</sup> TCE in V $\beta$ 8.1, for example, led to a 27% reduction in CD8<sup>+</sup> V $\beta$ 7 and a 30% reduction in an unrelated family, V $\beta$ 5, but to a >80% reduction in both V $\beta$ 8.2 and V $\beta$ 8.3 (Table II). CDR3 length analysis corroborated this finding (Fig. 1 C). This data strongly suggests that despite a degree of amino acid sequence similarity between V $\beta$ 7 and V $\beta$ 8 polypeptides, T cell subsets bearing them seem to be regulated largely independently of one another. Moreover, the results illustrate the age-associated reduction in TCR diversity at both the TCRV $\beta$  representation and the CDR3 $\beta$  length level and suggest that some level of homeostatic regulation might be specific for TCR structures, including V $\beta$ , as proposed previously in other models (27–29).

As all of the above analysis was performed on CD8<sup>+</sup> T cells, it was also of interest to evaluate the impact of TCE upon the CD4 compartment. Clearly there was no major reduction of CD4 T cell representation in animals bearing

**Table III.** Characteristics of the Lymphoid Compartment and of TCE in Mice Used in the Immunization Experiment

Mouse	TCE V $\beta$ (% of CD8)	CD8/CD4 ratio	% CD8 in young mice (range)
Sp3.4	V $\beta$ 8 (71)	NT	20.4 (16–23)
Sp2.2	V $\beta$ 8 (40)	NT	20.4 (16–23)
Sp1.5	V $\beta$ 8 (45)	NT	20.4 (16–23)
9216	V $\beta$ 8 (78)	1.3	20.4 (16–23)
8197	V $\beta$ 8 (59)	0.6	20.4 (16–23)
8624	V $\beta$ 10 (46)	0.5	6.5 (5–9)
8615	V $\beta$ 10 (42)	1.0	6.5 (5–9)
899	V $\beta$ 10 (36)	1.2	6.5 (5–9)
		0.9 $\pm$ 0.3	
404	V $\beta$ 4 (28)	1.4	3.2 (2–4)
409	V $\beta$ 5 (74)	1.2	12.9 (11–15)
6213	V $\beta$ 5 (71)	0.9	12.9 (11–15)
944	V $\beta$ 7 (45)	0.6	4.7 (2–5)
Sp1.4	V $\beta$ 11 (46)	NT	4.3 (3–5)
Sp1.1	V $\beta$ 11 (29)	NT	4.3 (3–5)
6198	V $\beta$ 13 (18)	0.5	3.1 (2–4)
		0.9 $\pm$ 0.4	

Summary of old B6 mice with TCE used in the immunization study (results of which are shown in Table IV and Fig. 4). All old mice used were 22–25 mo old at the time of functional testing, and were typed for the presence of TCE (as in Fig. 1) after hemisplenectomy (reference 9). After surgery, all mice were rested for at least 4 wk before immunization. Young mice and old mice without TCE (results from five animals/experiment shown in Fig. 4, A and B, and Table III, representative of 15 in total) were 2–3 and 22–26 mo old, respectively, and were treated exactly as the old counterparts. Overall, CD8/CD4 ratios were calculated for individual mice and also for groups of mice with TCE in V $\beta$ 8, V $\beta$ 10, and in other families (expressed as mean  $\pm$  SD). NT, not tested.

