

TOLERANCE-RELATED $V\beta$ CLONAL DELETIONS IN
NORMAL $CD4^{-}8^{-}$, $TCR-\alpha/\beta^{+}$ AND ABNORMAL
lpr AND *gld* CELL POPULATIONS

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Mice homozygous for the mutant *lpr* or *gld* genes develop a massive proliferation of an unusual subset of double-negative ($CD4^{-}8^{-}$), $TCR-\alpha/\beta^{+}$ T cells, associated with lymphoid organ enlargement, hypergammaglobulinemia, and production of diverse autoantibodies, as well as accelerated autoimmunity in strains with susceptible background genotypes, such as MRL-*lpr/lpr* (1). It seems likely that the abnormal *lpr* and *gld* cells are derived from a minor, normal thymocyte subset; and recently such a minor $CD4^{-}8^{-}$, $TCR-\alpha/\beta^{+}$ subset has been described (2-4). However, since both *lpr* and *gld* cells and their putative normal counterparts express an unusual combination of immature (i.e., $CD4^{-}8^{-}$) and mature (i.e., $TCR-\alpha/\beta^{+}$) surface markers, it has been difficult to incorporate them into currently accepted schemes of thymocyte maturation.

The phenomenon of clonal deletion of thymocytes expressing autoreactive TCR $V\beta$ genes (5) has provided a useful new marker of thymocyte maturation and a unique opportunity to readdress the developmental origin of *lpr* and *gld* and normal $CD4^{-}8^{-}$ $TCR-\alpha/\beta^{+}$ cells. In a number of independent studies, tolerance-related $V\beta$ clonal deletions in I-E⁺ mice (6), in mice with an active *Mls* allele (*Mls^a*, *Mls^c*, or *Mls^d*, but not *Mls^b*) (7-11), or in male mice expressing an anti-HY TCR transgene (12), have all been shown to occur at the transition from the double-positive ($CD4^{+}8^{+}$) cortical to the single-positive ($CD4^{+}8^{-}$ or $CD8^{+}4^{-}$) medullary stage of thymocyte maturation, and importantly, to require the expression of the CD4 (13, 14) or CD8 (12) accessory molecules. Here we show by multiprobe RNase protection analysis that such tolerance-related $V\beta$ clonal deletions are also present in *lpr* and *gld* cells and at least a portion of the $CD4^{-}8^{-}$ $TCR-\alpha/\beta^{+}$ subset of normal mice, indicating their derivation by secondary loss of CD4/CD8 accessory molecules from $CD4^{+}8^{+}$ precursors. Available evidence suggests that such a CD4/CD8 loss pathway may preferentially select cells with autoreactive TCR specificities, but that escape conventional clonal deletion. The exportation of these cells in large numbers to the periphery might contribute to the induction of systemic autoimmune syndromes.

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Materials and Methods

Mice. The origin and immunopathologic characteristics of the various *lpr* and *gld* congenic mice used in the present study have been detailed (1). Spleen and lymph node cells were derived from 4–5-mo-old mice. 6–8-wk-old C3H/HeJ mice were used for thymocyte preparations.

Isolation of Double-Negative Cells. *lpr* and *gld* lymph node cell suspensions were subjected to two rounds of killing with mAb GK1.5 (anti-CD4) and mAb 3.168 (anti-CD8) plus guinea pig complement (Cedarlane Laboratories, Ontario, Canada), followed by purification on Ficoll gradients. Normal CD4⁻8⁻ thymocytes were prepared as described above and then stained with FITC-conjugated mAb 145-2C11 (anti-CD3) and examined and sorted into CD3⁺ and CD3⁻ subsets by flow cytometry using a FACStar flow cytometer.

