

Apoptosis Mediated by Fas but Not Tumor Necrosis Factor Receptor 1 Prevents Chronic Disease in Mice Infected with Murine Cytomegalovirus

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Abstract

The role of Fas- and TNF-receptor 1 (TNF-R1)-mediated apoptosis in the clearance of virally infected cells and in the regulation of the immune response was analyzed after murine cytomegalovirus (MCMV) infection of C57BL/6 (B6)-+/+ mice, Fas-mutant B6-*lpr/lpr* mice, TNF-R1 knockout B6-*tnfr^{0/0}* mice, and double-deficient B6-*tnfr^{0/0} lpr/lpr* mice. There was approximately equivalent clearance of MCMV in B6-+/+, B6-*tnfr^{0/0}*, and B6-*lpr/lpr* mice, and by day 28 no infectious virus could be detected in the liver, kidney, lung, or peritoneal exudate. However, delayed virus clearance was observed in B6-*tnfr^{0/0} lpr/lpr* mice. An acute inflammatory response occurred in the liver, lung, and kidney of all mice, which was most severe 7 d after MCMV infection, but resolved by day 28 in B6-+/+ and B6-*tnfr^{0/0}* mice, but not in B6-*lpr/lpr* or B6-*tnfr^{0/0} lpr/lpr* mice. These results indicate that apoptosis mediated by either Fas or TNF-R1 is sufficient for rapid clearance of the virus. However, apoptosis induced by Fas, but not TNF-R1, is required for the downmodulation of the immune response to the virus and prevention of a chronic inflammatory reaction. (*J. Clin. Invest.* 1998. 102:1431–1443.) **Key words:** apoptosis • MCMV • Fas • *lpr* mice • TNF-R1 knockout mice

Introduction

Infection with human cytomegalovirus (CMV)¹ leads to widespread disease in immunocompromised patients including

hepatitis, pneumonitis, nephritis, retinitis, encephalitis, and colitis. Severe opportunistic CMV infections are especially common in transplant recipients and HIV-infected patients and are associated with significant mortality (1). Murine CMV (MCMV) has homology to certain regions of CMV (2) and MCMV infection in mice exhibits a similar course of infection to that in humans with regard to the establishment of acute and chronic infection, viral persistency and latency, and the host immune response (3, 4).

MCMV infection induces a primary immune response within the host involving macrophages, natural killer cells, and T and B lymphocytes (5–9). However, diverse mechanisms have evolved in MCMV, as well as other herpes viruses, to promote escape from immune surveillance and to enhance establishment of a latent infection (10–14). Prevention of apoptosis in the host cell has been proposed to be one important strategy by which viruses overcome the immune response (11, 15, 16). This concept is supported by the recent identification of an increasing number of virus-encoded proteins that can inhibit different pathways of apoptosis in vitro including p53/pRb, the bcl-2 family, and molecules of the tumor-necrosis-factor receptor (TNF-R) superfamily, notably Fas (CD95/Apo-1) and TNF-R1 (CD120a) (11, 15). Inhibition of these pathways may not only prevent viral-induced apoptosis, but also block the cytotoxic T cell response mediated by TNF- α , Fas ligand, and perforin (17–20). These data suggest that apoptosis induced by Fas and TNF-R1 is important for limiting virus replication and spreading and is regulated by both viral proteins and the host defense system.

The physiological importance of Fas-mediated apoptosis became evident through the identification of the *lpr* mutations of the *fas* gene and the *gld* mutation of the *fas ligand* gene, which lead to lymphoproliferation and systemic autoimmune disease in mice homozygous for these mutations (21). Although all strains of mice that are homozygous for the *lpr* gene develop autoimmune disease, the disease phenotype and severity also depend on the genetic background and environmental factors (21, 22). C57BL/6 (B6)-*lpr/lpr* mice develop a milder autoimmune disease of later onset. They exhibit glomerulonephritis after ~6–8 mo of age but do not develop significant lung or liver disease (23). TNF-R1 and Fas show homology and share a homologous intracellular death domain that uses some of the same cytoplasmic signaling proteins (24, 25). Interaction of TNF- α with TNF-R1 and TNF-R2 plays a bifunctional role during an immune response since TNF- α is involved in the clearance of cells either through direct lysis (26, 27), acting as a proinflammatory cytokine (28), or by synergism with antiviral activity of other cytokines including IFN- γ (29). Also, previous data indicate that TNF- α is very important in promoting the inflammatory process after infection with

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1. Abbreviations used in this paper: 7-AAD, 7-amino-actinomycin D; AICD, activation-induced cell death; B6, C57BL/6; CMV, cytomegalovirus; ds, double strand; LCMV, lymphocytic choriomeningitis virus; *lpr* mice, lymphoproliferation mutation mice; MCMV, murine CMV; RF, rheumatoid factor; TCR, T cell receptor; TDT, terminal deoxynucleotidyl transferase; TNF-R1, TNF receptor 1; *tnfr^{0/0}* mice, TNF-R1 knockout mice; TUNEL, TDT-mediated dUTP nick end labeling.

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MCMV (8). Both TNF-R1 and Fas have been shown to mediate activation-induced cell death (AICD) in T cells, B cells, and macrophages (19, 30–35). Fas and TNF-R1 double-deficient mice exhibit accelerated lymphoproliferation and increased autoimmune disease, which is associated with high mortality, indicating that the Fas and TNF-R1 signaling and apoptosis pathways may compensate for each other (23).

Previous studies using the murine model of lymphocytic choriomeningitis virus (LCMV) infection and mice carrying T cell receptor (TCR) transgenes suggest that the Fas–Fas ligand interaction is not required for peripheral deletion of CD8⁺ T cells, and the clearance of LCMV transgenic effector T cells has been found to occur in a Fas-independent fashion (36). However, T cells of LCMV-infected, Fas-deficient *lpr* mice exhibited decreased susceptibility to TCR-induced apoptosis *in vitro* (37). Furthermore, Fas-dependent control of peripheral clonal deletion of V β 6⁺CD4⁺ T cells has been demonstrated *in vivo* by infection of *lpr/lpr* and *+/+* mice with mouse mammary tumor virus expressing the viral superantigen SAG (38). Therefore, the role of Fas-mediated apoptosis in the peripheral deletion of activated T lymphocytes during a primary immune response after virus infections is controversial.

To determine the significance of apoptosis mediated by Fas and TNF-R1 in the clearance of virally infected cells and in the regulation of the immune response, B6-*+/+* mice, B6-*lpr/lpr* mice, TNF-R1 knockout (B6-*tnfr^{0/0}*), and TNF-R1 knockout Fas-deficient (B6-*tnfr^{0/0} lpr/lpr*) mice were inoculated intraperitoneally with MCMV. The present results indicate that apoptosis mediated by either Fas or TNF-R1 is sufficient for rapid elimination of MCMV-infected cells. However, apoptosis mediated by Fas, but not by TNF-R1, is critical for downmodulation of the immune response, and defective expression of Fas leads to a chronic inflammatory disease after MCMV infection despite the absence of infectious virus.

Methods

Animals. 10–14-wk-old, female B6-*+/+* mice and B6-*lpr/lpr* mice were obtained from The Jackson Laboratories (Bar Harbor, ME). B6-*tnfr^{0/0}* mice and B6-*tnfr^{0/0} lpr/lpr* were generated as described previously (23) and were bred and maintained in our own animal facility at the University of Alabama at Birmingham under pathogen-free conditions.

Virus and virus titration. Female Balb/c mice were inoculated intraperitoneally with MCMV strain Smith obtained from the American Type Culture Collection (Rockville, MD) and salivary glands were collected 12 d later. The salivary glands were homogenized in MEM (GIBCO BRL, Grand Island, NY) containing 10% FBS, and centrifuged. The supernatant was dispensed into aliquots, which were stored at –80°C and used as the MCMV stock virus pool (3×10^7 PFU/ml). For the determination of the virus titer in tissues, organs were removed and homogenized as 10% (wt/vol) suspensions in DME (GIBCO BRL) supplemented with L-glutamine, 10% FCS, penicillin/streptomycin, and amphotericin B. The homogenates were titrated as duplicates in log₁₀ dilutions on subconfluent primary murine embryo fibroblasts in 12-well plates. 7 d later, monolayers were stained with neutral red and the number of plaques was counted.

