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T-Lymphocyte Downregulation after Acute Viral Infection Is Not Dependent on CD95 (Fas) Receptor-Ligand Interactions

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Infection of mice with lymphocytic choriomeningitis virus (LCMV) causes a major expansion of $CD8^+$ T cells followed by a period of immune downregulation that coincides with the induction of lymphocyte apoptosis in the mouse spleen. CD95 (Fas) and its ligand are important for regulating peripheral T-lymphocyte numbers and can mediate apoptosis of mature T lymphocytes. We infected CD95- and CD95L-deficient mice (*lpr* and *gld*, respectively) with LCMV to determine if the immune downregulation that occurred following resolution of the LCMV infection was due to a CD95-dependent apoptotic mechanism. Lymphocytes from LCMV-infected *lpr* and *gld* mice were capable of normal T-cell expansion and cytolytic function but were, in contrast to activated cells from normal virus-infected mice, relatively more resistant to T-cell receptor-induced apoptosis in vitro. However, in vivo there were significant numbers of apoptotic cells in the spleens of *lpr* and *gld* mice recovering from the infection, and the T-cell number and cytolytic activity decreased to normal postinfection levels. Thus, CD95 is not required for the immune downregulation of the $CD8^+$-T-lymphocyte response following acute LCMV infection.

Viruses that cause generalized systemic infections lead to immune activation that can be characterized by a period of lymphocyte proliferation followed by a period of immune downregulation after the virus has been cleared. Only a small fraction of antigen-specific lymphocytes generated during clonal expansion survive this immune downregulation process and remain as providers of long-term immunologic memory (13, 26). We have recently demonstrated apoptotic T and B lymphocytes in the spleens of mice after the clearance of lymphocytic choriomeningitis virus (LCMV) and coincident with the decline in splenic T-cell numbers (18). This indicates that apoptosis of antigen-activated T cells is involved in the silencing of the immune response.

Mature activated lymphocytes receive a signal(s) to undergo apoptotic cell death from the surrounding tissue stroma or culture media. This apoptotic signal can be delivered either through the T-cell receptor (TCR) or by other means, such as growth factor withdrawal or cytokine signaling (6, 14, 35). Sustained strong signaling through the TCR of mature activated T cells can lead to apoptosis, an event termed activation-induced cell death (AICD) (24), and Fas and Fas ligand (CD95 and CD95L) interactions can mediate this process (1, 2, 4, 8, 11, 22, 23, 27, 32). Although CD95 is weakly expressed on resting T cells, its level of expression increases 10-fold on activated T cells; CD95 ligand can be found only on activated T cells (5, 17, 29, 34). The sensitivity of T cells to TCR-CD95-mediated apoptosis does not develop until 3 to 4 days after initial stimulation, implying that only continuously activated T cells are sensitive to this mechanism of cell death (17). Deficiencies in either CD95 or CD95L result in lymphoproliferative syndromes in mice and humans (7, 21, 30, 33), suggesting that these molecules are important for regulating peripheral T-lymphocyte numbers. Evidence to support the role of CD95 in controlling the number of antigen-specific T cells comes from the studies of lymphocyte responses in TCR-transgenic mice bred onto a wild-type or CD95-deficient background (27). When TCR-transgenic mice were challenged with the peptide specific for the TCR, the transgenic $CD4^+$ cells in the CD95-positive mouse were deleted in the periphery, whereas the transgenic $CD4^+$ cells in the CD95-deficient mice were not deleted (27).