RNAse Protection Assay. In this assay, sets of single-stranded, defined-length radiolabeled V β RNA probes are hybridized in solution to target mRNAs under conditions of large probe excess and high R₀t value, such that essentially all target mRNAs present become saturated with probe. After hybridization, excess probe is degraded by RNAse digestion, leaving the probe:mRNA duplex intact (i.e., protected). Protected probes are size-separated on polyacrylamide sequencing gels and autoradiographed, with the strength of the radioactive signal in a given band being directly proportional to the quantity of protected probe corresponding to that band, and hence to the quantity of target mRNA present in the original sample.

Assays were performed essentially as described by Melton et al. (15), with minor modifications. 5- μ g aliquots of cellular RNA, prepared by the micro-acid-guanidinium method (16), were lyophilized and dissolved in 4 μ l hybridization buffer (80% formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM Pipes, pH 6.7). Probes were prepared from mixtures of pGEM 3 templates (total 0.2 μ g template DNA) containing the appropriate V β inserts, using the Riboprobe Gemini system (Promega Biotec, Madison, WI), in 5- μ l reactions with 75 μ Ci (15 μ M) of α -[³²P]UTP as the labeled nucleotide, and after purification, were dissolved in hybridization buffer at $\sim 10^6$ cpm/ μ l. 1- μ l aliquots of probe were added to RNA samples in sterilized Eppendorf tubes, the solution was overlaid with liquid paraffin, and hybridization was allowed to proceed at 56°C for 16 h. Digestions were performed using 10 μ l of digestion buffer (10 mM Tris, pH 7.5/5 mM EDTA/0.3 M NaCl with RNAse A at 50 μ g/ml and RNAse T1 at 2 U/ μ l) per microgram of RNA for 1 h at 30°C. Samples were phenol extracted, ethanol precipitated, dissolved in sample buffer, and electrophoresed in standard polyacrylamide sequencing gels. Autoradiography of the dried gel was done on Kodak XRP film at -70°C with intensifying screens for ~ 24 h. Densitometric profiles from such autoradiographs were obtained using an LKB Ultrascan SL Densitometer.

Results

We first asked whether *lpr* and *gld* cells have undergone the tolerance-related V β clonal deletions appropriate to the background genotype on which they are found: i.e., V β 6 (7), V β 8.1 (8), and V β 7 (17) for *Mls^a*-positive AKR/J mice; V β 3.1 for *Mls^c*-positive C3H/HeJ mice (9–11); and partial deletion of V β 11-bearing thymocytes for I-E⁺ mice such as MRL, regardless of their *Mls* phenotype (17, 18; Table I). V β clonal deletions were detected at the RNA level using a multiprobe RNAse protection assay, which allows simultaneous quantitation of the relative levels of expression of multiple V β s in a single-tube hybridization reaction. Studies in our laboratory have documented that V β mRNA levels accurately reflect the percentages of V β -bearing cells detected by mAbs (not shown). A probe set consisting of probes for the V β 11, 7, 3.1, 6, and 8.1/8.2/8.3 genes was hybridized to total RNA extracted from either isolated double-negative *lpr* or *gld* lymph node cells, or from spleen cells of their normal congenics (as a control). From the autoradiographic result (Fig. 1) it can be seen that all appropriate tolerance-related V β clonal deletions were detected among *lpr* and *gld* cells. Thus, AKR/J (*Mls^a*) spleen and AKR-*lpr/lpr* double-

TABLE I
I-E/Mls Tolerance-related *V β* Clonal Deletions
 in *lpr* and *gld* Congenic Strains

	Haplotypes			<i>Vβ</i> deletions
	H-2	I-E	<i>Mls</i>	
AKR/J	k	+	a	6,7,8.1,11
C3H/HeJ	k	+	c	3.1,11
MRL- +/+	k	+	b	11
C57BL/6	b	-	b	None

negative lymph node cells were found to be essentially devoid of *V β* 6, 7, and 8.1 mRNA, whereas they expressed normal levels of the other *V β* s analyzed. Similarly, in C3H-*lpr/lpr* and *gld/gld* (*Mls^c*) lymph node cells, and C3H/HeJ spleen, *V β* 3.1 mRNA was selectively missing. MRL-*lpr/lpr* lymph node cells and MRL- +/+ spleen