Histological assessment and immunophenotyping. Organs were removed and fixed in 10% phosphate-buffered formalin. After paraffin embedding, tissue sections were cut (5 μ m) and stained with hematoxylin and eosin for morphological evaluation. Histopathologic scoring was performed by rating the severity of lesions and infiltration on a scale from 0 to 5. The following criteria were applied for the grading scale: 0: normal histology; 1: minimal mononuclear cell infiltration with

or without minimal cell destruction; 2: modest mononuclear cell infiltration with 1–2 clear foci per field of view with focal cellular destruction; 3: numerous aggregates (3–5) of mononuclear cells per field of view with destruction of liver, kidney, or lung tissue at several locations; 4: severe infiltration with mononuclear cells in multiple foci per lobe with destruction of liver, kidney, or lung tissue; and 5: diffuse infiltration with mononuclear cells with most severe cellular destruction. The histopathology was scored by three blinded observers, and the score represents the mean, with error bars representing the standard error of the mean (SEM).

For immunohistochemistry, slides were first incubated with a peroxidase-conjugated monoclonal antibody specific for TCR/CD3 (Dako, Glostrup, Denmark) or a biotin-conjugated monoclonal antibody specific for B220 (PharMingen, San Diego, CA). The biotinylated antibody specific for B220 was detected by application of peroxidase-conjugated streptavidin (Sigma Chemical Co., St. Louis, MO). Positive reactions were revealed using DAB substrate (Sigma) and methyl green was used for counterstaining.

Terminal deoxynucleotidyl transferase (TDT)-mediated dUTP nick end labeling (TUNEL). The method was modified slightly from that described previously (39). In brief, formalin-fixed and paraffin-embedded tissue sections were deparaffinized and rehydrated. After thorough washing with deionized water, the tissue sections were subjected to permeabilization with proteinase K (10 μ g/ml, room temperature for 15 min), and then incubated with freshly prepared TDT reaction mix (0.4 U/liter TDT, 10 nM digitonin modified-dUTP, and TDT buffer, which were purchased from Boehringer Mannheim, Indianapolis, IN) at 37°C for 60 min. The incorporated digitonin-dUTP was detected by incubation with alkaline phosphatase-conjugated antidigitonin antibody at room temperature for 60 min and positive reactions were revealed using NBT/BCIP substrate. Methyl green was used for counterstaining.

7-amino-actinomycin D (7-AAD) staining. For discrimination of apoptotic and nonapoptotic cells, tissue was harvested and single cell suspension was stained with 20 μ g/ml of 7-AAD (Calbiochem, San Diego, CA) in PBS on ice for 30 min in the dark as described previously (40, 41). After washing with PBS, cells were fixed in 1% paraformaldehyde supplemented with 10 μ g/ml actinomycin D. 10,000 nongated cells were analyzed using FACScalibur[®] (Becton Dickinson, Mountain View, CA).

Cytokine assays. Serum levels of IFN- γ and TNF- α were determined using specific standard ELISA (Endogen, Woburn, MA). The sandwich ELISA were performed according to the manufacturer's protocol. The sensitivities of the IFN- γ and TNF- α ELISA were < 15 and < 10 pg/ml, respectively.

Quantitation of autoantibodies. Serum levels of anti-double strand (ds) DNA antibodies and rheumatoid factor (RF) were determined by sandwich ELISAs as described previously (42). Sera samples were obtained from five mice per group of uninfected and infected mice. In brief, for quantitation of anti-dsDNA antibodies 96-well microtiter plates were precoated with 10 μ g/ml of poly-L-lysine followed by 10 μ g/ml of dsDNA or 10 μ g/ml of poly-L-glutamic acid as background control. For detection of RF, 96-well microtiter plates were coated with 4 μ g/ml of affinity-purified rabbit IgG (all reagents were obtained from Sigma). The sera were diluted at 1:100 and incubated at room temperature for 4 h. Bound anti-dsDNA antibodies or RF were detected by an IgG isotype-specific alkaline phosphatase-conjugated goat anti-mouse Ig (PharMingen). *p*-nitrophenylphosphate (Sigma) was used as substrate, and the color development was measured at 405 nm using an Emax microplate reader (Molecular Devices, Menlo Park, CA).

Results

Combined defect in Fas- and TNF-R1-mediated apoptosis delayed clearance of MCMV. To determine the role of Fas- and TNF-R1-mediated apoptosis in virus clearance, B6-*+/+* mice,

B6-*lpr/lpr* mice, B6-*tnfr^{0/0}*, and B6-*tnfr^{0/0} lpr/lpr* mice were inoculated intraperitoneally with MCMV. At different time points after infection, the presence of infectious MCMV in the organs was evaluated using a plaque assay. Consistent with previous reports, the clearance of MCMV occurred rapidly and efficiently in the liver, kidney, lung, and peritoneal exudate of B6-+/+ mice (4, 43, 44). Almost equivalent clearance of MCMV was found in B6-*lpr/lpr* and B6-*tnfr^{0/0}* mice since by day 14 only the lung of B6-*lpr/lpr* mice harbored small amounts of infectious MCMV. By day 28, infectious MCMV was not detectable in the liver, kidney, lung, or peritoneal exudate of B6-+/+, B6-*lpr/lpr*, or B6-*tnfr^{0/0}* mice (Fig. 1). However, higher titers of virus and delayed virus clearance were found in the lung, kidney, and peritoneal exudate of B6-*tnfr^{0/0} lpr/lpr* mice, indicating that either Fas or TNF-R1 is sufficient for rapid clearance of MCMV. They may compensate for each other in the elimination of MCMV-infected cells.

MCMV infection-induced chronic disease in Fas-mutant *lpr/lpr* mice. B6-+/+ mice, B6-*tnfr^{0/0}* mice, B6-*lpr/lpr* mice, and B6-*tnfr^{0/0} lpr/lpr* mice were inoculated intraperitoneally with MCMV. Histological examination performed 7 d after infection revealed an acute inflammatory response in the liver, kidney, and lung, which was most severe in B6-+/+ and B6-*lpr/lpr* mice (Fig. 2, A–C). Multifocal hepatitis, interstitial and mesangial nephritis, and interstitial pneumonitis were present in B6-+/+ and B6-*lpr/lpr* mice, whereas B6-*tnfr^{0/0}* mice exhibited only moderate inflammation, indicating the central role of TNF- α in mediating an inflammatory response. After 4 wk, the inflammation had resolved in the organs of B6-+/+ mice and B6-*tnfr^{0/0}* mice (Fig. 2, A–C). In contrast, moderate inflammation was still present in B6-*tnfr^{0/0} lpr/lpr* mice, and a severe chronic inflammatory response was observed in the liver, kidney, and lung of B6-*lpr/lpr* mice, which persisted for over 100 d after infection, and 70 d after the clearance of infectious

MCMV (Fig. 2 D). The chronic hepatitis in B6-*lpr/lpr* mice was characterized by moderate multifocal degeneration and regeneration of hepatocytes with infiltration of mononuclear cells. Typical features of interstitial pneumonitis were found during the chronic phase of disease in the lung including thickening of the alveolar septa, hyperplasia of alveolar macrophages, and perivascular and peribronchial lymphocytic infiltrates. Severe proliferative glomerulonephritis with multifocal tubular degeneration and a lymphocytic infiltration in the interstitium indicated a chronic nephritis (Fig. 2, A–C). Thus, Fas-mediated apoptosis is required for the downmodulation of the inflammatory response induced by MCMV.

Immunophenotype of infiltrating cells. There were numerous CD3⁺ T lymphocytes among the infiltrating cells found during the acute inflammatory response in liver and lung of B6-+/+ mice (Fig. 3 A) and B6-*lpr/lpr* mice. Furthermore, the infiltrates observed during the chronic phase of inflammation in the liver, kidney, and lung of B6-*lpr/lpr* mice consisted predominantly of CD3⁺ T lymphocytes (Fig. 3 B). The majority of these infiltrating T cells did not coexpress B220 and therefore do not correspond to the TCR- $\alpha\beta$ ⁺CD4⁺CD8⁺B220⁺ T cell subpopulation which causes lymphoproliferation in *lpr/lpr* mice. Only low numbers of cells stained positive for B220 in the liver and lung of B6-*lpr/lpr* mice 28 d after MCMV infection, indicating that the chronic inflammatory disease in B6-*lpr/lpr* mice was associated with an increased percentage of normal phenotype CD3⁺ T cells (Fig. 3 C).