We questioned whether the apoptosis and immune downregulation that occur following resolution of the LCMV infection of mice was due to a CD95-dependent mechanism. Using the terminal nucleotidyl transferase assay to detect apoptotic cells, we previously demonstrated that the reduction in both T- and B-cell numbers after LCMV infection correlates with high levels of apoptosis in the spleen (18). There were also significant numbers of apoptotic cells 10 days after LCMV infection in the spleens of mice deficient in CD95 (18). In this study we have undertaken a more detailed kinetic analysis of lymphocyte activation and downregulation during LCMV infection of wild-type and of CD95- and CD95L-deficient mice (*lpr* and *gld*, respectively). We show here that the T cells from the LCMV-infected *lpr* and *gld* mice were impaired in their ability to undergo AICD in vitro when stimulated with antibody to the TCR, but there were significant numbers of apoptotic cells in the spleens of the mice recovering from infection, and the immune response was silenced effectively. This suggests that neither AICD nor CD95-CD95L (Fas-FasL) mechanisms are required for the downregulation of the lymphocyte response to viral infection.

T lymphocytes in LCMV-infected *lpr* and *gld* mice expand and decline in numbers and activity with kinetics similar to those in control mice. The susceptibility of wild-type, CD95-deficient, and CD95L-deficient mice to LCMV infection was measured by examining lymphocyte phenotypic changes and cytotoxic-T-lymphocyte (CTL) activity over time after infection. Seven- to ten-week old male *H-2*^{b} C57BL/6J, *B6.*MRL-*Fas*^{lpr} (CD95-deficient), or B6Smn.C3H-*Fasl*^{gld} (CD95L-deficient) mice (Jackson Laboratory, Bar Harbor, Maine) were infected intraperitoneally with 5×10^4 PFU of LCMV, strain

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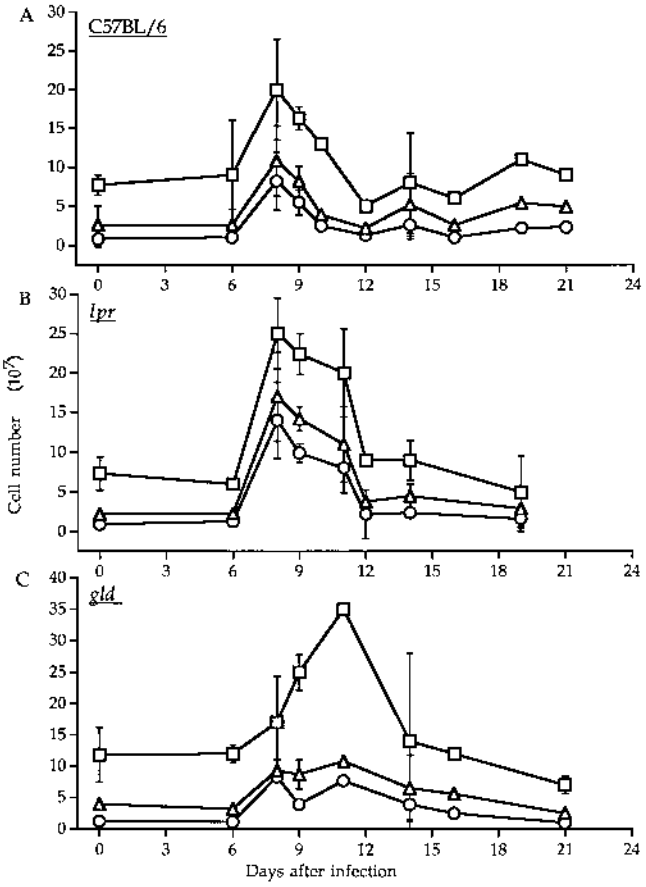

FIG. 1. Changes in the total splenic cell number, $CD3^+$-lymphocyte number, and $CD8^+$-lymphocyte number during LCMV infection were measured in C57BL6 (A), *lpr* (B), and *gld* (C) mice. The means ± standard deviations of total splenic leukocyte (squares), $CD3^+$ T cells (triangles), and $CD8^+$ T cells (circles) are plotted. A minimum of two mice per group were tested. Values for C57BL/6 mice were from a pool of two spleens. Values for the *lpr* and *gld* mice were averaged from a minimum of two individuals' spleens.

