

Mechanism of Interleukin 12-mediated Toxicities during Experimental Viral Infections: Role of Tumor Necrosis Factor and Glucocorticoids

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Summary

Interleukin 12 (IL-12) doses in excess of 100 ng/d have been shown to induce profound immunotoxicities in mice infected with lymphocytic choriomeningitis virus (LCMV). These immunotoxicities are characterized by almost complete inhibition of virus-induced CD8⁺ T cell expansion and CTL activation, and up to 2 log increases in viral replication. They are accompanied by induction of serum tumor necrosis factor (TNF). The studies presented here were undertaken to characterize mechanisms for the IL-12-induced toxicities and to examine expression and function of TNF in this context. Several physiological changes were induced in IL-12-treated uninfected and dramatically elevated in IL-12-treated virus-infected mice. IL-12 induced (a) decreases in body weights, >10% in uninfected and >20% in LCMV-infected mice; (b) elevation of circulating glucocorticoid levels to >10 µg/dl in uninfected and >20 µg/dl in infected mice; and (c) decreases in thymic mass, >30% in uninfected and up to 95% in infected mice. These changes are known to be associated with circulating TNF. Northern blot and in situ hybridization analyses demonstrated that IL-12 induced TNF-α expression and that LCMV infection synergized with IL-12 for induction of this factor. Antibodies neutralizing TNF reversed all of the IL-12-induced toxicities in LCMV-infected mice including the immunotoxicities against CD8⁺ T cells and anti-viral defenses. The TNF-mediated immunotoxicities appeared to result from an induced cellular sensitivity to the factor, as splenic leukocytes and CD8⁺ T cell subsets isolated from LCMV-infected mice were more sensitive to TNF-mediated cytotoxicity in culture than were equivalent populations prepared from uninfected mice. Experiments with the glucocorticoid type II receptor antagonist, RU486, demonstrated that endogenous glucocorticoids were secondary intermediaries in IL-12-induced thymic atrophy. Studies in IL-2-deficient mice showed that the synergism was dependent upon endogenous IL-2. The results delineate a unique mechanism of TNF-mediated toxicity. In addition, they have significant implications concerning potential detrimental consequences of in vivo TNF induction and of IL-12 administration for protective anti-viral responses.

IL-12 is a heterodimeric cytokine with potent biological effects in vitro and in vivo (reviewed in reference 1). IL-12 has been shown to (a) induce IFN-γ production, (b) augment cytotoxic function and proliferation of NK and activated T cells, and (c) promote Th-1 type cytokine responses. In vitro experiments have demonstrated that several IL-12-mediated functions require tumor necrosis factor (TNF) (2, 3). Endogenous IL-12 is clearly important for protective immune responses during acute infections with intracellular parasites (4) and bacteria (5). Murine studies have demonstrated that IL-12 administration at doses greater than or equal to 100

ng/d can enhance protective immune responses to these pathogens (4, 6, 7) as well as to tumors (8). Therefore, in the context of endogenous responses, administration of the factor can be beneficial in promoting protective responses against diseases resulting from these challenges.

Little is known about IL-12 expression and function for protective responses against acute viral infections. The early studies examining consequences of administering IL-12 during experimental murine infections indicate that effects of this factor can be very different in the context of the endogenous cellular and cytokine responses to viral infections (7, 9). We

have been studying IL-12 effects during infection with lymphocytic choriomeningitis virus (LCMV)¹ (9). The endogenous immune response to this virus is characterized by a profound increase in CD8⁺ T cell numbers, activation of virus-specific CTLs, restricted to class I histocompatibility molecules, and interleukin-2 (IL-2) expression at 7–10 d after infection (10, 11). In this model, the effects of IL-12 administration are pleiotropic and dose dependent (9). Low doses of IL-12 (1–10 ng/d) enhance, whereas high doses (100–1,000 ng/d) inhibit CD8⁺ T cell responses. Both CD8⁺ T cell expansion and presence of virus-specific CTLs are reduced with the higher doses. CD8⁺ T cell responses are required for LCMV clearance (12), and viral replication is increased in these mice (9). As high dose IL-12 administration induces numerous necrotic lesions and apoptotic cells in lymphoid tissue, the effects on T cell responses appear to be associated with cell death. Therefore, doses of IL-12 which are immune enhancing and resistance promoting in the context of other infections or tumors, can be detrimental during viral infection.

High doses of IL-12 also induce serum TNF levels of 50–125 pg/ml in uninfected and 85–200 pg/ml in LCMV-infected mice (9). TNF is known to mediate a variety of biological activities and elicit a number of physiological and pathological changes. At high concentrations, this factor can promote weight loss or wasting (13, 14). In addition, systemic TNF can induce circulating glucocorticoids (15, 16), and glucocorticoids can promote thymic atrophy (17, 18). Moreover, TNF can mediate cell killing directly (19), or indirectly through induction of glucocorticoids (17, 20, 21). As there is synergism for IL-12 induction of TNF during viral infections, these other toxicities also may be induced in virus-infected mice treated with IL-12.

The studies presented here were performed to further characterize IL-12-induced effects *in vivo* and to identify mechanisms for IL-12-induced toxicities. The results show that in addition to inducing profound reductions in responding CD8⁺ T cells, IL-12 administration acts synergistically with infection to induce wasting, thymic atrophy, and elevated corticosterone, the natural glucocorticoid of mouse. All of these parameters, including the CD8⁺ T cell immunotoxicities, are dependent upon endogenous TNF expression. The effects on thymic organs, but not CD8⁺ T cell reductions, are glucocorticoid-mediated. These data have significant implications concerning *in vivo* cytokine interaction, and consequences of administered cytokines in the context of ongoing immune responses. They also suggest a pathway by which infection with agents inducing IL-12 and/or TNF, during a viral infection, might dramatically limit protective anti-viral immune responses and induce physiologically adverse effects on the host.

Materials and Methods

Mice. Specific pathogen-free male 4-wk-old C57BL/6 mice were purchased from Taconic Laboratory Animals and Services (German-

town, NY). Animals were 6–12 wk old at time of experiments. Mice possessing a mutated IL-2 gene were a gift of Dr. Ivan Horak (Institute of Virology and Immunobiology, University of Wurzburg, Wurzburg, Germany) (22). Male founder mice were mated with C57BL/6 females (Taconic) and heterozygous progeny used to establish a mutant IL-2 colony in our pathogen-free facilities at Brown University. Heterozygous matings were set up and offspring genetically identified utilizing the polymerase chain reaction (PCR). IL-2^{+/-} and IL-2^{-/-} mice were between 4 and 7 wk when used. All animals were maintained in a 12-h light/dark cycle with lights on at 7 A.M. Mice were sacrificed by cervical dislocation. Procedures were carried out in accordance with institutional guidelines for animal care and use.