Apoptotic cells in situ after infection with MCMV. For detection of apoptosis in situ, liver tissue of MCMV-infected B6-+/+, B6-*lpr/lpr*, and B6-*tnfr^{0/0}* mice was obtained at days 3, 7, 28, and 100. Apoptotic cells were identified using the TUNEL technique. At day 3 after MCMV infection, numerous cells undergoing apoptosis could be observed in all of the mice. These apoptotic cells were mainly located in separated clusters,

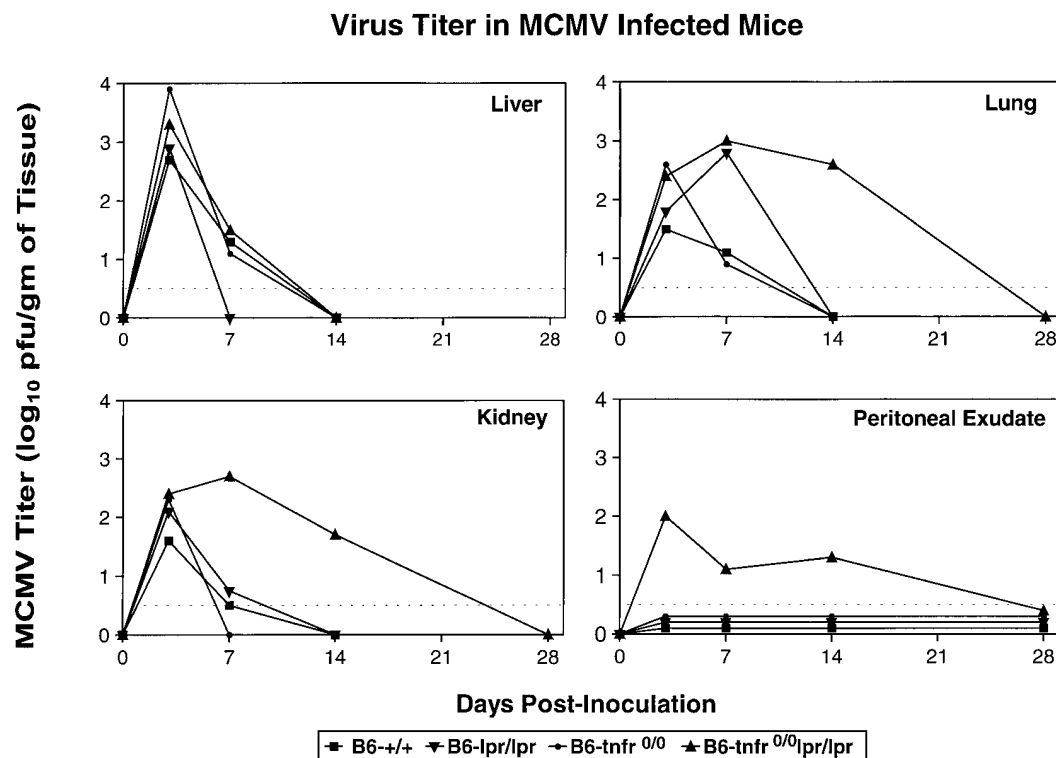


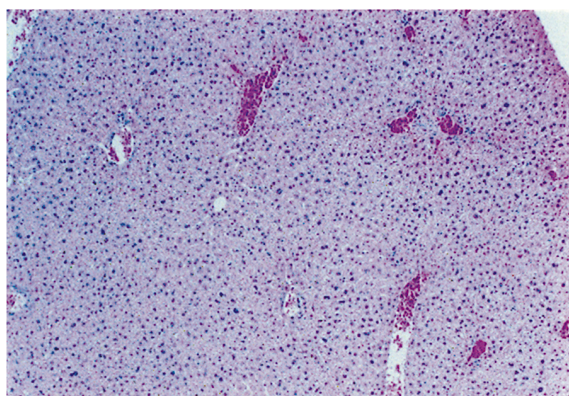
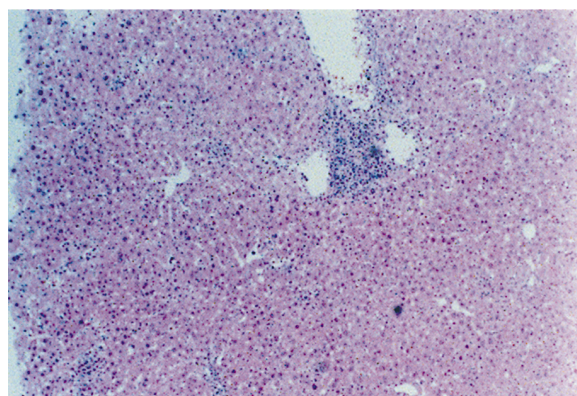
Figure 1. Delayed clearance of MCMV in Fas and TNF-R1 double-deficient mice. B6-+/+, B6-*lpr/lpr*, and B6-*tnfr^{0/0}*, and B6-*tnfr^{0/0} lpr/lpr* mice were inoculated intraperitoneally with MCMV (10^5 PFU). Quantitation of infectious MCMV in liver, kidney, lung, and peritoneal exudate was performed at different time points using a plaque assay.

A

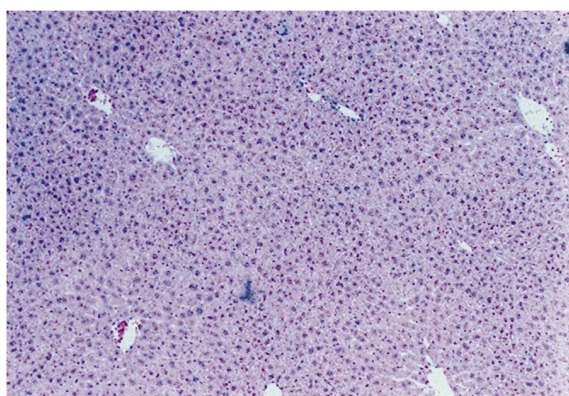
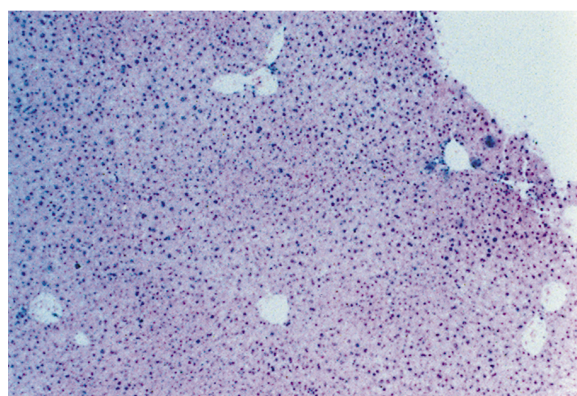
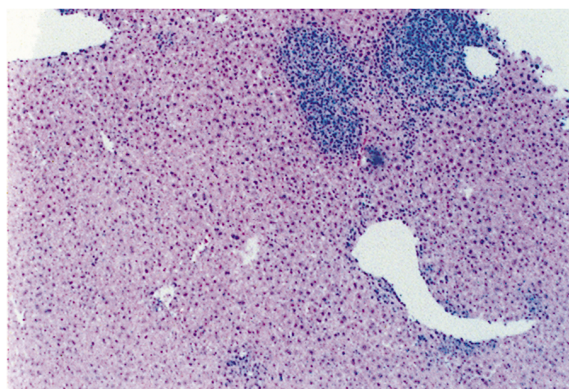
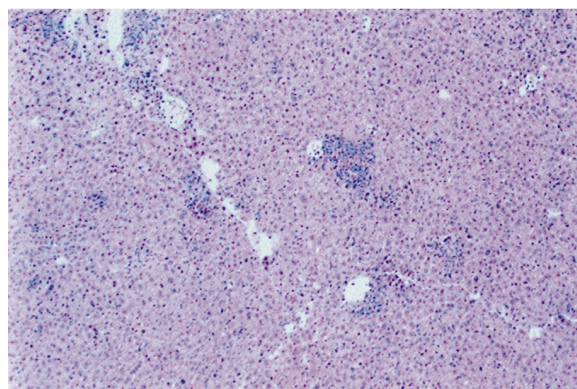
Liver (x 80)