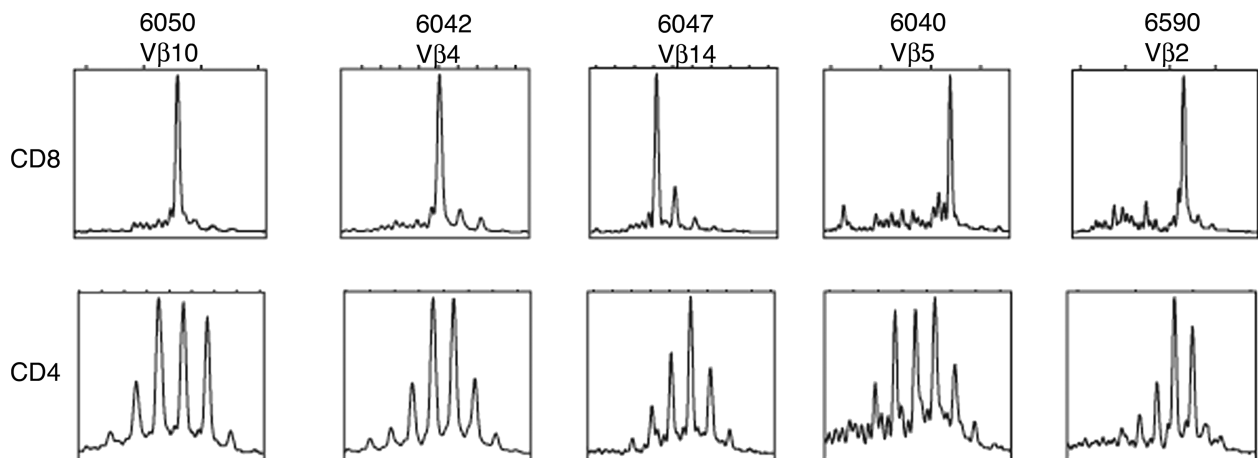


**Figure 2.** CD8<sup>+</sup> TCE preferentially impact cell numbers of other CD8<sup>+</sup>, but not of CD4<sup>+</sup>, cells. An illustrative example of the impact of a large CD8<sup>+</sup> TCE (Vβ5, making up 86.8% of all CD8<sup>+</sup> T cells) on other T cell families in mouse number 6040. Data are expressed as fold increase or decrease of absolute numbers of CD8<sup>+</sup> or CD4<sup>+</sup> T cells bearing different TCRVβ segments (other data for this and other mice, including percentage values, are shown in Table I). Note that even this large TCE shows little impact upon total CD4 counts (Table I) and that it exhibits most of its effects upon other CD8<sup>+</sup> T cells, whereas scarcely affecting even those CD4<sup>+</sup> T cells bearing the same TCR family (Vβ5).

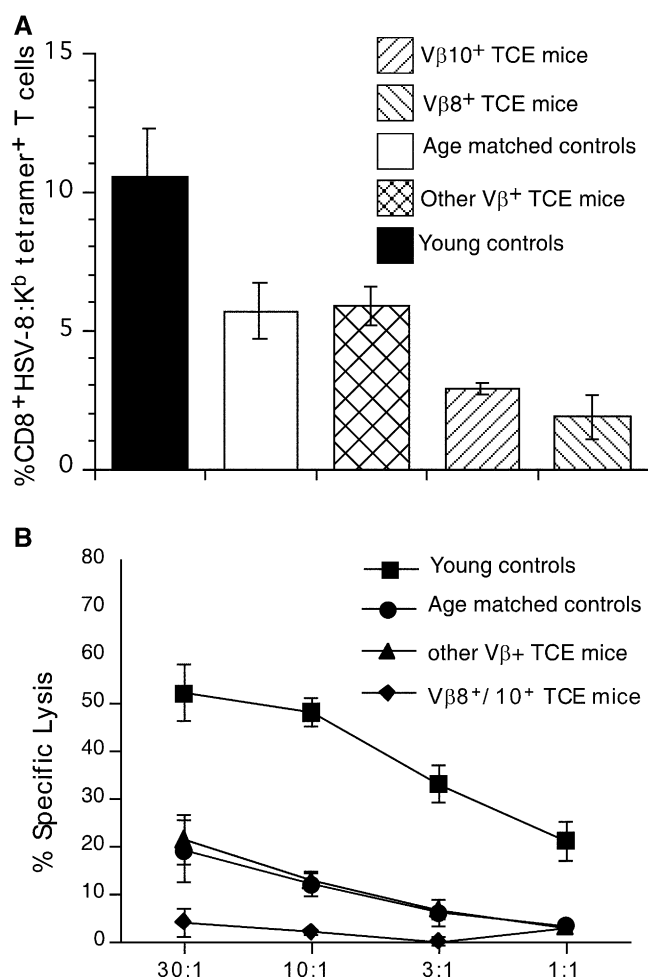
TCE compared with non-TCE controls (Table I), and the only effect, common to both groups, was the aforementioned increase in CD8/CD4 ratios, characteristic for the aging process and possibly related to differential proliferative characteristics of CD8 and CD4 T cells (30). The question was whether the CD4 T cells bearing the same Vβ family as the TCE would be influenced by the TCE. Results shown in Table I and Fig. 2 unequivocally show that there was no specific influence. Thus, in TCE-bearing animals, all of the CD8<sup>+</sup> Vβ families were reduced in representation, most of them to a similar extent, and that extent was proportional to the size of the TCE. For example,

the largest TCE analyzed in this manner, from the mouse 6040, increased more than sevenfold in representation (Fig. 2). This was followed by reduction in absolute numbers (Fig. 2) and relative representation (Table I) of other CD8<sup>+</sup> Vβ families. By contrast, not a single CD4<sup>+</sup> T cell family was affected more than its standard deviation, consistent with the fact that the ratio of CD8/CD4 cells, even in this drastic case, was not skewed more than in age-matched controls without TCE (Fig. 2 and Table I). Moreover, diversity of CD4<sup>+</sup> T cells bearing the same TCRVβ family as the TCE was also largely unperturbed by TCE (Fig. 3). In animals with less pronounced TCE, CD4<sup>+</sup> T cells remained unperturbed and the extent of constriction of other CD8<sup>+</sup> families was less than in animals with large TCE. Therefore, the regulation of TCE and TCR family homeostasis is separate for CD8 and CD4 subsets, consistent with other results indicating that the two represent separately regulated compartments (31–35).