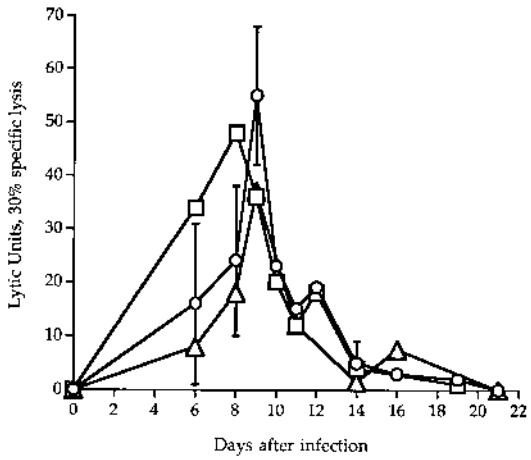

FIG. 2. Primary splenic CTL activity in C57BL/6 (circles), *lpr* (squares), and *gld* (triangles) mice infected with LCMV. CTL activity is expressed as mean lytic units per 10^6 splenic leukocytes. One lytic unit was defined as the number of lymphocytes necessary to obtain 30% specific lysis of 10^4 target cells (10). Each symbol represents at least two spleens per group. The standard deviation of the mean CTL response from C57BL/6 mice is shown for time points with more than two spleens per group.

Armstrong. Uninfected age- and sex-matched mice served as controls in each experiment. The percentages of splenic $CD3^+$ and $CD8^+$ T lymphocytes were identified by double surface immunofluorescence staining with anti-mouse CD3 conjugated with phycoerythrin (Gibco, Grand Island, N.Y.) and anti-mouse CD8 conjugated with fluorescein isothiocyanate (Gibco). The changes in splenic lymphocyte populations for C57BL/6, *lpr*, and *gld* mice are shown in Fig. 1. There was approximately a threefold expansion of the splenic leukocyte number in the C57BL/6 and *lpr* mice by 8 days postinfection (p.i.), and in both strains it declined to preinfection levels by 14 days p.i. (Fig. 1A and B). $CD8^+$ T cells contributed to the majority of the T-cell expansion in these mice. In the *gld* mice, the peak splenocyte expansion occurred by 12 days p.i., and the $CD8^+$ T cells constituted a smaller percentage of the total splenocyte expansion (Fig. 1C). Despite the differences in kinetics, the total splenic leukocyte, total T-cell, and $CD8^+$ T-cell numbers expanded and declined in all three strains of mice.

Primary CTL activity was measured on various days after LCMV infection against syngeneic LCMV-infected target cells (20). The LCMV-specific cytolytic activity peaked in the C57BL/6 mice 9 days after infection (Fig. 2). With the exception of a slightly accelerated CTL response in the *lpr* mice 6 to 8 days after infection, the kinetics of the rise and fall of the CTL response against LCMV-infected targets were similar in the infected *lpr* and *gld* mice compared with infected C57BL/6 mice. Consistent with the CTL response reported here, Kägi et al. found comparable clearance of virus in C57BL/6, *lpr*, and *gld* mice by 8 days after infection with LCMV-WE (12). Thus, the expansion, differentiation, and decline of antiviral T cells occur normally in the absence of CD95 receptor-ligand interactions.