Day 7

Day 28



+/+

tnfr^{0/0}

lpr/lpr

Figure 2. MCMV infection–induced chronic disease in B6-*lpr/lpr* mice. B6-+/+ mice, B6-*tnfr*^{0/0} mice, B6-*lpr/lpr* mice, and B6-*tnfr*^{0/0} *lpr/lpr* mice were inoculated intraperitoneally with MCMV (10⁵ PFU). Histological evaluation was performed at days 7 and 28 after infection (A–C) and day 100 (D) (original magnification of 10), and tissues were assessed for severity of inflammation and tissue damage on a relative scale ranging from 0 (not present) to 5 (most severe) (D). The severe chronic inflammatory response observed in B6-*lpr/lpr* mice was characterized by multifocal hepatitis (A), interstitial and mesangial nephritis (B), and interstitial pneumonitis (C).

which contained apoptotic hepatocytes and inflammatory cells. Some cells displayed morphological features characteristic of MCMV infection, including cell rounding and nuclear inclusions (Fig. 4). Higher numbers of apoptotic cells were present 7 d after MCMV infection, and these apoptotic cells were both localized in foci and also occurred as scattered, individual cells throughout the liver tissue of B6-+/+ and B6-*lpr*/

lpr mice. The number of scattered apoptotic cells was especially high in B6-*tnfr*^{0/0} mice. Large numbers of apoptotic cells also were observed in the areas of accumulation of infiltrating cells in B6-+/+ mice. In contrast, the amount of apoptosis among infiltrating cells remained low in B6-*lpr/lpr* mice. There were no inflammatory cells and few apoptotic cells in the liver tissue 28 and 100 d after MCMV infection in B6-+/+ and B6-

B

Kidney (x 80)

Day 7

Day 28

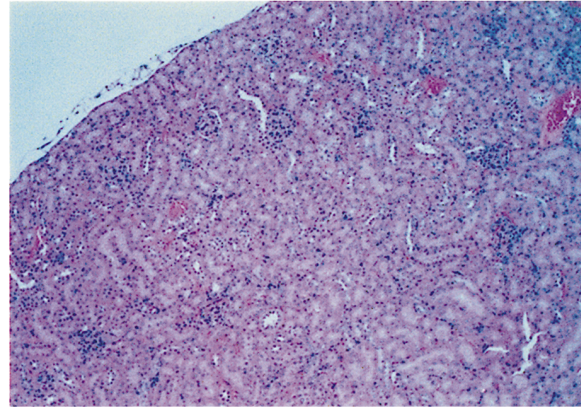
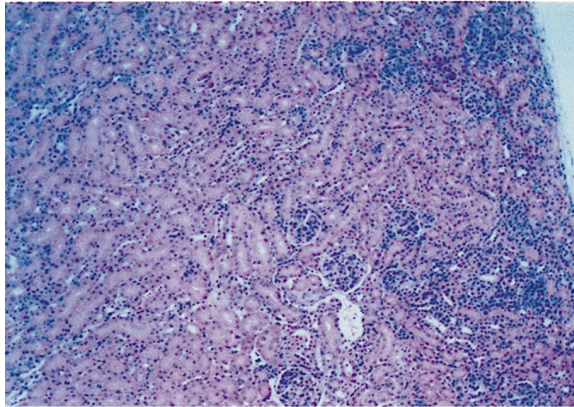
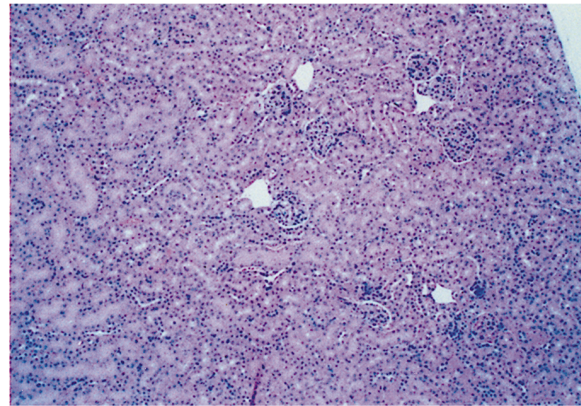
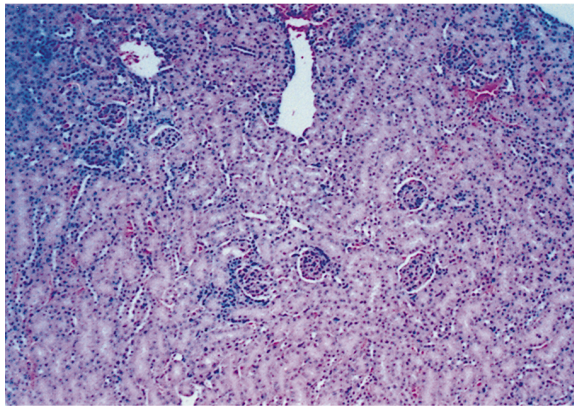
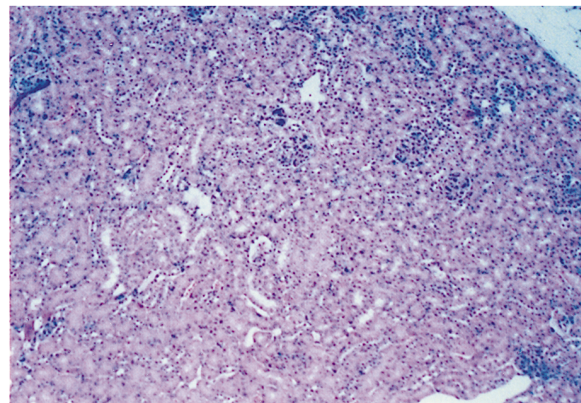
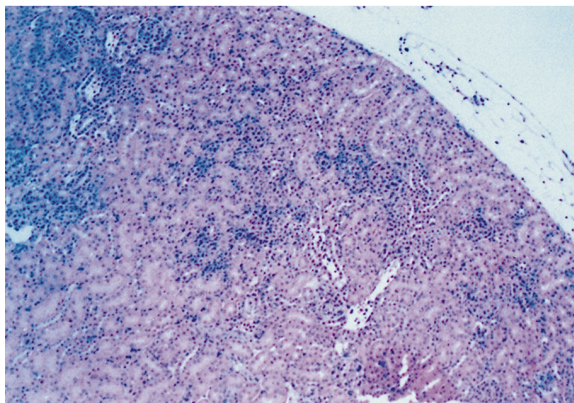
**+/+****tnfr^{0/0}****lpr/lpr**

Figure 2 (Continued)

tnfr^{0/0} mice. In contrast, extensive infiltration with inflammatory cells but very low apoptosis were present in B6-*lpr/lpr* mice at days 28 and 100 of MCMV-induced disease, suggesting that a defect in the AICD of lymphocytes in *lpr/lpr* mice leads to a chronic post-MCMV inflammation.

Apoptosis of peritoneal cells in MCMV-infected mice. Macrophages represent the first line of the host defense after intraperitoneal administration of viruses and it has been demonstrated in different mouse models that impaired macro-

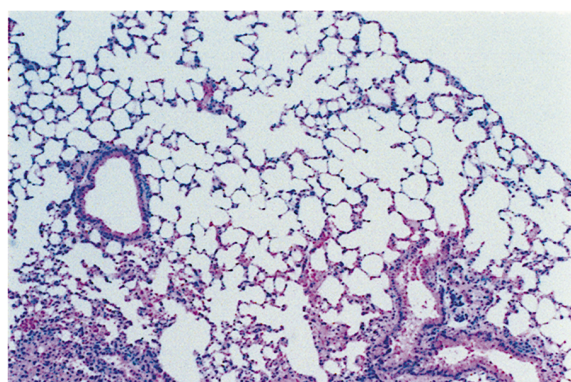
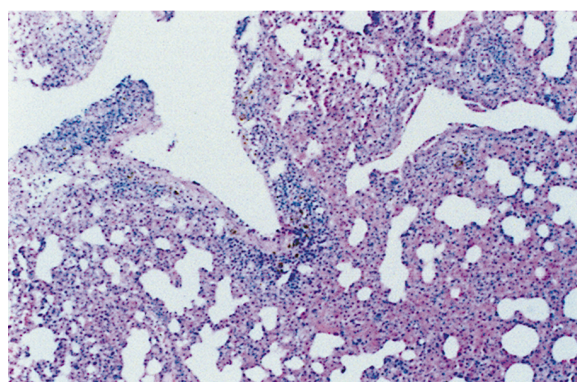
phage function leads to increased susceptibility of the host to virus infections (8, 45). The data thus far indicated that apoptosis is critical for the elimination of virally infected cells and high numbers of apoptotic cells were found in situ early after MCMV infection. To determine if apoptosis occurs at the site of infection and affects peritoneal macrophages, peritoneal exudate cells were obtained by lavage 7 d after MCMV infection of B6-+/+, B6-*lpr/lpr*, B6-*tnfr^{0/0}*, and B6-*tnfr^{0/0} lpr/lpr* mice, and analyzed for apoptosis using 7-AAD staining (Fig. 5).