*The Impact of TCE on Antigen-specific Responses.* Next, we showed that such large TCE impaired the ability of aging mice to mount a productive immune response against new antigenic challenge. To that effect, we used HSV-1, which in B6 mice elicits a vigorous CTL response. This response is almost entirely (>95% of CTL activity) directed against the immunodominant epitope gB-8p (gB<sub>495–502</sub>, SSIEFARL; reference 36). gB-8p-specific CTLs predominantly use Vβ10 (~50–70%) and Vβ8 (~20–25%) TCRs (37). To understand how the presence and the Vβ phenotype of TCE may impact the response to HSV-1 in aged mice, we performed extensive FCM screening of unimmunized old mice. Experimental animals (Table III) were accrued into four groups: (a) mice with TCE of CD8 Vβ8 and Vβ10 phenotypes (i.e., TCE affecting the T cell subsets from which the bulk of anti-gB-8p CTLs are recruited); (b) old mice with TCE bearing Vβ segments other than Vβ8 and Vβ10, which are not typically mobilized in response to HSV-1 (such as Vβ5, Vβ11, and Vβ7);



**Figure 3.** CD8<sup>+</sup> TCE do not reduce diversity of CD4<sup>+</sup> cells belonging to the same Vβ family. CDR3 length analysis was performed on CD8-enriched (>88% CD8<sup>+</sup>, >1% CD4<sup>+</sup>) and CD4-enriched (>87% CD4<sup>+</sup>, >1% CD8<sup>+</sup>) cells from animals carrying TCE. Only the polymorphism of the family to which the TCE belongs is shown; however, no skewing was observed in any other CD8 or CD4 family. Representative examples from five individual mice (out of >20 analyzed) are shown.



**Figure 4.** TCE drastically impair the immune response of old mice. (A) TCE that affect the gB-8p-responding TCRVβ families selectively reduce the number of gB-8p-specific CD8 cells after viral infection. Mice from Table II were infected i.p. with 10<sup>6</sup> PFU HSV-1 strain 17 and their ex vivo response against the immunodominant peptide assessed by gB-8p:K<sup>b</sup> tetramer staining.  $\bar{X} \pm$  SD are shown for all mice pooled from three experiments. Background tetramer staining of uninfected young and old controls was undetectable (<0.1%; not depicted). Differences between the Vβ8 and Vβ10 TCE, the age-matched and “other Vβ” TCE, and the age-matched and Vβ10 TCE groups were not statistically significant. Differences between all other groups were statistically significant at  $P < 0.05$  or lower. The absolute number of CD8 splenocytes obtained from young and old animals was superimposable. (B) TCE prevent development of a CTL response. Standard <sup>51</sup>Cr release assay was performed on cells analyzed in A after 5 d of in vitro restimulation with gB-8p-coated syngeneic spleen cells (reference 22). Data are shown as mean specific lysis  $\pm$  SD of individual data from all mice from Table II analyzed in three separate experiments, with background obtained on peptide<sup>-</sup> targets (<5%) subtracted.

(c) old mice with no detectable CD8 TCE; and (d) young mice with no TCE.

In three independent experiments summarized in Fig. 4 and Table IV, these mice were immunized with HSV-1. All young mice mounted a vigorous CTL response to HSV-1, as judged by the percent of cells stained ex vivo with gB-8p:K<sup>b</sup> tetramers ( $7.1 \pm 0.8\%$  of the total CD8<sup>+</sup> T cells; Fig. 4 A) and by cytolytic activity after 5 d of in vitro restimulation with gB-8p (Fig. 4 B). Both old mice without manifest

TCE and those with TCE not affecting Vβ8 or Vβ10 subsets mounted an anti-gB-8p CTL response. As expected, the response of aged mice was less vigorous than that of young counterparts, with a ~25% reduction in the gB-8p:K<sup>b</sup> CD8 T cells ex vivo ( $5.3 \pm 0.9\%$  CD8<sup>+</sup> cells scoring as tetramer<sup>+</sup>; Fig. 4 A) and an almost 30-fold reduction in CTL activity (Fig. 4 B) upon one in vitro restimulation, as judged by the CTL effector/target ratios required to produce comparable levels of lysis. This indicated that the presence of clonally expanded T cells in analyzed mice did not lead to generalized immunosuppression. However, the presence of a TCE among either the Vβ10<sup>+</sup> or Vβ8<sup>+</sup> cells before immunization further reduced the numbers of ex vivo-detectable antigen-specific CD8 cells (by ~60% to  $2.2 \pm 0.6\%$ ; Fig. 4 A), and completely prevented the development of a detectable virus-specific CTL response after one in vitro restimulation in all animals tested (anti-gB-8p response; Fig. 4 B and Table IV; anti-HSV-1 response, specific lysis <1% at any effector/target ratio tested).