T lymphocytes from LCMV-infected *lpr* and *gld* mice are partially resistant to AICD in vitro. The sensitivity to AICD of T cells from LCMV-infected mice was determined by the combined methods of lymphocyte proliferation in response to stimulation by plate-bound anti-CD3 and by the detection of apoptotic cells in these cultures by flow cytometry. Splenic T lymphocytes were enriched by nylon wool purification, and dendritic cells were isolated from the nonadherent cell fraction collected after overnight incubation of splenic adherent cells (19). Tissue culture plates (48-well plates; Falcon) were coated overnight with phosphate-buffered saline (PBS) or anti-CD3 monoclonal antibody (clone 145-2C11) diluted 1:1,000 in PBS. Anti-CD3 was prepared from an NH_4SO_4-precipitated ascitic fluid, and the dose to be used was selected after titration on naive and activated T cells. Proliferative responses were determined from 2×10^6 purified T cells cultured 48 h in the presence of dendritic cells in the anti-CD3-coated plates. Individual wells were resuspended gently, and three aliquots of 200 μl each were transferred to a 96-well flat-bottomed plate (Falcon) for incubation with 1 μCi of [^{3}H]thymidine (Amersham, Arlington Heights, Ill.) per well for 6 h prior to harvesting. Aliquots from the wells sampled for [^{3}H]thymidine uptake were used to determine the percentages of apoptotic cells as identified by their reduced forward light scatter and uptake of propidium iodide (PI) (19, 31). Unfixed cells were stained with 20 μg of PI (Sigma, St. Louis, Mo.) per ml and analyzed immediately on a FACStar Plus (Becton Dickinson, San Jose, Calif.).

Table 1 shows the results from six independent experiments. In experiments 1 and 2 and experiments 4 and 5, T lymphocytes from C57BL/6 mice taken 8 days p.i., at the peak of the

TABLE 1. Resistance of T cells from LCMV-infected *lpr* and *gld* mice to activation-induced cell death in vitro

Expt	Mouse strain	Culture condition	Proliferation			Apoptosis		
			Rate[a] on p.i. day		Fold change[b]	Rate[c] on p.i. day		Fold change[d]
			0	8		0	8	
1	C57BL/6	Medium	72 ± 46	340 ± 110				
		Anti-CD3	18,455 ± 1,870	10,823 ± 2,361	−1.7	1.0	6.6	+6.6
	B6 *lpr*	Medium	55 ± 14	170 ± 98				
		Anti-CD3	12,659 ± 2,212	19,387 ± 1,505	+1.5	1.2	5.3	+4.4
2	C57BL/6	Medium	88 ± 18	158 ± 42				
		Anti-CD3	62,391 ± 17,920	28,838 ± 8,198	−2.6	0.6	4.9	+8.2
	B6 *lpr*	Medium	120 ± 29	153 ± 8				
		Anti-CD3	67,014 ± 13,214	73,295 ± 22,885	+0.1	0.8	1.6	+2.0
3	C57BL/6	Medium	ND[e]	ND				
		Anti-CD3	ND	ND		1.7	3.0	+1.7
	B6 *lpr*	Medium	ND	ND				
		Anti-CD3	ND	ND		2.1	2.2	+1.1
4	C57BL/6	Medium	62 ± 7	79 ± 25				
		Anti-CD3	36,175 ± 4,976	16,703 ± 1,978	−2.2	1.5	5.5	+3.7
	B6 *gld*	Medium	43 ± 5	53 ± 13				
		Anti-CD3	36,971 ± 4,188	27,540 ± 7,062	−1.3	2.3	3.6	+1.5
5	C57BL/6	Medium	118 ± 32	3,068 ± 111				
		Anti-CD3	87,599 ± 20,029	33,656 ± 8,007	−2.6	2.0	4.5	+2.2
	B6 *gld*	Medium	63 ± 18	929 ± 65				
		Anti-CD3	91,293 ± 1,460	66,596 ± 1,431	−1.3	1.7	3.1	+1.8
6	C57BL/6	Medium	ND	ND				
		Anti-CD3	ND	ND		1.2	3.3	+2.7
	B6 *gld*	Medium	ND	ND				
		Anti-CD3	ND	ND		1.2	1.9	+1.6

[a] Presented as the mean counts per minute ± 1 standard deviation of [^{3}H]thymidine incorporation. Two mice were tested per group in each experiment. The results for the C57BL/6 mice are generated from the pool of two mice per group. The results for the *lpr* and *gld* mice are averaged from the values for individual mice (experiments 1 and 4) or from the pool of two mice per group (all others).