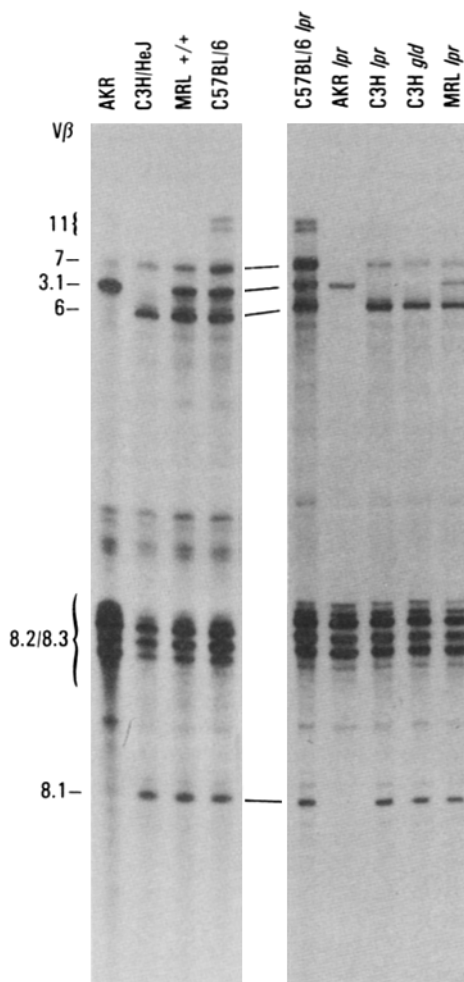


FIGURE 1. Detection of *V β* clonal deletions in abnormal *lpr* and *gld* lymph node cells on different genetic backgrounds. Total cellular RNA was prepared from splenocytes (*left*) and purified *CD4⁻8⁻* cells from the enlarged lymph nodes of *lpr/gld* mice (*right*) and analyzed by RNAse protection assay in a single-tube hybridization, using the *V β* probes indicated. Band intensities are proportional to the amount of each *V β* mRNA present in the sample. The multiple-banding patterns seen for the *V β* 8s and *V β* 11 were a consistent feature of the assay system. Unlabeled minor bands are artefacts caused by partial protection of some *V β* probes. Protected probe lengths: *V β* 11 (324nt), *V β* 7 (274nt), *V β* 3.1 (250nt), *V β* 6 (222nt), *V β* 8.2/8.3 (~95nt), and *V β* 8.1 (~66nt).

(*Mls^b*) expressed normal levels of V β 3.1, 6, 7, and 8.1/8.2/8.3, but shared a significant reduction in expression of V β 11 with the other I-E⁺ strains (i.e., AKR and C3H). As expected, the I-E⁻, *Mls^b* C57BL/6-*lpr/lpr* lymph node cells (and normal C57BL/6 spleen), included as a control, showed no *Mls*-associated clonal deletions or reduction of V β 11 mRNA. Thus, the abnormally proliferating *lpr* and *gld* cells are derived from a thymocyte population that has already undergone tolerance-related V β clonal deletions.

We next turned to similar analysis of the minor CD4⁻⁸⁻ TCR- α/β^+ subset found in normal mice. Double-negative thymocytes from 6–8-wk-old C3H/HeJ mice were prepared by two rounds of killing with anti-CD4 and anti-CD8 plus complement, resulting in a CD4⁻⁸⁻ population that was <1% contaminated with single- or double-positives. Such double-negative thymocytes (~5% of the total) can be further subdivided with respect to expression of the TCR-associated CD3 marker, having: (a) a CD3⁺ subset, consisting of TCR- α/β^+ CD4⁻⁸⁻ cells (the subject of this investigation) and TCR- γ/δ^+ CD4⁻⁸⁻ cells; and (b) a CD3⁻, TCR⁻ subset, made up of the earliest known thymocyte precursors (4). To enrich our double-negative thymocyte preparation for CD4⁻⁸⁻ TCR- α/β^+ cells, we stained and sorted the cells with