Only upon three consecutive in vitro restimulations did CTL activity become detectable in one half of the cultures. Even then the efficacy of lysis was far inferior (only ~40% of the control) to that observed in old mice without TCE (Table IV and not depicted). Moreover, once the TCE-bearing mice developed a response, this response never contained antigen-specific T cells bearing the Vβ segment expressed by the TCE (Table IV and not depicted). In fact, in the case of two mice with Vβ8 TCE where the cell culture after the third restimulation contained Vβ8 cells, these cells likely belonged to the original TCE, as they did not stain with gB-8p:K<sup>b</sup> tetramers. (mice Sp1.5 and 8197; Table IV and its legend).

This leads to two conclusions. First, in the Vβ8<sup>+</sup> or Vβ10<sup>+</sup> TCE-bearing mice, the non-TCE cells from the Vβ8<sup>+</sup> or Vβ10<sup>+</sup> populations that would normally respond to gB-8p were either absent or below the numeric threshold that would allow a productive response. We favor the latter explanation, based upon the observations that in some experiments we could detect infrequent Vβ8 or Vβ10<sup>+</sup> cells amongst ex vivo-isolated pMHC<sup>+</sup> cells (not depicted). This is consistent with the ability of such cells to expand after three, but not after one, round of restimulation (Table IV). Second, compensation for the lost responding Vβ8<sup>+</sup> or Vβ10<sup>+</sup> populations in TCE-bearing old mice was either nonexistent in the case of Vβ10 TCE or incomplete and functionally inadequate in the case of most Vβ8 TCE (Table IV). This difference between the Vβ10 TCE- and Vβ8 TCE-bearing mice is discussed further below.

As shown in Table IV, the cells that occasionally could respond to the virus in four out of eight Vβ8/Vβ10 TCE-bearing mice became detectable only after three restimulations in vitro. These cells were functionally less active (lytic activity ~60% lower) than the cells expanded from animals bearing other TCE or from those not carrying detectable TCE (Table IV). Experiments are in progress to elucidate the reasons for this suboptimal response, which could include the defects in recruitments of the critical signaling mediators to the sites of TCR-pMHC contact (synapse),

**Table IV.** CTL Responses to gB-8p after In Vitro Restimulation

Mouse	TCE	% Lysis first restim <sup>a</sup>	% Lysis third restim <sup>a</sup>	% gB-8p:K <sup>b+</sup> at third restim <sup>b</sup>	% Vβ8 <sup>c</sup>	% Vβ10
Young control						
(H, n = 9)	—	49	68	>97	22	67
Old control						
(H, n = 9)	—	21	51	>98	24	52
Old with TCE						
Sp3.4	Vβ8	<1	22	>97	2	74
Sp2.2	Vβ8	<1	25	>97	7	66
Sp1.5	Vβ8	<1	18	54	40 <sup>d</sup>	53
8197	Vβ8	<1	<1	0	70 <sup>d</sup>	5
9216	Vβ8	<1	23	>96	2	90
8624	Vβ10	<1	<1	NA	NA	NA
8615	Vβ10	<1	<1	NA	NA	NA
899	Vβ10	<1	<1	NA	NA	NA
404	Vβ4	17	43	>97	21	64
7197	Vβ5	24	48	>97	26	72
6213	Vβ5	14	38	>98	16	73
944	Vβ7	15	49	>97	18	58
Sp1.4	Vβ11	27	58	>99	19	65
Sp1.1	Vβ11	25	53	>97	29	48
6198	Vβ13	22	47	>97	30	67

Evolution of the in vitro CTL response in old mice bearing TCE. Summary of TCE status, Vβ use and lytic activity after one and three in vitro restimulations with the immunodominant gB-8p determinant (E/T ratio of 30:1).

<sup>a</sup>Ex vivo isolated spleen cells were restimulated for one (a) or three (b) weekly cycles using syngeneic irradiated and peptide (gB-8p)-coated splenocytes and lytic activity was tested 5 d after the last restimulation.