c

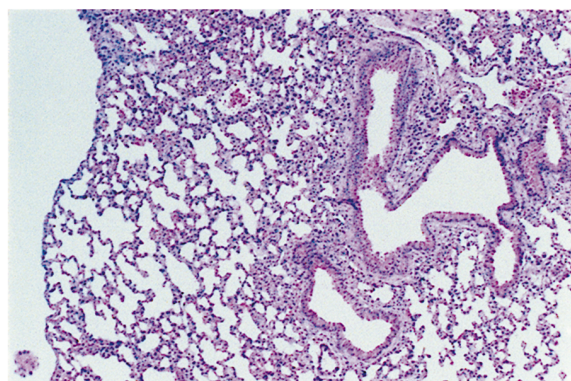
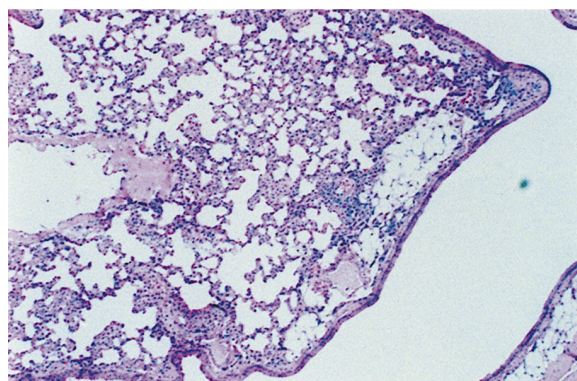
Lung (x 80)

Day 7

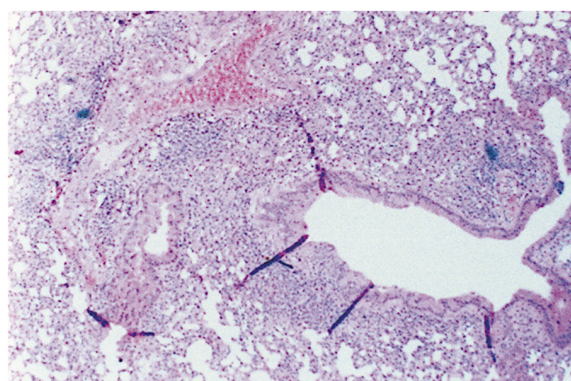
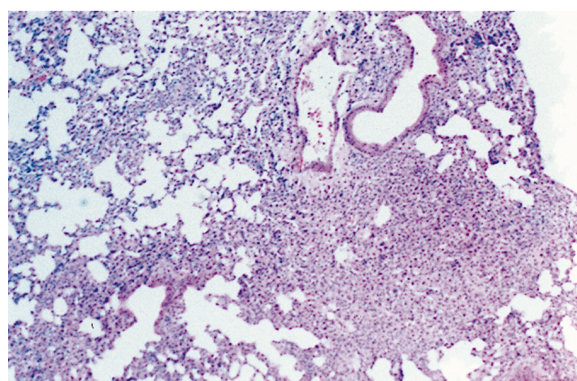
Day 28



+/+



tnfr^{0/0}



lpr/lpr

Figure 2 (Continued)

Large numbers (39%) of peritoneal cells were undergoing apoptosis in B6-+/+ mice at this time as indicated by increased 7-AAD uptake. Lower numbers of apoptotic peritoneal cells were present in B6-*lpr/lpr* mice (15%) and B6-*tnfr^{0/0}* mice (11%). In contrast, very few apoptotic peritoneal cells were found in B6-*tnfr^{0/0} lpr/lpr* mice (3%) at the same time point, indicating that substantial apoptosis is associated with rapid clearance of MCMV.

Serum levels of IFN- γ and TNF- α after MCMV infection. Analysis of serum levels of cytokines revealed high levels of IFN- γ (ranging from 400 to 1,200 pg/ml), which were produced

early (day 3) after infection, in B6-+/+, B6-*lpr/lpr*, and B6-*tnfr^{0/0}* mice (Fig. 6). Only low levels of IFN- γ were found in the same mice by day 7, which is consistent with studies in B6-+/+ mice by other investigators (9, 46). However, production of IFN- γ was about fourfold higher at day 28 in B6-*lpr/lpr* mice than in B6-+/+ and B6-*tnfr^{0/0}* mice, which might be due to the activation of T cells during the chronic inflammatory response. In contrast, IFN- γ was produced at very high levels (2,700 pg/ml) in B6-*tnfr^{0/0} lpr/lpr* mice and remained high through 28 d after infection (1,400 pg/ml). Induction of TNF- α by MCMV was detected early after infection in all mice. However, levels

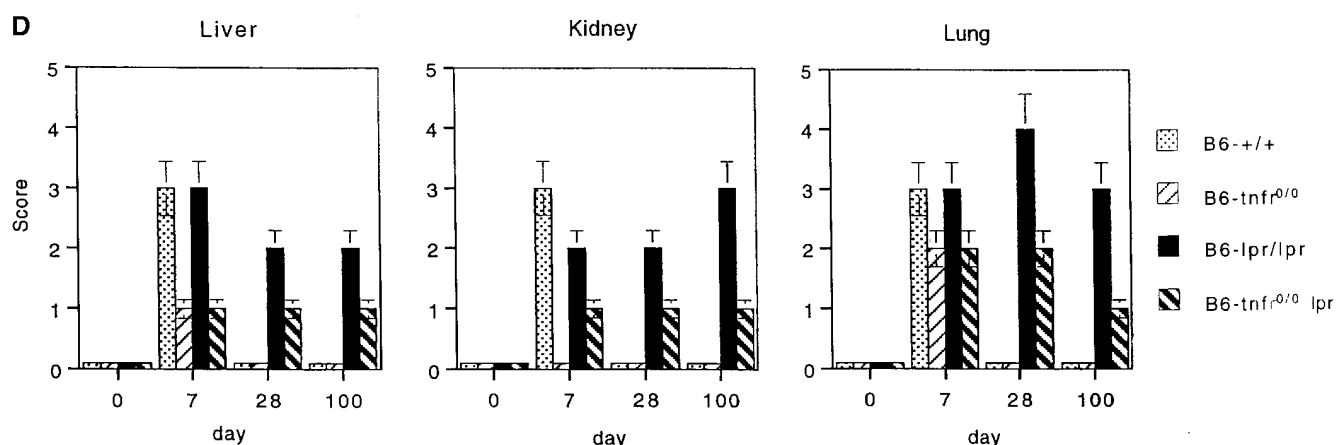


Figure 2 (Continued)

of TNF- α were ~ 10 -fold higher in the sera of B6-*lpr/lpr*, B6-*tnfr^{0/0}*, and B6-*tnfr^{0/0} lpr/lpr* mice than that of B6-*+/+* mice on day 3 and twice as high on day 7 (Fig. 6). This is consistent with our report that production of TNF- α is increased in *lpr/lpr* mice after stimulation with staphylococcal enterotoxin B (47). Remarkably, TNF- α continued to be expressed at high levels (80 pg/ml) in B6-*lpr/lpr* and B6-*tnfr^{0/0} lpr/lpr* mice up to day 28 after MCMV infection.

Enhanced autoantibody production in B6-*lpr/lpr* mice. To determine if MCMV infection also stimulates autoimmunity, the production of RF and anti-dsDNA antibodies, which are characteristic markers of autoimmune disease, was evaluated 28 d after MCMV infection. There was a significant induction of RF after MCMV infection in B6-*lpr/lpr* and B6-*tnfr^{0/0}* mice (Fig. 7 A). There was also a two- to threefold increase in the production of anti-dsDNA antibodies in MCMV-infected B6-*lpr/lpr* mice and B6-*tnfr^{0/0}* mice compared with uninfected mice (Fig. 7 B). The expression of RF and anti-dsDNA was increased in uninfected B6-*tnfr^{0/0} lpr/lpr* mice, compared with B6-*+/+* mice, and was not significantly increased after infection with MCMV (Fig. 7).