Cytokines and Cytokine Assays. Murine rIL-12 (a gift from Genetics Institute, Inc., Cambridge, MA) was expressed from cloned cDNAs (23) provided by Drs. David Schoenhaut and Ueli Gubler (Hoffman-La Roche, Inc., Nutley, NJ). All experiments presented here were performed with lot MRB630717292A which had a specific activity of 8.4×10^6 U/mg (24). Endotoxin contamination, as measured in the *Limulus* ameobocyte assay, was <1.8 EU/mg. Concentrated aliquots of IL-12 were stored at -90°C and diluted in PBS containing 5% heat-inactivated male C57BL/6 serum (Taconic) immediately before use. Murine rTNF- α (endotoxin contamination of <0.1 ng/ μg), purchased from R & D Systems, Inc. (Minneapolis, MN), was reconstituted according to manufacturer specifications. Serum TNF was measured by ELISA using either commercially prepared kits from Genzyme Corporation (Cambridge, MA) or commercially available reagents described as follows. Capture hamster anti-mouse TNF antibody, TN3.19-12 (25), was used at 10 $\mu\text{g}/\text{ml}$ to coat Immulon 4 microtiter plates (Dynatech Laboratories, Chantilly, VA). After sample incubation at 37°C , bound TNF was detected with rabbit polyclonal anti-TNF (Endogen, Inc., Cambridge, MA) at 5 $\mu\text{g}/\text{ml}$, peroxidase-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) at 3 $\mu\text{g}/\text{ml}$, and the substrate ABTS (2,2'-azino-di [3-ethylbenzthiazoline sulfonate 6]) (Kirkegaard and Perry Labs, Gaithersburg, MD). Colorimetric changes in ELISAs were read at either 490 or 410, respectively, using a Dynatech MR 4000 plate reader. rIL-2 was a gift of Cetus Corp. and had a specific activity of 1.8×10^7 IU/mg.

In Vivo Treatment Protocols. Mice were either uninfected or infected *i.p.* with 2.5×10^4 PFU LCMV Armstrong strain clone E350. Except where specified, animals were treated with 100 μl vehicle, or vehicle containing 500 ng IL-12 (this dose of IL-12 is also referred to as “high dose”). In LCMV-infected mice, first injections were given 6 h after infection. Mice were treated with IL-12 or vehicle on each subsequent day for a total of seven injections. IL-2-treated mice were given 1,000 ng IL-2 per day. Where specified, mice were weighed using a portable digital scale (Ohaus, Florham Park, NJ) starting on day 0 before any injections were given, and then on each day after infection. Individual mice were identified by ear and/or toe markings. Samples for serum corticosterone were obtained retroorbitally, after methoxyflurane anesthesia (Pittman-Moore, Mundelein, IL), at the beginning of the light cycle (7–8 A.M.) under low stress conditions, *i.e.*, in less than 4 min from time of animal handling.

To evaluate functions of endogenous glucocorticoids in IL-12-induced toxicities, the type II steroid receptor antagonist, mifepristone (RU-38 486, hereafter called RU486) (Research Biochemicals International, Natick, MA) was formulated into 20-mg, 8-d release pellets (Innovative Research of America, Toledo, OH) giving 100 mg/kg/d. The dose was selected based on previous studies by others (26, 27) and our *in vivo* titrations examining thymic type II receptor occupancy by RU486 in a steroid receptor binding assay

¹ *Abbreviations used in this paper:* DIG, digoxigenin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LCMV, lymphocytic choriomeningitis virus; SSC, sodium chloride sodium citrate buffer.

as described (18, 28). Under the conditions used, >66% of the type II receptors were occupied by the antagonist. Pellets containing RU486 or placebo control were implanted into mice subcutaneously, in the subscapular region, one day prior to infection. In vivo activity of TNF was evaluated utilizing a neutralizing chimeric hamster/murine anti-TNF monoclonal antibody which has the Fab of TN3.19-12 combined with a murine IgG1 Fc region (29). Mice were treated with either 125 or 250 μg of purified antibody in PBS on days 0, 2, 4, and 6, relative to infection. The day 0 injection was given 3 h before infection with LCMV. Control animals were treated with equivalent amounts of similarly purified MOPC21, a monoclonal antibody with no known specificity (IgG1) derived from X63P3. Under the conditions used, circulating TNF was inhibited by ~50–60% as evaluated in ELISA.

Preparation and Analysis of Cells and Tissues. Leukocytes were isolated from macerated whole spleen after ammonium chloride lysis of erythrocytes. Flow cytometric analyses were performed at the Roger Williams Cancer Center of Brown University using a FACScan (Becton Dickinson, San Jose, CA) with argon laser output at 15 mw. Analyses of splenic T cell subsets were performed as described (9) using phycoerythrin-conjugated rat monoclonal RM-4-5 specific for mouse L3T4 to detect CD4 and FITC-conjugated rat monoclonal 53-6.7 specific for mouse Ly-2 to detect CD8 (PharMingen, San Diego, CA). Blast size cells were determined by analyzing forward scatter plots of gated CD8⁺ CD4⁻, or CD8⁻ CD4⁺ cells. Thymus glands were removed intact, trimmed of fat and connective tissue, and weighed on a Mettler PC 220 digital scale (Highstown, NJ). After weighing, glands were sectioned, fixed in 10% neutral buffered formalin for at least 15 h, Bouins fixative for 6 h, and paraffin embedded. 5- μm sections were stained with hematoxylin and eosin, and photographed with an Olympus OM-1N camera at 30 \times using Kodak Tungsten 64T Film and a Hoya neutral density filter.

Analysis of Viral Replication. Viral titers were measured in kidneys because these organs were readily available in all animals tested, and we have shown that changes in renal viral titers reflect the changes in other compartments (9). Tissue samples were frozen at -80°C , thawed, homogenized, and LCMV measured by plaque assays on Vero cells as described (9).

Preparation of Cellular RNA and Probes for Hybridization. Total splenic leukocyte RNA was prepared using RNazol-B (BIOTECX LAB., Houston, TX) according to manufacturer's recommendations. TNF- α and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probes were made from PCR-amplified cloned plasmid inserts (30, 31) or cDNA generated by reverse transcription of 1 μg of total RNA from C57BL/6 mice using Moloney Murine Leukemia Virus reverse transcriptase (GIBCO BRL, Gaithersburg, MD). 5 μl of cDNA template were then added to 1 \times PCR buffer (50 mM KCl, 10 mM Tris-HCl), 0.5 mM dNTPs, 1.5 mM MgCl₂, 0.25 U *Taq* DNA polymerase (Perkin-Elmer Corp., Norwalk, CT) and 50 pmol of TNF- α or GAPDH-specific primers (Clontech, Palo Alto, CA) in a final volume of 50 μl . The 692-base pair TNF- α and the 983-base pair GAPDH-specific cDNAs were amplified with the following cycling conditions: 30 s at 94°C , 30 s at 60°C , 1 min at 72°C for 30 cycles, followed by a 10-min extension at 72°C . Specificity was verified by Southern hybridization using internal oligonucleotide probes (Clontech). Specificity of the cDNA for TNF- α was verified by demonstration of hybridization to cloned TNF- α but not to cloned TNF- β sequences. An additional negative control probe prepared for in situ hybridizations was a 611-base pair GC low region amplified from the baker's yeast *SPR1* gene (32) (gift from Dr. Don Primerano, Marshall University School of Medicine, Huntington, WV). Probes were labeled by random priming (Prime-it, Stratagene, La Jolla, CA) with [³²P]dCTP for

Northern blot analysis or with digoxigenin-dUTP using the Genius System Labeling Kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) for in situ hybridization. PCR reactions were executed in a PTC-100 Programmable Thermal Cycler (MJ Research, Inc., Watertown, MA).