<sup>b</sup>After the third restimulation, cells were also analyzed for CD8 T cell specificity using the gB-8p:Kb tetramer. >99% of all cells analyzed were CD8<sup>+</sup>. OVA-8p:Kb was used as a control, revealing <0.1% nonspecific staining.

<sup>c</sup>After the third restimulation, cells were stained for the expression of TCRVβ segments. Results indicate percent of CD8<sup>+</sup> cells bearing Vβ8 or Vβ10. There were <0.5% CD4<sup>+</sup> cells present at this time.

<sup>d</sup>These cells failed to stain with the gB-8p:Kb tetramer and were likely the surviving TCE population.

The lysis and Vβ percentages of control young and old animals without identified TCE are the average of 5 experiments, but representative of 15 (SD was never >5%). Note that no CTL lines were established from mice bearing Vβ10 plus TCE.

NA, not applicable (as no response was detectable at any time point).

recently identified to be compromised in aged T cells by Garcia and Miller (38).

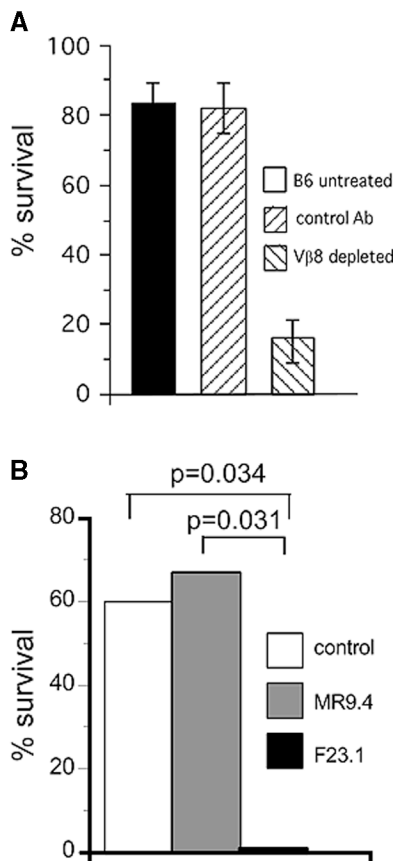
*Experimentally Induced Vβ8 TCE Compromises Viral Resistance.* Overall, the above data indicate that CD8<sup>+</sup> TCE can produce an effect akin to a functional “hole” in the T cell repertoire, which rather specifically affected the T cell subset using the same Vβ segment as the TCE. This, of course, does not preclude the possibility that these cells could have other, more general effects on the immune system, as suggested by the recent findings of Zhang et al. and Ortiz-Suarez et al. (16, 17). However, in our model, such effects did not prevent a response in those animals bearing TCE affecting TCRVβ families not “relevant” for this response.

The germane question, then, is what are the physiological consequences of a TCE-related hole in TCR repertoire in vivo? If the TCE-related holes in the repertoire are detrimental to generating certain immune responses, we deduced that they could affect antipathogen resistance. If so,

one would expect that mice with TCE that affects the population of HSV-1-responding T cells may have lower resistance against that virus. Unfortunately, due to the combined requirements for TCE screening, animal accrual, viral titration, and precise age and weight matching, it was impossible to obtain sufficient numbers of mice with Vβ8<sup>+</sup> and Vβ10<sup>+</sup> TCE to perform properly controlled viral resistance experiments, and thus directly test this hypothesis.

However, because Vβ8<sup>+</sup>/Vβ10<sup>+</sup> TCE were associated with the functional elimination of those Vβ families from the HSV-1-specific response, we used mAb-induced in vivo elimination of T cells bearing the Vβ8 family to mimic the effect of TCE and thus indirectly test the above hypothesis (Fig. 5 A). Injection of an anti-Vβ8 mAb resulted in a profound inability to use CTLs bearing the relevant Vβ, as assessed by proliferation and CTL assays in response to allogeneic spleen cells or to immobilized anti-TCRVβ mAbs, but did not affect the response of T cells