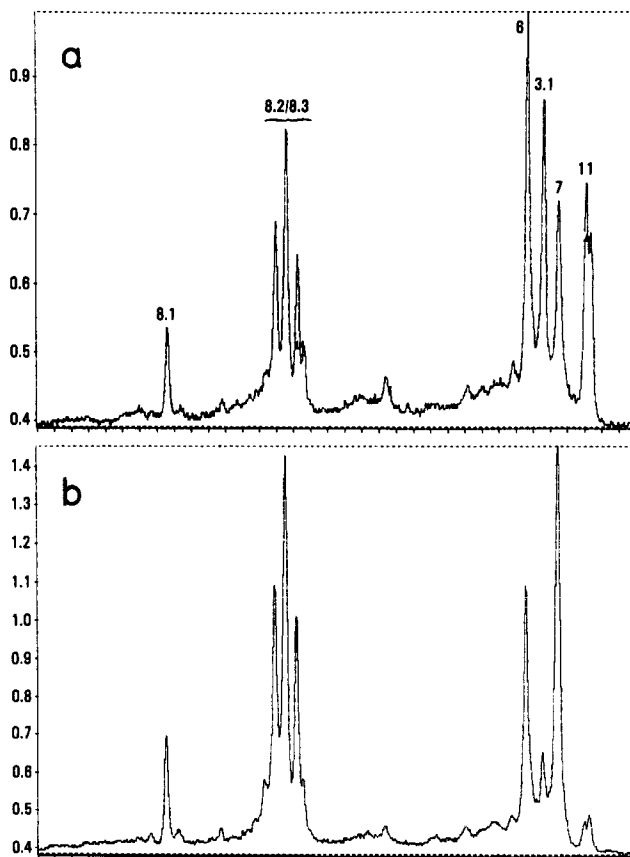


FIGURE 2. Detection of V β 3.1 and V β 11 clonal deletions in the normal CD4⁻⁸⁻ TCR- α/β^+ subset of C3H/HeJ mice. Total RNA was prepared from sorted CD4⁻⁸⁻CD3⁻ (a) and CD4⁻⁸⁻CD3⁺ (b) cell populations, and 2- μ g aliquots analyzed by RNase protection assay. Shown are densitometric tracings of the resulting autoradiograph, prepared using an LKB Ultrosan XL Densitometer. Numbers above peaks refer to respective V β gene products.

FITC-labeled anti-CD3 and obtained a relatively high yield of 40% CD3⁺ cells with negligible contamination by CD3⁻ cells (data not shown).

Total RNA was prepared from the FACS-sorted double-negative CD3⁺ cells and CD3⁻ cells (as a control), and hybridized to the same V β probe set used earlier for the *lpr* and *gld* cells. Densitometric tracings of the results of this analysis are shown in Fig. 2. Since the immature CD4⁻8⁻ CD3⁻ thymocyte population expresses only cytoplasmic, and hence unselected, β chain mRNA, this RNA sample should reveal a V β expression pattern unaffected by clonal deletions. As shown in Fig. 2 *a*, this was indeed the case. In striking contrast, however, the profile obtained from the CD4⁻8⁻ CD3⁺ population (Fig. 2 *b*) revealed clear deletion of the majority of V β 3.1- (*Mls*⁻-reactive) and V β 11- (I-E-reactive) bearing thymocytes in these C3H/HeJ mice. We therefore conclude that a significant portion of (but not all) TCR- α/β -bearing cells in the CD4⁻8⁻ CD3⁺ subset of normal mice, like their *lpr* and *gld* counterparts, are derived from thymocytes that have previously undergone tolerance-related clonal deletions.