Discussion

Almost three decades ago, Dixon et al. (48, 49) and other investigators (50–53) discovered that certain chronic virus infections accelerate autoantibody production and induce immune complex glomerulonephritis in NZB/W mice. T cell tolerance was shown to be critical in maintenance of B cell nonreactivity in a vesicular stomatitis virus transgenic mouse (54). However, the molecular mechanism describing how a viral infection can lead to a chronic inflammation is not known.

Apoptosis of virally infected cells has been recognized as a critical mechanism by which the host limits virus spread, and the presence of apoptotic cells has been reported during several viral infections, including MCMV (11, 55, 56). The important role of Fas- and TNF-R1-mediated apoptosis in the elimination of virally infected cells is emphasized by the discovery of potent inhibitors of these pathways by several viruses, which interfere with signaling molecules at different levels of the signal transduction process and thereby prevent apoptosis of the host cell. These inhibitors include the cowpox virus CrmA pro-

tein (57, 58), the baculovirus protein IAP (59), the E1B 19K protein encoded by adenovirus (60), the immediate early genes 1 and 2 of CMV (61), and the recently discovered new family of viral inhibitors (v-FLIP) expressed by several herpes viruses (62). The Fas-Fas ligand and perforin-granzyme pathways also are used by cytotoxic T lymphocytes during the cytotoxic response for the elimination of virally infected cells (17, 18).

These results demonstrate that early apoptosis of virally infected cells is important for the clearance of an acute virus infection in vivo, and involves either the Fas or TNF-R1 pathway. The clearance of MCMV was significantly delayed and low numbers of apoptotic peritoneal cells were observed in B6-*tnfr^{0/0} lpr/lpr* mice. However, the clearance of MCMV in the liver, kidney, lung, and peritoneal exudate was not greatly impaired in B6-*lpr/lpr* mice or B6-*tnfr^{0/0}* single mutant mice, suggesting a compensatory mechanism involving these apoptosis-inducing molecules. Apoptosis correlated with viral clearance since large numbers of apoptotic cells could be detected in situ in target organs at an early stage during MCMV infection, but no apoptotic cells were present after the clearance of infectious MCMV in B6-*+/+*, B6-*lpr/lpr*, or B6-*tnfr^{0/0}* mice. Apoptosis in the liver tissue occurred in scattered cells, and also in clusters containing hepatocytes and T cells, and some hepatocytes exhibited morphological features characteristic of MCMV infection including cell rounding and nuclear inclusions. It has been reported previously that different virus infections, including herpes viruses, induce increased expression of both Fas and Fas ligand in host cells in vitro (16, 63). Together, these results suggest that cell suicide and paracrine killing of infected cells mediated by Fas and TNF-R1 that occur within 1 wk after MCMV entry are important mechanisms for the clearance of MCMV-infected cells. We propose that the first phase for Fas or TNF-R1-mediated apoptosis in response to virus infections is elimination of virally infected cells as depicted in Fig. 8 A.

Fas and TNF-R1 have been demonstrated to mediate apoptosis in activated T cells in vitro (19, 31–34, 64–67). Although it has not yet been demonstrated formally, it is generally accepted that activation-induced apoptosis exists to maintain immune tolerance (67). The present data extend this concept to the downmodulation of the immune response after resolution of an infectious process. Histological examination of the liver, kidney, and lung at different time points after MCMV infec-

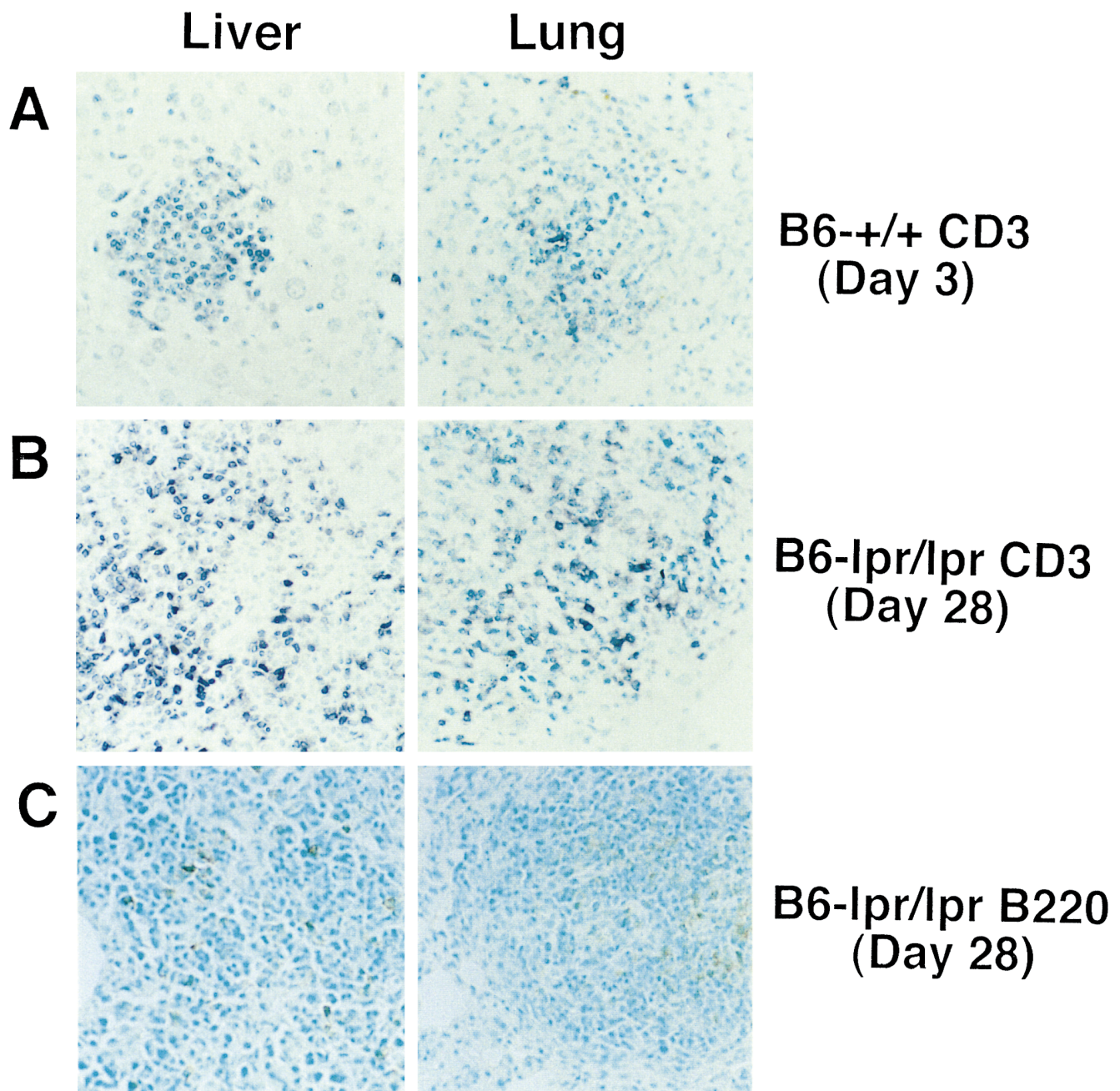


Figure 3. Immunophenotype of infiltrating cells. Infiltrating T cells in liver and lung of B6-+/+ mice 7 d after MCMV infection (A). Infiltrating T cells in liver and lung during the chronic phase (day 28) of MCMV-induced disease in B6-lpr/lpr mice (B). Expression of B220 antigen in lymphocytes 28 d after MCMV infection in liver and lung of B6-lpr/lpr mice (C). Original magnification of 320.

tion revealed a severe, chronic inflammatory disease in B6-lpr/lpr mice, which was characterized by large numbers of infiltrating T cells. Since chronic disease did not develop in MCMV-infected B6-+/+ or B6-tnfr^{0/0} mice, it can be concluded that Fas-mediated, but not TNF-R1-mediated, apoptosis is required for the second phase of apoptosis after the clearance of virus, which eliminates inflammatory cells and leads to the downmodulation of the immune response (Fig. 8B). This concept is supported by the observation that a large percentage of infiltrating T cells was undergoing apoptosis in B6-+/+ mice 7 d after MCMV infection, whereas few apoptotic cells were detected in B6-lpr/lpr mice. Excessive inflammation and low

apoptosis were not due to a persistent infection in B6-lpr/lpr mice since the clearance of MCMV was not delayed as no infectious MCMV could be detected in the liver, kidney, lung, or peritoneal fluid of these mice at day 28. These results indicate that a defect in Fas-mediated apoptosis may predispose to a chronic postviral inflammatory disease process.