**Figure 5.** Elimination of one responding TCRVβ family significantly reduces antiviral resistance. (A) 25 mice/group (two experiments) were treated with experimental (anti-Vβ8) or isotype-matched control mAbs (19E12, anti-Thy1.1) and infected with an otherwise sublethal dose of HSV-1 ( $1.5 \times 10^7$  PFU/mouse, strain 17). Survival after 100 d is shown as average percent survival  $\pm$  SD. All morbidity and mortality ensued within 7–14 d after infection. Survival after 100 d is shown as percent mean survival  $\pm$  SD. (B) C57BL/6 mice were thymectomized at 5 wk of age and then depleted either of Vβ5 or Vβ8 T cells using a single injection of mAbs (MR9.4 and F23.1 clones, respectively), or left untreated (control group). The efficiency of T cell depletion was verified by FCM 2 wk after the antibody injection and immediately before viral challenge (4 mo later). At 5 mo of age, the animals depleted of Vβ5 T cells received a transfer of  $20 \times 10^6$  OT-1 T cells, and those depleted of Vβ8 received  $20 \times 10^6$  2C T cells. Transfer success was assessed using FCM 24 h later. The animals were rested for an additional 24 h before viral challenge with  $6 \times 10^7$  HSV-1 strain 17, clone syn2<sup>+</sup>. This dose was shown reliably in our laboratory to kill ~40% unmanipulated, 4-mo-old C57BL/7 mice. A total of 12 animals/group was used. Survival after 100 d is shown as average percent survival  $\pm$  SD as explained above.

bearing other Vβs (refer to Materials and Methods). Treated and control mice were then challenged with a sublethal dose of HSV-1 ( $1.5 \times 10^7$  PFU of this particular lot of the strain 17). Although <15% of the mice receiving the control antibody succumbed to this dose of virus (2/15), this number rose to >80% (13/15) amongst the mice treated with anti-Vβ8 (Fig. 5 A). Of interest, the surviving αVβ8-treated mice recruited almost exclusively the Vβ10<sup>+</sup> CTLs in response to HSV-1 challenge as detected by tetramer staining (not depicted), similar to the situation in three times-restimulated cells from Vβ8 TCE-bearing ani-

mals. This was also found in three out of three moribund mice analyzed from the same group. In contrast, surviving mice in the group without depletion recruited both Vβ8 and Vβ10.

Finally, to better experimentally mimic the TCE situation, we experimentally induced TCE in young ATX animals by depleting Vβ8 or Vβ5 T cells in vivo using antibodies and replacing them with transgenic 2C or OT-1 CD8 T cells. A third group of mice was treated with an isotype mAb control. Both mAbs used caused a complete depletion of the targeted T cell subset as described in Materials and Methods. Moreover, the groups represented internal controls for each other: Vβ8-depleted animals contained a polyclonal Vβ5 population and vice versa. After transgenic T cell transfer, all three groups were challenged with HSV-1 strain 17 clone syn2<sup>+</sup>, using a dose that reliably resulted in 60% survival in adult C57BL/6 mice ( $6 \times 10^7$  PFU). While 60–67% of the control and the Vβ5-TCE (OT-1) groups survived this dose, none of the Vβ8 TCE group survived (Fig. 5 B). The results were statistically significant, at the level of  $P < 0.03$ .

Thus, inactivation of T cells expressing a TCRVβ family that participates in a CTL response against HSV-1 reduced the resistance to that pathogen. Because the presence of TCE amongst a particular TCRVβ<sup>+</sup> T cell subset effectively abolishes the participation of naive cells from that subset in a productive immune response, it is likely that TCE have the potential to similarly impair the pathogen resistance in aged individuals.

## Discussion

The above results demonstrate the potential of TCE to impair new immune responses, suggesting that they contribute to the immune deficiency of the elderly. We documented that this defect is focused, which would be expected given that the TCR diversity is heavily reduced only in the CD8<sup>+</sup> population bearing the same Vβ as the TCE. This strongly suggests that the regulation of TCE and TCR family homeostasis is separate for CD8 and CD4 subsets, and is consistent with other results indicating that the two represent separately regulated compartments (31–35), including the fact that CD8 and CD4 T cells do not compete with one another in homeostatic proliferation (31, 32) and that in animals deficient in one of these subsets or in transfer experiments, the other subset does not “fill the void” (33, 34, 35).

The finding that TCE preferentially affect the immune responses that normally recruit T cell populations bearing the same Vβ family appears somewhat unexpected. However, the explanation of how TCE preferentially affect certain immune responses can be found in the numerical analysis of T cell diversity and frequencies. Analysis by Casrouge et al. (39) estimated that the murine T cell repertoire contains  $\sim 6 \times 10^5$  Vβ sequences, each distributed on 30–50 cells. This allows one to estimate TCE-associated reduction of antigen-specific precursor diversity and frequency. For example, in the case of mouse Sp3.4 (Table I),