Northern Blot Analysis. Northern blot analyses were carried out using minor modifications of previously reported techniques (33). Briefly, samples were denatured, and separated by electrophoresis in a 1.2% agarose formaldehyde gel. The RNA was transferred to Hybond-N filters (Amersham Corp., Arlington Heights, IL), baked, and prehybridized as recommended by the manufacturer. Hybridizations were carried out overnight at 65°C . Filters were washed with 2 \times SSC with 0.1% SDS at room temperature and 0.1 \times SSC and 0.1% SDS at 65°C . Films were exposed without intensifying screens for linear quantitation. Where stated, densitometry was carried out with the Fotodyne Image Analysis System (Fotodyne, Hartland, WI).

In Situ Hybridization. Samples were prepared and analyzed using modifications of previously published procedures (34). Briefly, 10- μm cryostat splenic sections were prepared, fixed for 20 min in ice-cold 4% paraformaldehyde in PBS, dehydrated in ethanol, and stored at -80°C until hybridization. Sections were warmed to room temperature before prehybridization as follows: 15 min 0.3% Triton X-100 in 0.1 M Tris base, pH 7.5; 5 min 0.01 M Tris base; 10 min in a solution of 1 $\mu\text{g}/\text{ml}$ of proteinase K in Tris-EDTA (10 mM Tris base, pH 7.5, 1 mM EDTA, pH 8.0) at 37°C ; rinsed in nuclease-free water; rinsed in 0.1 M Triethanolamine, pH 8.0; 10 min 0.25% acetic anhydride in 0.1 M Triethanolamine; 5 min 0.2 \times SSC (Sodium chloride, Sodium Citrate buffer); and dehydrated with ethanol. Sections were immediately hybridized with 25 μl of labeled TNF- α probe in hybridization buffer overnight at 42°C in a humidified chamber. Sections were then washed twice for 10 min in 2 \times SSC, twice for 10 min in 0.1 \times SSC, and once for 15 min in 0.1 \times SSC at 55°C . Hybridization was detected by enzyme immunoassay using anti-digoxigenin-alkaline phosphatase with the substrate, nitrobluetetrazolium (NBT) salt, as described by the manufacturer (Nucleic Acid Detection Kit; Boehringer Mannheim Biochemicals). Control hybridizations with the PCR amplified yeast probe (see above) demonstrated specificity of the TNF- α probe.

In Vitro Assays. To evaluate TNF production, conditioned media were prepared by suspending splenic leukocytes from IL-12- or control-treated mice (uninfected or day 7 LCMV-infected) at 1×10^7 cells/ml in RPMI media (GIBCO) supplemented with 2 mM glutamine, sodium bicarbonate, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (GIBCO) and 10% FBS (HyClone Laboratories, Inc. Logan, UT). Cell suspensions were incubated in microtiter plates (100 $\mu\text{l}/\text{well}$) for 24 h at 37° in a 5% CO₂ incubator. To test for sensitivity to TNF, splenic leukocytes from day 6 LCMV-infected or uninfected mice were seeded, at 5×10^6 cells/ml, in complete RPMI media and 0, 0.1, 1, 10, 100, or 1,000 ng/ml TNF- α with or without 10 ng/ml IL-12. After incubation at 37°C in 5% CO₂, cells were removed from plates using successive rounds of ice cold 100% FBS and media washes. Cells were counted and examined for viability by trypan blue exclusion. T cell subsets were analyzed by flow cytometry as described above. CTL activity was determined by chromium release assay as described (35). Briefly, ⁵¹Cr (Dupont-New England Nuclear, Boston, MA) was used to label uninfected or LCMV-infected histocompatible MC57G target cells (36). Virus-specific lysis was determined as the difference between the lysis of infected and uninfected cells. Lysis was calculated as $100 \times (\text{cpm test sample supernatant} - \text{cpm spontaneous release}) / (\text{cpm maximum release} - \text{cpm spontaneous release})$. Effector to target cell ratios of 100:1, 33:1, 11:1, 3:1, and 1:1 were examined and individual assays were performed in quadruplicate; replicates of individual

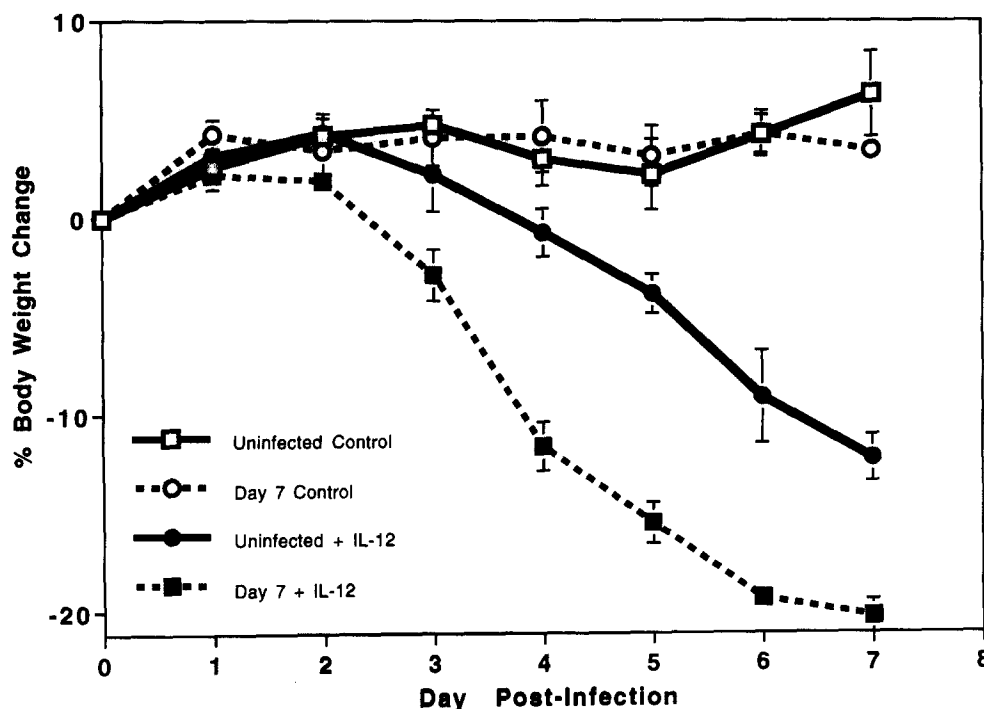


Figure 1. Body weight changes in response to IL-12 treatment and/or LCMV infection. Animals were weighed before any treatments were given and weight change each day was calculated based on the initial weight measurement. Animals were either uninfected (solid lines), or LCMV-infected (dashed lines) and IL-12-treated (solid symbols), or treated with vehicle (open symbols). Error bars represent SE, $n = 4$.

samples had SD of <10%. Samples were quantified in an Isoflex Automatic Gamma Counter (ICN Micromedex Systems, Huntsville, AL). Lytic units were determined as the number of leukocytes required to perform 10% virus specific lysis on 10^4 target cells.

Serum Corticosterone Analysis. Total serum corticosterone was measured by radioimmunoassay (RIA) using rabbit antiserum raised against corticosterone-21-hemisuccinate BSA as described (37). Assay sensitivity was 10 pg corticosterone.

Statistics. The Student's *t* test was performed where indicated.