Viral persistence and the inflammatory response are regulated differently by TNF-R1 and Fas. TNF-R1 and Fas signaling are synergistic in regard to viral elimination. Viral elimination can be mediated by either TNF-R1 or Fas since single mutant mice, but not double mutant mice, exhibit nearly normal elimination of virus. TNF-R1 and Fas signaling are antagonistic

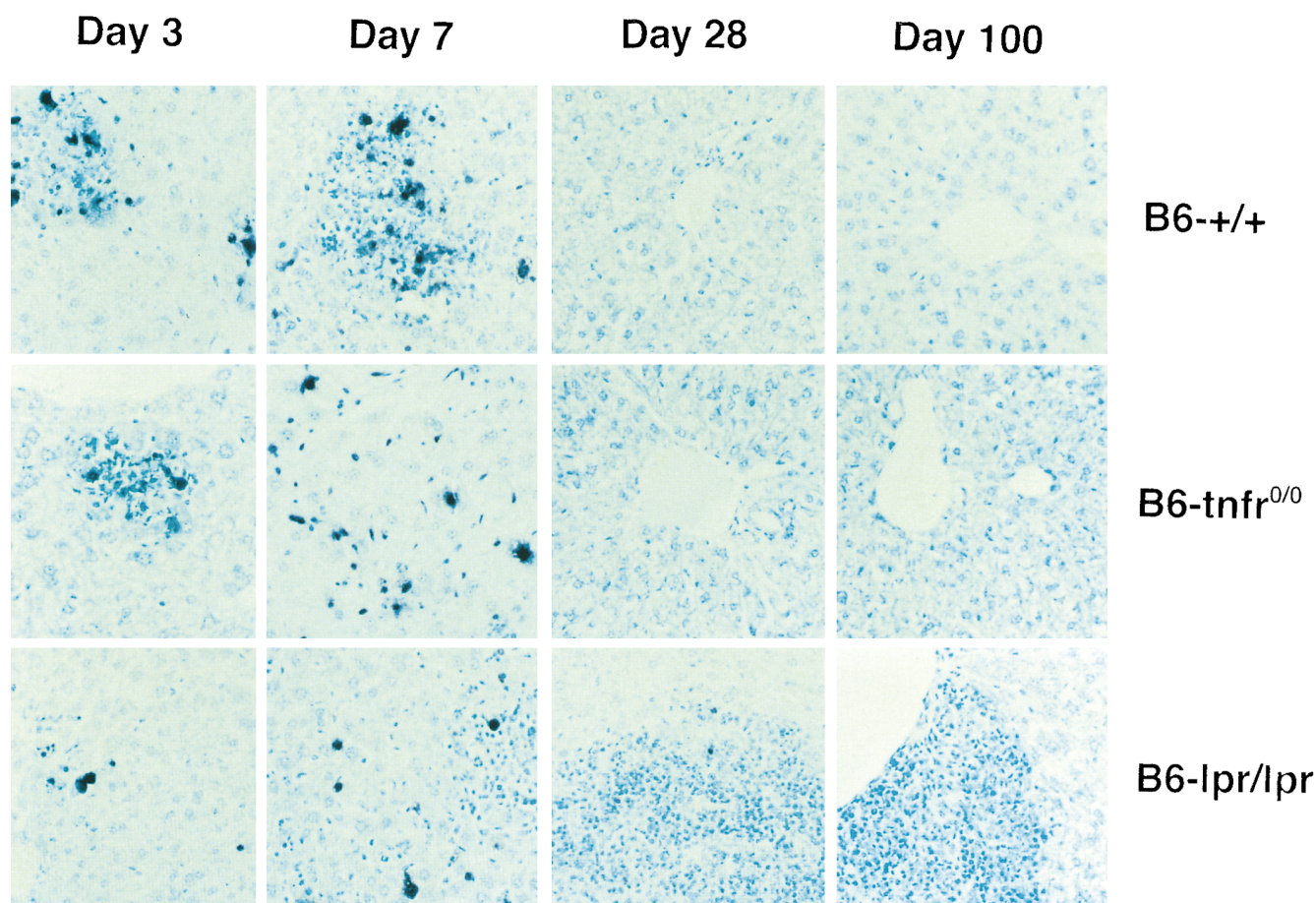


Figure 4. Apoptotic cells in situ. Apoptotic cells were identified by labeling the characteristic DNA-strand breaks using the TUNEL reaction in liver tissue of MCMV-infected B6-+/+, B6-*lpr/lpr*, and B6-*tnfr*^{0/0} mice at days 3, 7, 28, and 100. Original magnification of 320.

onistic in regard to inflammation. The inflammatory response to the virus is dependent, in part, upon signaling through TNF-R1. This is illustrated by a decreased inflammatory response in both the TNF-R1 single knockout mouse and in the B6-*tnfr*^{0/0} *lpr/lpr* double knockout mouse. The lack of TNF-R1 signaling results in lower inflammation despite higher virus levels in B6-*tnfr*^{0/0} *lpr/lpr* mice. In contrast, the absence of Fas is associated with a prolonged inflammatory response. Therefore the lack of Fas signaling, combined with higher virus levels, results in higher inflammation in the B6-*tnfr*^{0/0} *lpr/lpr* mice compared with the B6-*tnfr*^{0/0} mice.

There is increasing evidence that autoimmune disease can be initiated and promoted by infections, and several hypotheses have been proposed to explain the mechanism by which infectious agents could induce autoimmunity (68). CMV infection has been implicated in several autoimmune syndromes in humans including diabetes mellitus (69), systemic lupus erythematosus (70), Sjögren's syndrome (71), graft versus host disease (72), and rheumatoid arthritis (73). The induction of autoantibodies of various specificities also has been reported after MCMV infection (74, 75). High titers of autoantibodies in the B6-Tnfr1^{0/0} mice are consistent with previous results by Jacob et al. that TNF- α treatment decreases autoantibody production in NZB/W strains of mice (76, 77). There were high levels of RF and anti-dsDNA in uninfected B6-Tnfr1^{0/0}-*lpr/lpr* mice, which were not further increased 28 d after viral infec-

tion. This is consistent with our previous observation that B6-Tnfr1^{0/0}-*lpr/lpr* mice develop very high levels of autoantibodies spontaneously which we propose was due to a severe defect in apoptosis to the absence of both TNF-R1 pathway and Fas pathway of apoptosis (23).

Autoimmune disease after CMV and EBV has been described previously (78). However, autoimmune disease is rarely diagnosed in cases in which isolation of infectious virus has been possible (79). Isolation of live virus is difficult since productively infected cells and viral antigens are cleared at different rates by different individuals (80). Moreover, whereas EBV is known to have the capacity to establish a latent infection in B cells, the cell population becoming eventually latently infected during the course of CMV as well as MCMV infection is not clearly defined, and the mechanisms by which latency is established are poorly understood (81, 82). Evidence indicates that blood cell progenitor cells in the bone marrow may be a source of latent CMV infection (83, 84), and endothelial cells appear to be a potential site of latent MCMV infection (80). Furthermore, MCMV DNA has been detected in several tissues including salivary glands in the absence of infectious virus, again suggesting latent MCMV infection (83–86). Therefore, latent virus may underlie the chronic inflammatory response after MCMV infection.