a V $\beta$ 8 TCE takes up  $\sim 71\%$  of the CD8 repertoire. V $\beta$ 8 normally makes up  $\sim 20.4\%$  of total CD8 T cells, or  $0.4\text{--}0.6 \times 10^5$  different sequences (assuming CD8/CD4 ratios to be between 1:2 and 1:1), each present on 10–50 cells (39). We know that young and old mice have comparable numbers of CD8 T cells in secondary lymphoid organs, with old spleens being larger, but containing lower relative CD8 T cell numbers. Therefore, a major expansion in one V $\beta$  population should reduce representation of T cells belonging to other populations across the board. Indeed, our results show that this is true within the CD8 T cell compartment, and the fact that the CD4 compartment remains mostly unperturbed is irrelevant for the case of TCE affecting class I-restricted responses. In the case of mouse SP3.4, where the V $\beta$ 8<sup>+</sup> cells increase  $\sim 3.5$ -fold (from 20 to 71%), it follows (refer to Materials and Methods) that T cells bearing other V $\beta$  populations will decrease in representation by 2.74-fold each, assuming that such reduction occurs independently of TCR chain sequence. In that case, the average population of 30–50 cells expressing a gB-8p-reactive V $\beta$ 10 sequence in this mouse would be reduced to  $\sim 4\text{--}12$  cells. Although many T cells bearing gB-8p-reactive V $\beta$ 10 sequences would fail to react to gB-8p due to TCR- $\alpha$  chain influences, a fraction of them (2–10% according to our preliminary data from TCR- $\beta$  transgenic mice) would recognize gB-8p:K<sup>b</sup>, particularly given the crucial role of TCR- $\beta$  chain in recognition of gB-8p:K<sup>b</sup> (40). Thus, 11–18 cells/sequence and several “responding sequences” or T cell clones (40) would make up for a sufficient number of T cell precursors bearing TCRV $\beta$ 10 to respond to pathogen challenge.

A very different situation pertains to the CD8<sup>+</sup> V $\beta$ 8 cells if the TCE is of the V $\beta$ 8 type. In the mouse Sp3.4 (71% of the CD8 repertoire), almost the entire V $\beta$ 8 family (>97% sequences) is taken up by the TCE. In the CD8 subset, that leaves <3% of T cells in that family to represent all memory and naive cells, including those with potential to respond to gB-8p. Simple calculation reveals that the maximal percentage of diverse V $\beta$ 8 cells ( $71 \times 3\% = 2.1\%$ ) is reduced by about tenfold compared with animals without TCE that contain  $\sim 20\%$  V $\beta$ 8<sup>+</sup> cells. Therefore, the representation of cells bearing diverse V $\beta$ 8 sequences in mouse Sp3.4 with a V $\beta$ 8 TCE would drop from 30–50 to 3–5 or less, with a realistic possibility that none would be specific for gB-8p (due to TCR- $\alpha$  effects, maintenance of memory cell in the same pool, etc.). Of note, the difference in diversity reduction between the T cells outside the TCE and those that share the V $\beta$  family with TCE is even more drastic in animals with lower absolute expansion levels, where the TCE still takes >97% of all sequences (i.e., in mouse Sp2.2, the reduction is  $\sim 20$ -fold within V $\beta$ 8, but only 1.3-fold for all other families). Even if no other mechanisms were contributing to the delayed and reduced immune response of the old animals, this reduction in precursor frequency would easily explain the extremely weak and delayed response of non-TCE clones coexisting in the same V $\beta$  family with the TCE. Note that this discussion assumes an even and balanced reduction in all T cells regard-

less of sequence or immunological experience. If, however, memory cells are selectively maintained in the repertoire of the aged animals (1, 3, 4, 18, 41) and if one takes into the account the increased CD8/CD4 ratios described in old animals (1, 3, 18), then diversity would be expected to undergo even more reduction.

In our experiments, there seemed to be better compensation for the TCE-related loss of V $\beta$ 8<sup>+</sup> gB-8p precursors than for the loss of V $\beta$ 10<sup>+</sup> T cells. We explain this by different participation of CTLs from these populations in the anti-gB-8p response, and by the unique dependence of this response in B6 mice upon germline VDJ elements (42). Thus, if gB-8p-specific V $\beta$ 10 CTL precursors (CTLp) outnumber the V $\beta$ 8 gB-8p-specific CTLp by  $\sim 3:1$  (as suggested by dominant recruitment of CTLs from this T cell population in young mice), then in V $\beta$ 8 TCE-bearing animals the V $\beta$ 10 CTLp can belatedly compensate for the depressed or nonexistent response of gB-8p-specific V $\beta$ 8 CTLp (as their number would remain sufficient for expansion/detection after several stimulations), but in V $\beta$ 10 TCE-bearing animals frequencies of V $\beta$ 8 gB-8p-specific CTLp would be insufficient to mount a detectable response. Indeed, in many cases compensation between different T cell populations after the loss of TCR diversity is incomplete or absent (for review see reference 43) and in the specific case of this HSV-1 response in H-2<sup>b</sup> mice, it was shown previously that the absence of germline j $\beta$ 2-D $\beta$ 2 segments knocks the V $\beta$ 10 family from this response, but that V $\beta$ 8<sup>+</sup> CD8 T cells only feebly compensate for the loss; the mobilized response reaches <40% of the response in the presence of V $\beta$ 10 participation (42). An alternative explanation, that V $\beta$ 10<sup>+</sup> T cells have higher avidity for the cognate pMHC than those bearing V $\beta$ 10<sup>+</sup>, is highly unlikely, as we failed to detect any such differences using pMHC tetramers to evaluate the kinetics of TCR-pMHC interaction in these T cell populations (unpublished data). Most importantly, it is crucial to note that this compensation, which is slowed and rendered even less efficient by the intrinsic defects in activation of old T cells, is belated and fails to ensure responsiveness to the virus in a timely manner.