These experiments revealed substantial V β 3.1 clonal deletion in 6–8-wk-old C3H/HeJ mice where an unusually high proportion (~40%) of cells isolated from the CD4⁻8⁻ population were CD3⁺. Of interest, in other preliminary experiments in our laboratory using the *Mls*^a/I-E⁺ strain AKR/J, where the proportion of CD3⁺ cells in the double-negative population was lower, we detect somewhat less clonal deletion of V β 6⁺ and V β 11⁺ thymocytes (data not shown); and other investigators have similarly reported equivocal clonal deletion of V β 6⁺ cells in the related B2A2⁻ CD4⁻8⁻ population of BALB.D2 *Mls*^a mice (19). We would suggest that such variable findings are not unexpected, and simply reflect known strain- and age-dependent differences in the size of the CD4⁻8⁻ TCR- α/β ⁺ subset (4). The apparent relationship between the level of V β clonal deletion and the proportion of CD4⁻8⁻ CD3⁺ cells in our experiments is consistent with this possibility.

Discussion

The presence of I-E- and *Mls*-related V β clonal deletions in *lpr* and *gld* cells, together with the established requirement for CD4 participation in V β clonal deletions (13, 14), supports the conclusion that these abnormal cells arise through a novel CD4/CD8 loss pathway of late thymic differentiation. That is, at or subsequent to the double-positive stage in which tolerance-related clonal deletions occur, cells in this lineage must in some way be induced to downregulate both CD4 and CD8 accessory molecules and become, in effect, "secondary" double-negative, but TCR- α/β ⁺. An alternative explanation, i.e., that these cells represent expansion of a TCR- α/β ⁺ subset that never expressed CD4/CD8 is generally not supported by current evidence, including (*a*) the inability to detect such CD4⁻8⁻ TCR- α/β ⁺ cells concurrently with the appearance of the earliest CD4⁺8⁺ cells (4), and (*b*) the aforementioned studies indicating the requirement of accessory molecules for clonal deletion (12–14). With regard to *lpr* cells, a similar conclusion has also recently been reached by Kotzin et al. (20), who demonstrated tolerance-related V β clonal deletions using anti-V β -specific antibodies, and is further supported by earlier experiments by these investigators (21) in which in vivo treatment of MRL-*lpr/lpr* mice with anti-CD4 antibody inhibited the accumulation of abnormal CD4⁻8⁻, TCR- α/β ⁺ cells.

Since a portion of the normal CD4⁻8⁻ TCR- α/β ⁺ subset is also shown here to express tolerance-related V β clonal deletions, we conclude that it too derives from the putative CD4/CD8 loss pathway. This possibility has been raised previously (4), and is consistent with observations indicating that these cells represent a late rather than early stage in T cell development (4). The detection of V β clonal deletions in these cells also strengthens their proposed lineage relationship to *lpr* and *gld* cells (2). It is interesting, however, that the double-negative TCR- α/β ⁺ subset in normal mice is known to be heterogeneous, with the apparent contribution of thymocytes having an earlier stage surface phenotype (22). Indeed, the lack of complete V β 3.1 deletion in our present experiments supports the possibility that other, pre-tolerized, TCR- α/β ⁺ populations contribute to this subset; however, formal demonstration of this will probably require complete removal of TCR- γ/δ ⁺ cells from analyzed populations, since a portion of γ/δ -bearing cells may also express cytoplasmic, and hence unselected, 1.3-kb β chain mRNA (22).

What might the factors be that induce the downregulation of CD4/CD8 in the CD4⁻8⁻ TCR- α/β ⁺ lineage? Current concepts on the mechanism of positive and negative selection offer several possibilities, which we have recently proposed in detail elsewhere (17). Concerning the function of CD4/CD8 downregulation in post-negative selection thymocyte lineages (i.e., *lpr* and *gld* or clonally deleted normal CD4⁻8⁻ TCR- α/β ⁺), we suggest that the recently reported findings of Kisielow et al. (12) on the fate of autoreactive T cells expressing an anti-HY TCR transgene in male mice may provide an important clue. In these experiments, "conventional" tolerance-related clonal deletion of CD8⁺ anti-HY TCR-bearing thymocytes was observed (CD8 being required for efficient HY-antigen recognition). In addition, however, significant numbers of peripheral transgene-bearing T cells, which apparently escaped the clonal deletion process, were detected, and importantly, found to be largely (58%) of the double-negative phenotype. These findings provide a logical connection between autoreactive cells and putative tolerance-induced accessory molecule downregulation, and therefore suggest a model in which CD4/CD8 loss serves as a novel, "back-up" form of negative selection of autoreactive thymocytes, distinct from conventional clonal deletion.