These results suggest that MCMV infection could trigger autoimmune disease in B6-*lpr/lpr* mice since significantly in-

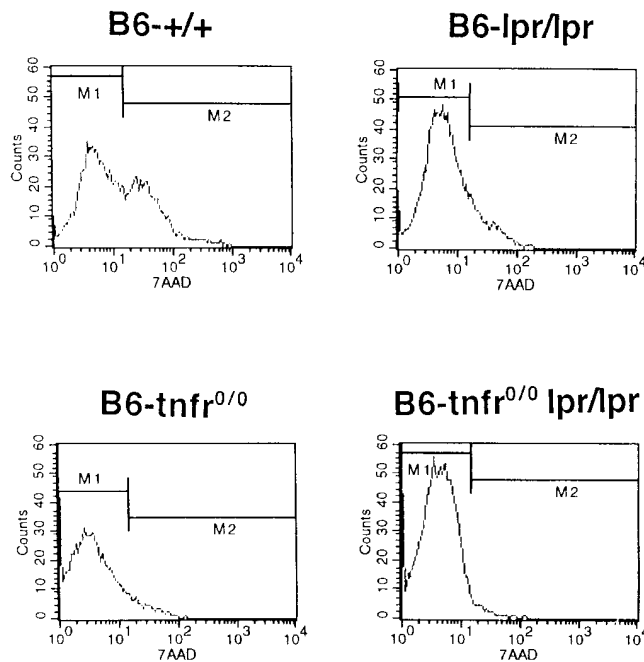


Figure 5. Apoptosis of peritoneal exudate cells in MCMV-infected mice. Apoptosis of peritoneal exudate cells was determined 7 d after MCMV infection in B6-+/+, B6-lpr/lpr, B6-tnfr^{0/0}, and B6-tnfr^{0/0} lpr/lpr mice by 7-AAD staining. Living cells were 7-AAD negative and are marked as the M1 cell population. Increased 7-AAD uptake indicates apoptotic cells, marked as the M2 cell population. The percentages of apoptotic cells (M2) were: 39% in B6-+/+ mice, 15% in B6-lpr/lpr mice, 11% in B6-tnfr^{0/0} mice, and 3% in B6-tnfr^{0/0} lpr/lpr mice.

creased levels of RF and anti-dsDNA autoantibodies were detected, and the histopathological features of the MCMV-induced chronic disease in B6-lpr/lpr mice resembled autoimmune syndromes observed in humans. These results are in accordance with a previous study demonstrating increased numbers of CD4⁺8⁺B220⁺ T cells in B6-lpr/lpr mice after infection with murine leukemia virus suggesting an accelerated progression of the lpr-associated lymphadenopathy (76). In such a scenario, the Fas apoptosis defect in B6-lpr/lpr mice would lead to the accumulation of activated inflammatory cells

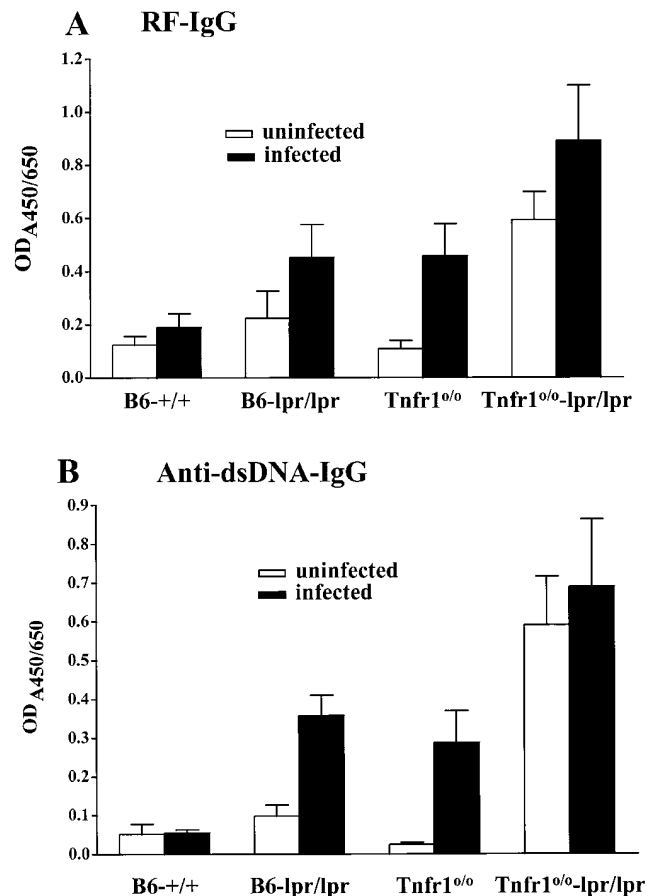


Figure 7. Enhanced autoantibody production in lpr/lpr and tnfr^{0/0} mice. Serum levels of RF (A) and anti-dsDNA (B) antibodies were evaluated 28 d after MCMV infection using specific ELISA assays as described. Each value represents the mean \pm SEM of at least five mice per group analyzed separately.

after the virus infection, resulting in an overproduction of cytokines, which may lead both to a persistent polyclonal activation, including activation of autoreactive T and B cells, and to the expansion of the CD4⁺8⁺B220⁺ T cell population causing lymphadenopathy. This model combines a genetic predisposi-

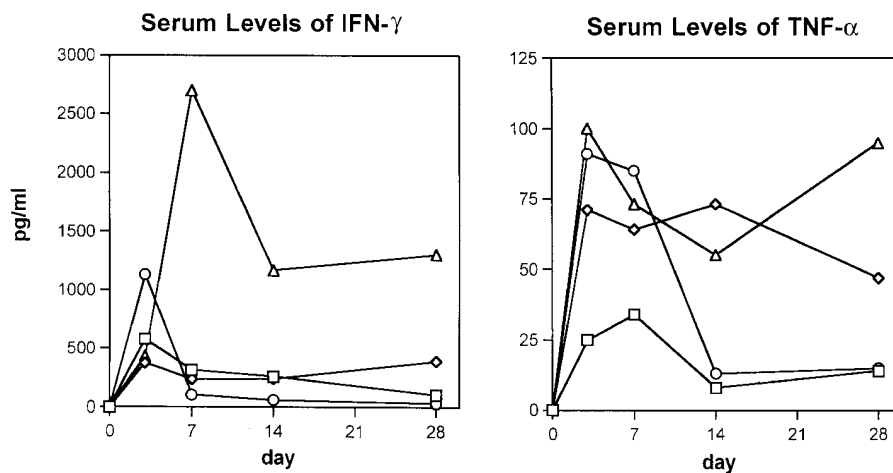


Figure 6. Serum levels of IFN- γ and TNF- α . Analysis of IFN- γ and TNF- α induction was determined in B6-+/+ (boxes), B6-lpr/lpr (diamonds), B6-tnfr^{0/0} (circles), and B6-tnfr^{0/0} lpr/lpr (triangles) mice at days 3, 7, 14, and 28 after MCMV infection using standard ELISA assays.

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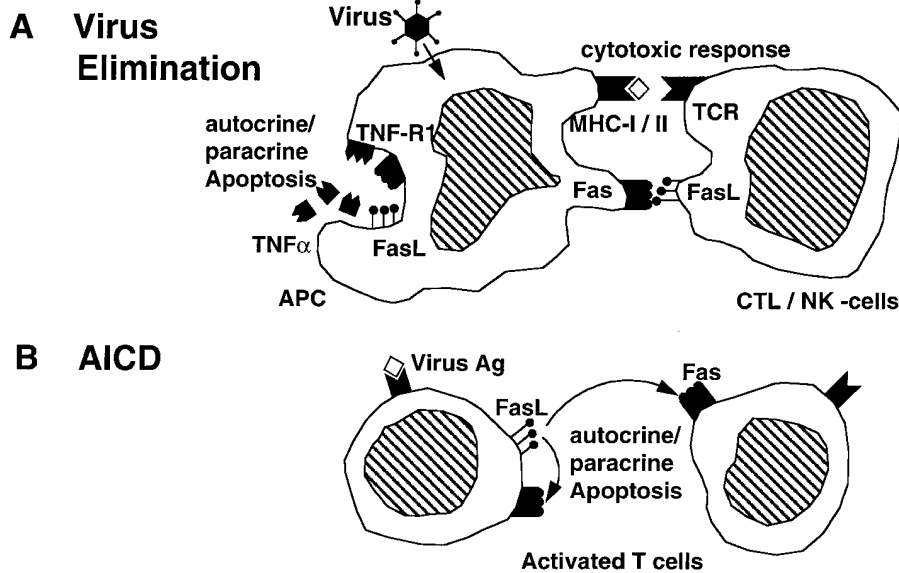


Figure 8. Potential roles of Fas and TNF-R1 apoptosis molecules in virus infections. Phase 1 (A): Clearance of virally infected cells by Fas- and TNF-R1-induced suicide, or paracrine killing of infected cells, or apoptosis of the host cell mediated by CTL. Phase 2 (B): Fas-mediated AICD is required for downmodulation of the immune response to maintain homeostasis of the immune system.

tion (apoptosis defect) with an environmental trigger (virus infection) and may therefore form an excellent model for analysis of the interplay between these factors in the induction of autoimmune disease in humans.

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