Results

Physiological Toxicities Resulting from IL-12 Treatment. Our earlier report demonstrated IL-12 induced immunotoxicities in LCMV-infected but not uninfected mice, and induction

of circulating TNF in both groups (9). Gross and histological examinations were carried out to evaluate IL-12-elicited physiological changes. Uninfected C57BL/6 mice receiving high dose IL-12 (500 ng/d) had slightly ruffled fur and displayed mild lethargy after 7 d of treatment. In contrast, LCMV-infected, IL-12-treated mice displayed mild lethargy and ruffled fur by day 4 after infection and treatment, which became increasingly severe. By day 7 after infection, treated mice were hunched and inactive. Internal examination of the IL-12-treated animals revealed blanched internal organs. The alterations were more significant in LCMV-infected mice. These changes were accompanied by decreases in body weight. After 6 d of treatment, the IL-12-treated, uninfected mice displayed weight loss of >10% of total body weight (Fig. 1). Weight loss in infected IL-12-treated mice occurred more rapidly and to a

Table 1. Effects of High Dose IL-12 and LCMV Infection on Thymic Mass

| Experiment* | Uninfected [†] | Uninfected [§] + IL-12 | % Reduction uninfected | d7 LCMV [†] | d7 LCMV [§] + IL-12 | % Reduction infected |
|-------------|-------------------------|------------------------------------|---------------------------|----------------------|---------------------------------|---------------------------------------|
| | mg | mg | | mg | mg | |
| 1 | 49 ± 2 | 31 ± 6 | 37 | 47 ± 6 | 14 ± 1 | 70 |
| 2 | 46 ± 5 | ND | ND | 48 ± 3 | 12 ± 2 | 75 |
| 3 | 70 ± 20 | ND | ND | 67 ± 3 | 3 ± 3 | 95 |

* Experiments 1 and 2, $n = 4$; Experiment 3, $n = 3$. Results shown ± SE.

† Mice received daily vehicle injections as control treatment. Injections of infected mice were initiated at 6 h post-infection. A total of seven injections were given.

§ Mice received 500 ng/d IL-12. Injections of infected mice were initiated at 6 h after infection. A total of seven injections were given.

|| Percent reduction of thymic mass by IL-12 and LCMV infection compared to control LCMV infection was significant to $p < 0.0005$ for all values.

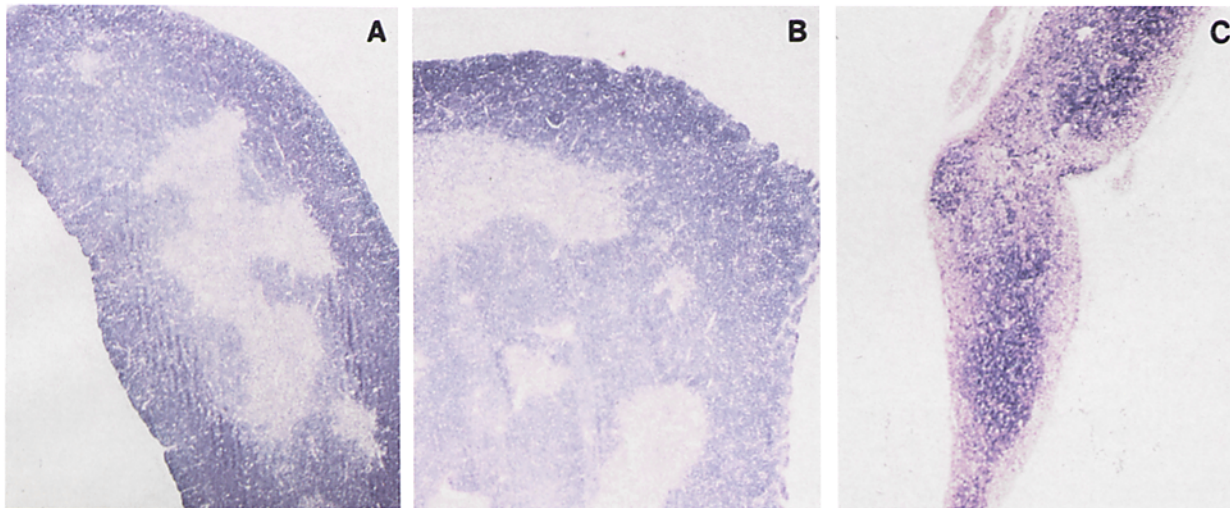


Figure 2. Effects of LCMV infection and IL-12 treatment on thymic morphology. Thymus glands were removed from uninfected, vehicle-treated animals (a), day 7 LCMV-infected, vehicle-treated animals (b), and day 7 LCMV-infected, IL-12-treated (500 ng/d) animals (c). Sections of fixed, paraffin embedded glands were stained with H & E. $\times 30$.

greater extent such that these animals lost >20% of body weight by day 6 after infection.

Another change observed in IL-12-treated mice was striking thymic atrophy (Table 1). In IL-12-treated, infected animals the thymus had atrophied such that only a small fibrous and largely acellular organ with little evidence of cortical or medullary structure remained (Fig. 2). In three different experiments the percent decrease in thymic mass in treated, infected animals was always statistically significant and greater than 70% (Table 1). Thymic atrophy evidenced in IL-12-treated, uninfected animals was also significant but less extensive than in infected animals. These observations demonstrate that IL-12 induces physiological toxicities and LCMV infection dramatically synergizes to exacerbate these toxicities.

IL-12 Induction of TNF Expression In Vivo. The aforementioned physiological toxicities are known to be associated with circulating TNF. We have previously shown that in vivo administration of high dose IL-12 induces serum TNF in unin-

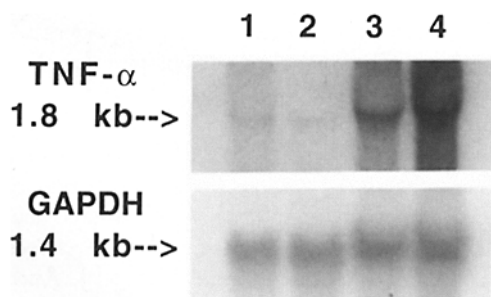


Figure 3. Northern blot analysis of TNF- α from splenic mRNA. Total cellular RNA was prepared from splenic leukocytes isolated from mice that were uninfected (lane 1), day 7 LCMV-infected (lane 2), uninfected and treated with 500 ng/d IL-12 (lane 3), or infected and treated with 500 ng/d IL-12 (lane 4). Blots were prepared and hybridizations were carried out as described in Materials and Methods. Results of hybridization with cDNA probes specific for murine TNF- α and GAPDH are shown.

fected mice and LCMV infection synergizes with IL-12 for serum TNF induction (9). In vitro experiments were carried out to examine TNF production by splenic leukocytes. Leukocytes from LCMV-infected C57BL/6 mice that had been control treated or treated with 500 ng/d IL-12 were used to condition media for analysis by ELISA. On a per cell basis, cells from IL-12-treated LCMV-infected animals produced 128–328 pg/million cells TNF whereas those from control-treated LCMV-infected animals produced 34–122 pg/million cells. These studies show that IL-12 induces TNF production by splenic leukocytes.

Because antibodies against TNF generally react with both TNF- α and TNF- β (LT), it is difficult to distinguish the two molecules using these reagents. To specifically examine induction of TNF- α , mRNA was prepared for Northern blot hybridization analysis with a probe specific for TNF- α (see Materials and Methods). Splenic leukocytes from uninfected or day 7 LCMV-infected C57BL/6 mice given either vehicle or IL-12 were used. Extremely low levels of TNF- α were detected in mRNA from uninfected (Fig. 3, lane 1) or day 7 infected mice (Fig. 3, lane 2). Administration of 500 ng/d IL-12 to uninfected animals dramatically induced TNF- α (Fig. 3, lane 3). Administration of IL-12 to LCMV-infected animals induced substantially more TNF- α (Fig. 3, lane 4) than did either LCMV infection alone or IL-12 administration to uninfected mice. Densitometric scanning revealed that IL-12 induced a four- to fivefold increase in TNF- α mRNA expression in uninfected and a 7- to 10-fold increase in LCMV-infected mice. These data demonstrate that the accumulation of TNF- α mRNA is induced by IL-12 and that there is synergy between IL-12 and LCMV infection for this induction.