In advancing the above concepts, therefore, it might be suggested that double-negative TCR- α/β ⁺ *lpr* and *gld* cells and their normal counterparts have essentially autoreactive TCR specificities. In the normal setting, the few moderately self-reactive cells that escape clonal deletion, but downregulate their accessory molecules, will slowly accumulate in the thymus to constitute a portion of the minor late-developing, double-negative TCR- α/β ⁺ population. Some of these cells might migrate to the periphery, but their numbers will be insufficient to exert detectable adverse effects. In contrast, such cells, when massively produced and exported into the periphery as in *lpr* or *gld* homozygous mice, could clearly enhance autoimmune manifestations by virtue of either: (a) their retention of sufficient autoreactivity, in spite of their lack of CD4/CD8 (23); (b) secretion of appropriate B cell differentiation promoting lymphokines, as in fact has been shown for both *lpr* and normal double-negative TCR- α/β ⁺ cells (24, 25); or (c) their subsequent reacquisition of accessory molecules to become fully functional and autoreactive (25-27). Despite their postulated common developmental origin and functional status, it should be noted that the *lpr* and *gld* mutations are independent (17).

The above-postulated autoreactive potential for double-negative TCR- α/β^+ cells is further supported by the induction of autoimmune disease by transfers of such cells isolated from normal BALB/c spleen into syngeneic nude mice (28), or thymic cells from cyclosporin-treated mice severely depleted of CD4 $^+$ 8 $^-$ and CD8 $^+$ 4 $^-$ cells (29). Furthermore, in (NZB \times W)F $_1$ lupus mice (30), and in humans with lupus (31), substantial in vitro helper activity for anti-DNA production was found in CD4 $^-$ 8 $^-$ spleen and peripheral T cells, respectively. Moreover, >10-fold increases in such cells were found in peripheral blood of lupus patients compared with normals (32). Interestingly, murine strain-specific differences in the proportion of double-negative TCR- α/β^+ cells have been noted (2, 4, 25, 33). Such genetically imposed expansions of these bona fide autoreactive double-negative TCR- α/β^+ cells may contribute in an important way to the induction of murine, and even human systemic autoimmune disease.

Summary

We have analyzed tolerance-related clonal deletion of *Mls*- and I-E-reactive thymocytes at the RNA level using a multi-V β probe RNase protection assay, and used this phenomenon to identify the maturation stage of the abnormally expanded CD4 $^-$ 8 $^-$, TCR- α/β^+ subset in *lpr* and *gld* homozygous mice, and of the phenotypically similar minor thymocyte subset found in normal mice. Essentially complete V β clonal deletions were detected in *lpr* and *gld* cells of all appropriate background strains. Substantial, but not complete, V β clonal deletions were also detected in the CD4 $^-$ 8 $^-$ TCR- α/β^+ subset of normal mice. Since expression of CD4/CD8 is required for V β clonal deletions to occur, we conclude that *lpr* and *gld* cells, and at least a portion of CD4 $^-$ 8 $^-$ TCR- α/β^+ thymocytes in normal mice, are derived by secondary loss of CD4/CD8 accessory molecules from more mature CD4 $^+$ 8 $^+$ precursors. One possible interpretation of these findings is that such CD4/CD8 loss may affect a class of self-reactive thymocytes that have escaped direct clonal deletion. Exportation and expansion of such cells in the periphery may be an important contributory factor in the induction of systemic autoimmunity.

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