At the present, we do not understand many of the details of the age-related dysregulation of peripheral T cell diversity and of the TCE ontogeny, including the reason why a TCE accumulates in a fashion to initially preferentially dilute out the T cells bearing the same family of TCRV $\beta$  proteins, while affecting other neighboring T cell populations bearing different TCRs (V $\beta$ s) to a lesser extent. This, together with the surprisingly tight control of the representation of T cell populations bearing different TCRV $\beta$ , suggests that T cell diversity might be under homeostatic control. Neither the existence nor the mechanistic details of this control are firmly established at this time. However, one could speculate that there are two mechanisms to control peripheral T cell homeostasis: a general homeostatic mechanism, supported by our current knowledge (44), regulates total number of T cells in a compartment (likely based on contact inhibition, TCR-self-pMHC interaction, and/or general survival/maintenance factors such as IL-15;

reference 15), and another, presently speculative, mechanism, that regulates the relative abundance of T cell subsets based upon TCR sequence diversity. That second mechanism could operate via a finite number of distinct survival-mediating ligands, each of which is occupied by CD8 or CD4 T cells (separate niches) bearing TCRs with different structural characteristics. This mechanism could operate based on TCRV $\beta$  family expression, similar to regulation mechanisms proposed before (27–29), but still incompletely understood. A distinct ligand/niche would attract TCRV $\beta$ 8 cells and would provide them with survival signals based upon TCR binding, perhaps not unlike low avidity, sub-activation threshold interaction between TCR and pMHC or MHC-endogenous superantigen. TCE would originate from T cells that became independent of the ligand-mediated survival signal (and that thus have survival advantage over other T cells), but that still effectively bind the survival-promoting ligand, thus progressively out-competing other cells bearing the same V $\beta$  (15). Meanwhile, the constriction of the rest of the TCR repertoire would occur later, beginning only when a TCE fully occupies the space allocated to the pool of cells bearing the same TCR structural element and starts to accumulate above this level. At this point, TCE would act as a “sink” for the general maintenance factors, thus competing out other T cells in a TCR-independent manner (general homeostatic mechanism). Alternatively, TCE could differ from other memory T cell subsets by secreting (and also being resistant to antiproliferative effects of) IFN- $\alpha$  and possibly IFN- $\gamma$ , which then inhibits the ability of other, non-TCE cells to proliferate. This is consistent with the findings of Zhang et al. (16) that aged non-TCE CD8 T cells respond poorly to IL-15 due to environmental IFN-I, and with findings of Ortiz-Suarez and Miller (17) that an actively proliferating subset of aged CD8<sup>+</sup> CD28<sup>hi</sup> T cells secrete high levels of IFN- $\gamma$ . Inasmuch as Ku et al. (15) showed that TCE have higher CD28 levels than average memory CD8 T cells, the subset identified by Ortiz-Suarez and Miller (17) would be likely to contain TCE. All of these cytokines would work at the general level, as could IL-7, recently implicated in the maintenance of memory CD8 cells (44).

Although experiments are underway to test the existence and operation of the hypothetical V $\beta$ -specific mechanism, as well as the reliance of this regulation on TCR specificity (45), exact TCR CDR region sequence and specificity, and regulatory cytokines, their results would not affect the major conclusions of this study. Indeed, regardless of mechanistic issues, which are outside the scope of this study, results presented here suggest that the functional knockout of one TCR family by the age-related TCE can lead to potentially serious impairment of immune resistance. We conclude that CD8<sup>+</sup> TCE can clearly constrict the available CD8<sup>+</sup> T cell repertoire and are likely to not only be a consequence of, but also a contributing factor to, the immunodeficiency of the senescence. Understanding the harmful effects of these cells on a productive immune response should stimulate new strategies to prevent the onset or eliminate the existing clonal ex-

pansions as means to treat and ameliorate the immunodeficiency of the senescence.

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