To evaluate levels of TNF- α message per cell and frequency of positive cells, in situ hybridizations were carried out on splenic sections as described in Materials and Methods. Uninfected, control-treated mice showed little to no detectable TNF- α expression (Fig. 4 a). On day 7 after infection with

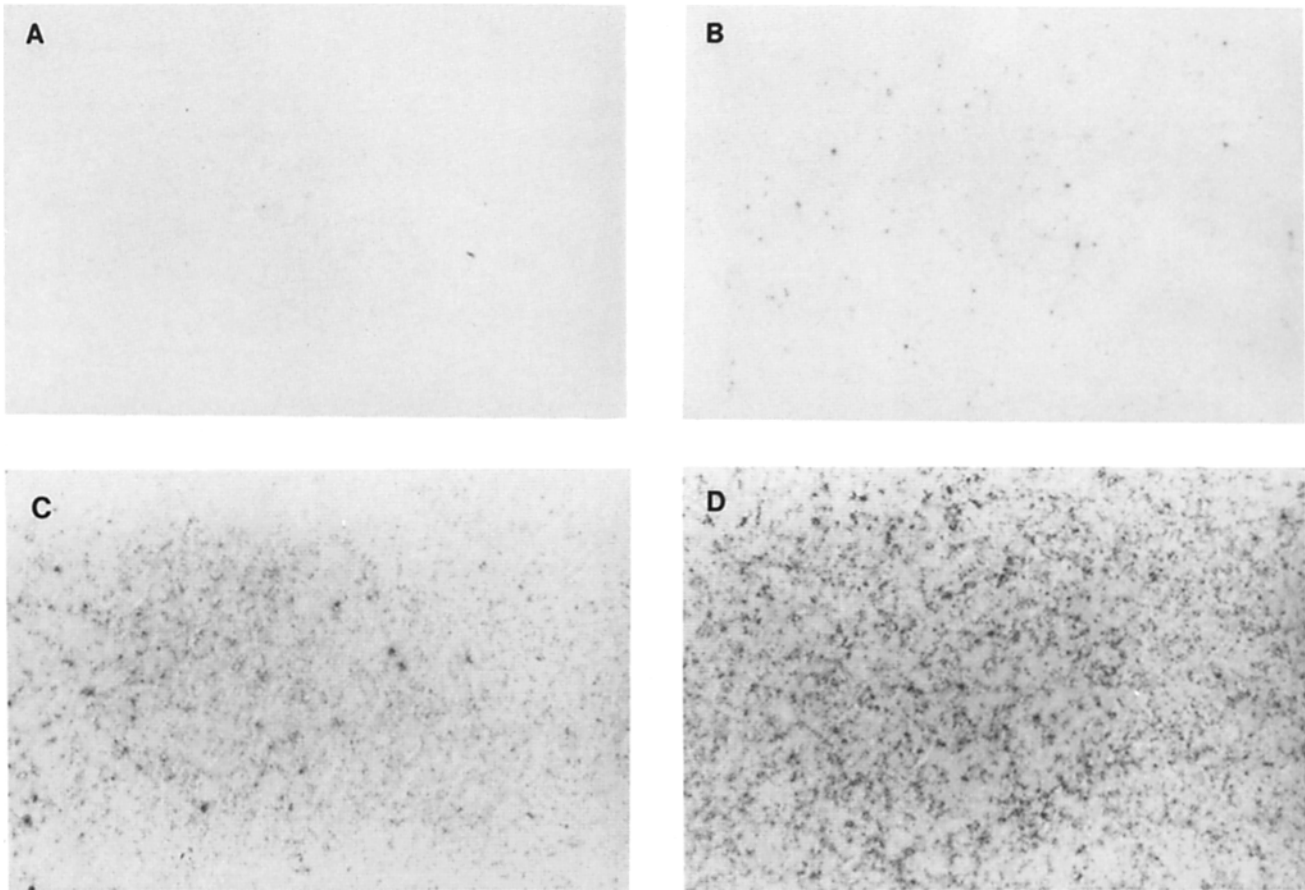


Figure 4. In situ hybridization for TNF- α mRNA. Spleens were harvested from animals that were uninfected, control treated (a), day 7 LCMV-infected, control-treated (b), uninfected, 500 ng/d IL-12-treated (c), and day 7 LCMV-infected, 500 ng/d IL-12 treated (d). Frozen sections were hybridized with TNF- α cDNA probe as described in Materials and Methods. Positive signals were detected in an enzyme immunoassay using alkaline phosphatase and nitrobutetrazolium substrate. Tissues were not counterstained. $\times 30$.

LCMV, there was low TNF- α expression in an infrequent population of cells (Fig. 4 b). Compared to LCMV infection, 500 ng/d IL-12 given to uninfected animals induced substantially more TNF- α expression than did LCMV infection; there were more positive cells with greater intensities in these spleens (Fig. 4 c). LCMV-infected animals treated with IL-12 showed a synergistic induction of TNF expression (Fig. 4 d). Individual cells producing TNF in the spleens of IL-12-treated animals were significantly more intense and more frequent. Thus, IL-12 treatment not only increased the levels per cell, but also the proportions of cells expressing detectable levels of TNF- α mRNA. Taken together, these studies show that IL-12 induces TNF- α expression in vivo and that LCMV infection synergizes with IL-12 for TNF- α expression.

IL-12 Induction of Serum Corticosterone. Because TNF can stimulate the production of elevated levels of endogenous glucocorticoids (16), the levels of corticosterone were measured in IL-12-treated mice. Serum corticosterone was measured in samples drawn at early times in the light cycle to quantitate changes at the nadir of the diurnal cycle. Administration of IL-12 to uninfected animals induced significant levels of serum corticosterone (Fig. 5 a). IL-12 administration and

LCMV infection induced a threefold increase in corticosterone levels compared to uninfected, IL-12-treated animals. Mice receiving the first doses of IL-12 on day 1 before infection, on the day of infection, 0, or 1 or 2 after infection all had high serum corticosterone levels (Fig. 5 b). These high levels of corticosterone were specific to IL-12 treatment; equivalent amounts of IL-2, on a weight basis, induced no detectable steroid response (Fig. 5 b). These data demonstrate that IL-12 elicits a significant serum corticosterone response and LCMV infection synergizes for this effect.

Role of Endogenous TNF in IL-12-Induced Toxicities during Infection. To characterize the role of TNF in IL-12-induced toxicities, anti-TNF monoclonal antibodies were used to neutralize TNF in vivo. The neutralization protocols cleared $\sim 60\%$ of serum TNF (see Materials and Methods). Antibody treatments did not inhibit TNF production as conditioned media levels of TNF were comparable to control animals. Serum corticosterone in LCMV-infected, IL-12-treated mice was substantially decreased by anti-TNF treatment (Fig. 6). This demonstrated that IL-12 induced serum corticosterone was largely TNF dependent.