[b] Fold change in the proliferative response to anti-CD3, calculated as day 8 counts per minute divided by day 0 counts per minute.

[c] Ratio of apoptotic cells to viable cells as determined by the ratio of PI^{high} uptake (>10 mean fluorescence units) to PI^{low} uptake (<10 mean fluorescence units). Two mice were tested per group in each experiment. The results for the C57BL/6 mice are generated from the pool of two mice per group. The results for the *lpr* and *gld* mice are averaged from the values from individual mice (experiments 1 and 4) or from the pool of 2 mice per group (all others).

[d] Fold change in apoptosis, calculated as level of death in anti-CD3-stimulated cultures on day 8 divided by the level of death in anti-CD3-stimulated cultures on day 0.

[e] ND, not done.

in vivo T-cell expansion, had 1.7- and 2.6-fold reductions in thymidine uptake upon anti-CD3 stimulation compared with lymphocytes from uninfected C57BL/6 mice. In addition to the reduction of proliferation on day 8, there were 1.7- to 6.6-fold more apoptotic cells than viable cells in the anti-CD3-treated cultures. In contrast to the C57BL/6 mice, anti-CD3 stimulation of the day 8 T cells from the *lpr* mice had no negative effect on proliferation compared with the response of day 0 cells from the *lpr* mice (experiments 1 and 2). The direct levels of apoptosis in the cultures of anti-CD3-stimulated T cells taken from the day 8 *lpr* mice were always lower than the levels observed in cultured lymphocytes from control day 8 C57BL/6 mice (5.3 versus 6.6, 1.6 versus 4.9, and 2.2 versus 3.0). In experiments 4 through 6, anti-CD3-stimulated lymphocytes from the day 8 *gld* mice had a 1.3-fold reduction in proliferation, but this was less inhibition than that seen with C57BL/6 T cells in the same experiment. As observed in the *lpr* mice, the direct levels of apoptosis in the *gld* mice were lower than the levels observed in the C57BL/6 control cultures (3.6 versus 5.5, 3.1 versus 4.5, and 1.9 versus 3.3). Thus, activated lymphocytes from LCMV-infected *lpr* and *gld* mice were resistant to, but not completely protected from, triggering of apoptosis through the mechanism of AICD.

Significant numbers of apoptotic cells are present in the

TABLE 2. Detection of apoptosis in situ in LCMV-infected C57BL/6, *lpr*, and *gld* mice

No. of days after LCMV infection	No. of apoptotic nuclei/HPF[a]/spleen (n)[b]		
	C57BL/6	B6 *lpr*	B6 *gld*
0	33 ± 14 (8)	29 ± 10 (5)	46 ± 14 (3)
8	73 ± 20 (9)	73 ± 24 (6)	71 ± 38 (4)
11	123 ± 56 (5)	78 ± 18 (6)	84 ± 21 (4)
14	84 ± 25 (5)	93 ± 35 (4)	78 ± 16 (8)
19	45 ± 22 (3)	26 (1)	45 (1)

[a] Total magnification of high-powered field (HPF) × 1,000.

[b] The total number of C57BL/6 spleens include previously published values from three mice infected for 0, 8, 11, and 14 days (18). The total number of B6 *lpr* spleens include previously published values from two mice infected for 0 and 11 days (18). For all mouse strains, values on days 8, 11, and 14 were found to be significantly different ($P < 0.001$) from those on day 0 based on a two-factor analysis of variance and a multiple-comparison analysis using Tukey's honestly significant difference test.