Anti-TNF treatment had numerous other protective effects

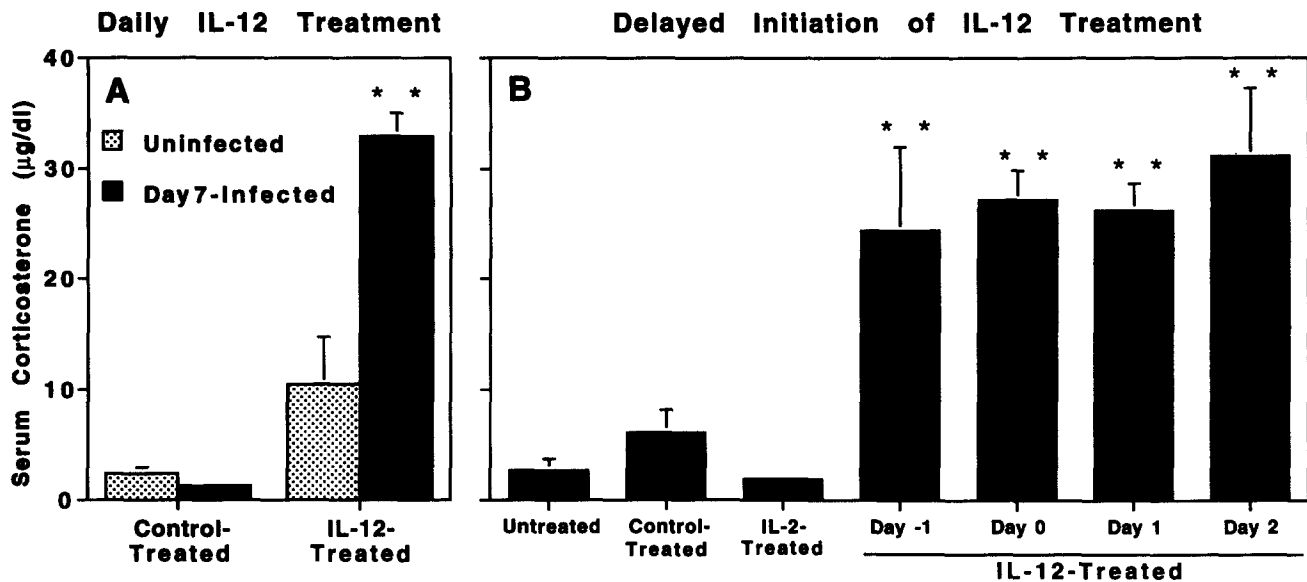


Figure 5. Induction of serum corticosterone by IL-12 administration. Mice were bled after daily IL-12 treatments under low stress conditions as described in Materials and Methods. Serum was tested for presence of corticosterone by RIA. Results presented are means of 3-4 animals \pm SE. (a) Comparison of uninfected and LCMV-infected animals treated with 500 ng/d IL-12 or equivalent volumes of vehicle for 7 d. The synergy in corticosterone induction between IL-12 treatment and IL-12 treatment plus LCMV infection is significant $p < 0.005$ (**). (b) Delayed treatment with IL-12 was examined by beginning IL-12 treatment on day -1, 0, 1, or 2 relative to infection. Levels induced were greater than levels in vehicle-treated control animals $p < 0.05$ (**). IL-2 was administered diluted in vehicle as a cytokine control.

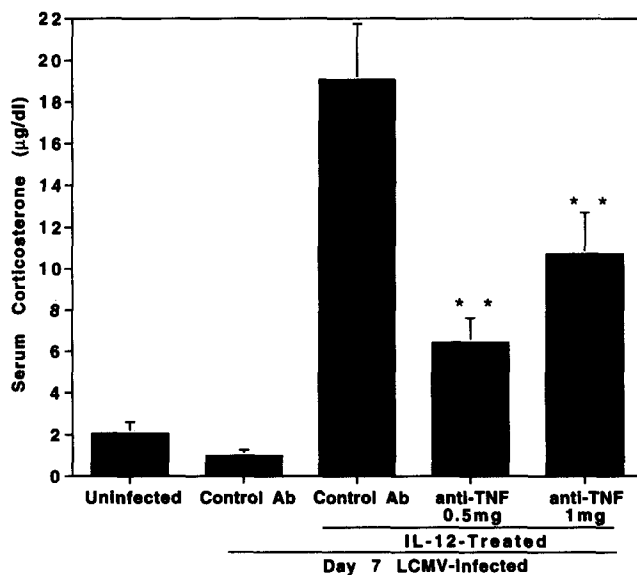


Figure 6. Effects of anti-TNF treatment on IL-12-mediated induction of serum corticosterone. LCMV-infected animals were treated with 500 ng/d IL-12 or vehicle and given a total of either 0.5 mg or 1 mg chimeric hamster/murine anti-TNF TN3.19-12/IgG1 or 1 mg MOPC21 IgG1 in four equivalent doses on days 0, 2, 4, and 6. Animals were bled under low stress conditions on day 7 and serum was evaluated for the presence of corticosterone by RIA. Results presented are the means of 6 animals \pm SE. Reduction in serum corticosterone in IL-12-treated animals given anti-TNF compared to those given MOPC21 was significant $p < 0.01$ (**).

in infected, IL-12-treated animals. First, anti-TNF-treated animals displayed less lethargy and fur ruffling at day 7 after infection. These animals also displayed significantly less cachexia and thymic atrophy. Mice given either a total of 0.5 or 1.0 mg of anti-TNF had about one-half the body (Fig. 7 a) and thymic (Fig. 7 b) weight losses of control-treated infected animals.

In addition to providing systemic protection from IL-12-mediated physiological toxicities, anti-TNF treatment reversed much of the immunotoxicity against CD8⁺ T lymphocytes. Administration of anti-TNF to LCMV-infected, IL-12-treated mice allowed for a greater expansion of the CD8⁺ T cell population (Fig. 7 c); anti-TNF-treated animals had greater than five million more CD8⁺ T cells per spleen than control-treated animals. CTL activity, measured as virus-specific lytic units per spleen, was also improved; anti-TNF-treated animals had greater than twofold more lytic units per spleen than IL-12-treated antibody control-treated animals. The anti-TNF-mediated improvements in immune parameters were accompanied by decreased viral replication (Fig. 7 d). Anti-TNF treatment allowed for up to a 2 log reduction in renal LCMV titers. Taken together, these data demonstrate that endogenously produced TNF contributed to all measured toxicities induced by IL-12 in LCMV-infected animals, and that TNF was pivotal for the IL-12 induction of serum corticosterone.

Sensitivity of CD8⁺ T Cell Responses to TNF-mediated Inhibition. To characterize sensitivities of in vivo elicited T cell populations to TNF-mediated toxicity, experiments were carried out in culture with splenic leukocyte populations isolated from uninfected mice and mice on day 6 after infection.

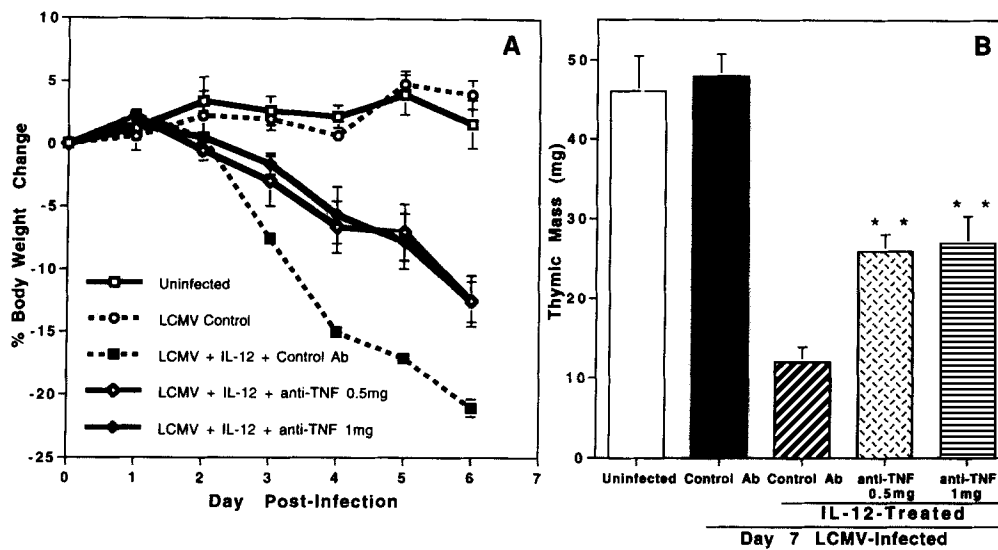


Figure 7. Effects of anti-TNF treatment on parameters of IL-12-mediated toxicity. LCMV-infected animals were treated with 500 ng/d IL-12 or vehicle and given a total of either 0.5 mg or 1 mg chimeric hamster/murine anti-TNF TN3.19-12/IgG1 or 1 mg MOPC21 IgG1 in 4 equivalent doses on days 0, 2, 4, and 6. Mice were weighed before any treatment was given and then weighed on each subsequent day before daily injection. Percent weight change was calculated using the initial reading as standard (a). On day 7 after infection animals were killed and thymus glands were weighed (b), splenic CD8⁺ T cells were quantitated by flow cytometry (c), viral replication was measured by plaque assay on Vero cells (d). Animals were treated five different ways: uninfected with vehicle control (□); LCMV-infected with 1 mg MOPC21 and vehicle control (■); LCMV-infected with 1 mg MOPC21 and 500 ng/d IL-12 (▨); LCMV-infected with 0.5 mg chimeric anti-TNF and 500 ng/d IL-12 (▧); and LCMV-infected with 1 mg chimeric anti-TNF and 500 ng/d IL-12 (▩). Means of four animals are shown ± SE. IL-12-treated animals given anti-TNF were statistically different from IL-12-treated animals given control antibody $p < 0.05$ (**).

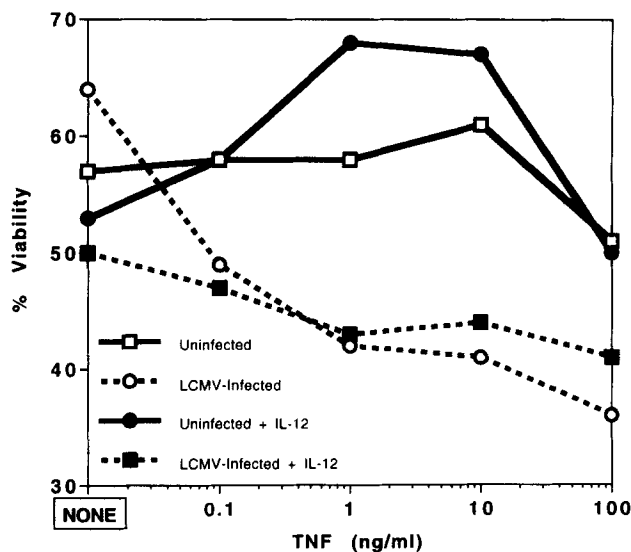
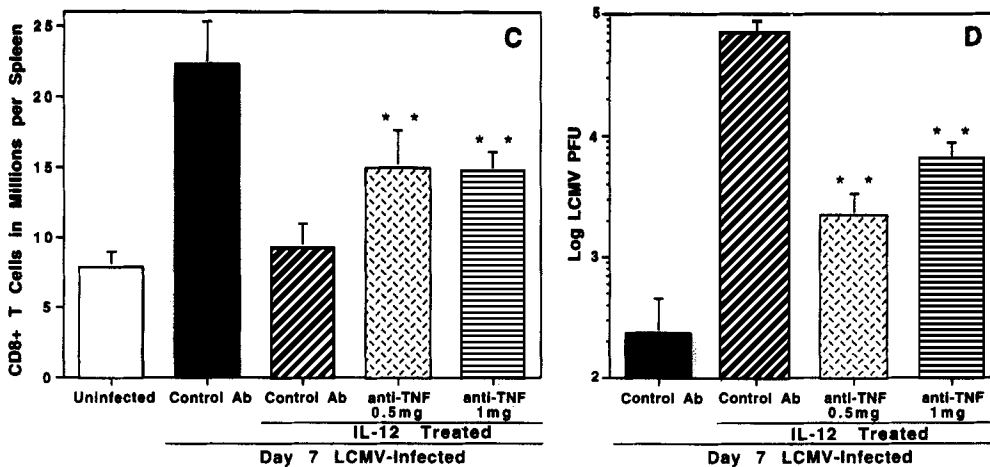


Figure 8. In vitro effects of TNF and/or IL-12 on splenic leukocytes. Splenic leukocytes were isolated from uninfected (solid lines) or LCMV-

infected (broken lines) animals on day 6 after infection. Populations were incubated for 24 h with either 0, 0.1, 1, 10, or 100 ng TNF- α /ml, with (solid symbols) or without (open symbols) 10 ng/ml IL-12. Cells were harvested from culture plates and viability was measured by trypan blue exclusion.

Effects of TNF were examined after 24 h in culture, i.e., day 7 after infection. TNF concentrations used were 0, 0.1, 1, 10, 100 ng/ml. Enhancing effects of IL-12 on TNF activity were investigated by adding 10 ng/ml IL-12 along with TNF. TNF induced a dose-dependent decrease in cell viability of populations from infected but not uninfected mice (Fig. 8). Addition of IL-12 did not substantially change TNF-mediated cytotoxicity. The decrease in viability was accompanied by a specific inhibition of total and blast-sized CD8⁺ T cells in populations from infected but not uninfected mice (Table 2). This inhibition was decreased further by addition of IL-12 (Table 2). Virus specific lytic units per culture were also decreased by TNF and further inhibited by addition of IL-12 (Table 2). These data demonstrated that the virus-activated

Table 2. *In Vitro* Effects of TNF or TNF and IL-12 on CD8+ T Cells from LCMV-Infected Animals*

| Cells | Cytokines | CD8+ [†] T Cells | Blast CD8+ [†] T Cells | Virus Specific Lytic Units |
|------------|--------------------------|------------------------------|------------------------------------|-------------------------------|
| Uninfected | | 6.49 | 0.41 | 0 |
| Uninfected | TNF [‡] | 6.68 | 0.48 | 0 |
| Uninfected | TNF + IL-12 [§] | 8.90 | 0.64 | 0 |
| Uninfected | IL-12 | 6.59 | 0.40 | 0 |
| Day 6 LCMV | | 18.71 | 11.50 | 424 |
| Day 6 LCMV | TNF [‡] | 14.31 | 5.89 | 276 |
| Day 6 LCMV | TNF + IL-12 [§] | 9.71 | 2.85 | 135 |
| Day 6 LCMV | IL-12 | 24.40 | 9.73 | 153 |

* Splenic leukocytes were removed from uninfected, or LCMV-infected animals on day 6 after infection, and placed in culture with or without cytokine for 24 h.

‡ TNF was added to culture at 100 ng/ml.