spleens of mice recovering from infection. DNA fragmentation associated with apoptotic cells in the spleens of infected mice was detected by in situ hybridization of a digoxigenin-labeled probe to the free 3′-OH ends of DNA using the ApopTag kit (Oncor, Gaithersburg, Md.), according to the manufacturer's instructions. The mean number of apoptotic nuclei per high-powered field (total magnification, ×1,000) was generated by counting apoptotic nuclei per tissue section and expressing this value per high-powered field. Statistically, the means per high-powered field were normally distributed when analyzed for assumption of normality with the Kolmogorov-Smirnov one-sample test. The data were then tested for significance with a two-factor analysis of variance. Significant differences between individual means were determined by a stringent multiple-comparison test, Tukey's honestly significant difference (3). We have previously shown that LCMV-infected C57BL/6 mice have a peak incidence of splenocyte apoptosis on day 11 p.i., and this declines to preinfection levels by 14 days p.i. (18). As shown in Table 2, spleens from LCMV-infected C57BL/6, *lpr*, and *gld* mice all had a significant 1.8- to 3.7-fold increase in the number of apoptotic cells identified on days 8, 11, and 14 p.i. compared with spleens from uninfected mice or mice 19 days p.i. ($P < 0.001$). However, there was no significant difference between the groups of mice, indicating that the incidence of apoptosis in the spleens of mice recovering from LCMV infection is independent of the presence of CD95 and its ligand.

lpr mice are impaired in their ability to eliminate antigen-activated $CD4^+$ lymphocytes in a TCR-transgenic system (27), and, as a consequence, we predicted that lymphocytes that proliferated in response to LCMV infection would persist in these mice. However, we have shown here that T cells from LCMV-infected *lpr* and *gld* mice were capable of clearing the infection and that the immune response was downregulated in a manner comparable to that observed in wild-type mice. Thus, CD95-CD95L (Fas-FasL) interactions are not necessary for either the antigen-specific expansion of T cells, the decline in T-cell numbers, or the induction of apoptosis in vivo.

A recent report has shown that apoptosis of mature activated lymphocytes after signaling through the TCR can occur through two possible mechanisms that segregate by lymphocyte subset. $CD4^+$ lymphocytes are more susceptible to CD95-mediated apoptosis, whereas TNF-TNF receptor interactions could substitute for the CD95-CD95L system for induction of apoptosis of some activated $CD8^+$ lymphocytes (35). Although the focus of our study was on $CD8^+$ lymphocytes, we did not observe an enhanced number of $CD4^+$ lymphocytes, i.e., $CD3^+$ $CD8^-$ lymphocytes, in the LCMV-infected *lpr* and *gld* mice (Fig. 2). Apoptosis of $CD8^+$ cells in vivo in the *lpr* and *gld* mice may be compensated by the TNF pathway in the absence of CD95. The contribution of TNF to immune silencing of LCMV was not investigated in this study. These observations may indicate that there are multiple pathways for the induction of apoptosis in vivo of antigen-activated T lymphocytes, and the CD95-CD95L system is not required for silencing of either the T- or the B-cell response to infection.

AICD has been postulated as a mechanism for the removal of activated lymphocytes once the antigen has been cleared from the host (9, 15, 28). Induction of AICD, however, requires strong stimulation through the TCR (24). In the LCMV infection examined here, detectable viral antigen is mostly cleared 2 to 3 days prior to the decline in T-cell number and the major apoptotic event. The lack of sufficient viral antigen to trigger AICD and the result that *lpr* and *gld* mice, whose T cells are resistant to AICD, are still capable of immune downregulation suggest that AICD is not the mechanism for eliminating activated T cells following resolution of acute LCMV infection. Recent detailed analysis of LCMV-specific CTL precursors per $CD8^+$ T cell demonstrates that the frequencies and specificities of the CTL precursors are only marginally affected by the decline in T-cell number during immune silencing, suggesting that elimination of activated T cells is not TCR specific (25). The immunological setting in which AICD may be involved in the deletion of activated T cells occurs under conditions of very high antigen load and results in clonal exhaustion (16). After mice are infected with a high dose of a disseminating strain of LCMV, both primary and memory CTL are eliminated, and viral replication persists (16). It would be interesting to investigate the difference between the apoptotic mechanism of immune downregulation after viral clearance and the mechanism that leads to clonal exhaustion under conditions of viral persistence.

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