§ TNF was added to culture at 100 ng/ml and IL-12 was added at 10 ng/ml.

|| IL-12 was added to culture at 10 ng/ml.

† CD8+ T cells and blast CD8+ T cells are expressed as cells recovered from culture × 10⁵.

** CTL activity is expressed as LCMV specific lytic units per culture at 10% lysis.

populations were particularly sensitive to TNF-mediated inhibition.

Role of Endogenous Glucocorticoids in IL-12-induced Toxicities during Infection. To evaluate the role of TNF-induced glucocorticoids in the IL-12-induced physiological and immunological toxicities during LCMV infection of C57BL/6 mice, the type II corticosteroid receptor antagonist, RU486, was used. For these studies, RU486 was delivered at 100 mg/kg/d in time-released pellets. Under these conditions two-thirds of the available type II receptors were occupied after RU486 treatment (see Materials and Methods). The overall appearance of the infected, IL-12-treated mice given RU486

was not noticeably different than that of infected placebo controls. Both sets of animals had the previously mentioned signs of toxicity, i.e., lethargy, hunching, and ruffled fur. However, IL-12-treated animals given RU486 had statistically significantly larger thymus glands compared to placebo-treated animals (Fig. 9 a). Moreover, histological examination of thymus from animals receiving RU486 revealed a cellular gland with recognizable cortical and medullary regions (data not shown).

Although RU486 reversed thymic atrophy, it did not restore normal immune function or anti-viral status. In contrast to the anti-TNF treatments (Fig. 7), there was no

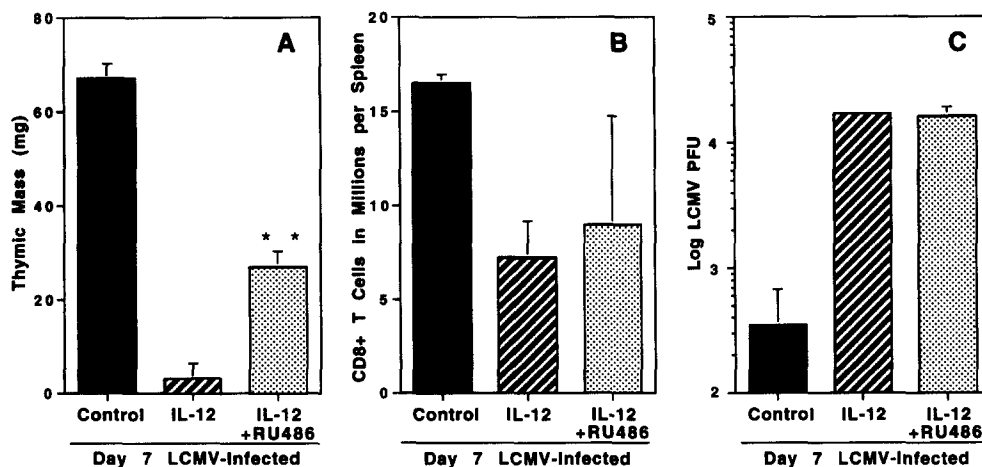


Figure 9. Effects of RU486 on parameters of IL-12-mediated toxicity. An 8-d time release pellet containing either RU486 or placebo was surgically implanted in mice the day before LCMV infection. RU486 was administered at ~100 mg/kg/d from pellets releasing 20 mg over 8 d at 0.1 mg/h. Mice were either vehicle-treated with placebo pellets (■), IL-12-treated (500 ng/d) with placebo pellets (▨), or IL-12 treated with RU486 pellets (▩). Thymic mass was measured on an analytical balance (a), CD8+ T cells were quantitated by flow cytometry (b), and viral titers were measured by plaque assay on Vero cells (c). Values are averages of four mice per group and error bars represent SE. RU486-treated animals were significantly different from IL-12-treated control animals, $p < 0.01$ where noted (**).

Table 3. Requirement for IL-2 in Synergy between Viral Infection and IL-12

| Mice | % Enhancement of IL-12-Induced* Changes by LCMV Infection | | | |
|---------------------|---|------------------|------------------|----------------------|
| | Weight loss | Thymic atrophy | Serum TNF | Serum corticosterone |
| IL-2 ^{-/-} | 4.1 [†] | 20 | 9 [‡] | 23 [‡] |
| IL-2 ^{+/-} | 16.4 [§] | 43 | 586 [§] | 389 [¶] |

* Percent change in IL-12-treated, LCMV-infected mice as compared to IL-12-treated, uninfected littermates. Groups of five to eight animals were used.

[†] Synergy is not significant.

[§] Synergy is significant $p < 0.0001$.

^{||} Synergy is significant $p < 0.05$.

[¶] Synergy is significant $p < 0.005$.

significant increase in numbers of CD8⁺ T cells (Fig. 9 *b*) or in LCMV titers (Fig. 9 *c*) resulting from RU486 treatment of IL-12-treated infected mice. There was also no significant difference in the CTL-mediated virus specific lytic units per spleen (data not shown). In IL-12-treated, infected animals administered RU486 there was still greater than 200 pg/ml TNF. Taken together, these results demonstrate that, in IL-12-treated mice, serum glucocorticoids were not required for the specific immunotoxicities on CD8⁺ T cell responses but did contribute to thymic atrophy.

IL-2 Requirement for Synergism between IL-12 and LCMV Infection. LCMV infection induces IL-2 (10, 11), and IL-2 has potent synergy with IL-12 in vitro (38, 39). Therefore, the involvement of endogenous IL-2 in the synergistic toxicity between LCMV and exogenous high dose IL-12 was evaluated. For these experiments effects of IL-12 administration were examined in mice made IL-2 deficient by homozygous mutation of the IL-2 gene, or their heterozygous littermates (22). There was a decreased synergy for toxicity between LCMV infection and IL-12 administration in IL-2^{-/-} as compared to IL-2^{+/-} littermates (Table 3). There was 75% less decrease in body weight loss and 50% less decrease in thymic atrophy between IL-12 treatment and LCMV infection in IL-2^{-/-} mice as compared to the +/− littermates. There was also 95% less increase in serum TNF and corticosterone induction. IL-2^{-/-} mice were less lethargic and showed less ruffling of fur than IL-2^{+/-} littermates. These data demonstrate that IL-2 is required for synergistic induction of endogenous TNF and corticosterone as well as synergistic physiologic toxicity between LCMV infection and high dose IL-12 administration.

Discussion

The studies presented here used uninfected and LCMV-infected mice to investigate effects of IL-12 treatment. IL-12-induced TNF factor expression and TNF- α mRNA expres-

sion in vivo. Viral infection synergized with administered IL-12 in this induction. As the synergism was a result of increased frequency of TNF- α positive cells and dependent upon endogenous IL-2, it appeared to be a consequence of endogenous immune responses to infection. A panel of physiological changes, known to be associated with TNF expression, were induced in IL-12-treated mice. These included: (a) wasting with significant decreases in body weights, (b) elevated circulating glucocorticoids, and (c) thymic atrophy. Changes in all of these parameters were dramatically elevated in virus-infected mice. An additional immunotoxicity was observed in the virus-infected mice receiving IL-12; CD8⁺ CTL responses were profoundly inhibited and viral replication was increased. As antibodies neutralizing TNF reversed the IL-12-induced immunotoxicity, wasting, circulating glucocorticoids, and thymic atrophy, TNF was responsible for all of the observed toxicities. Experiments with the type II receptor antagonist, RU486, indicated that glucocorticoids were intermediaries in the induction of thymic atrophy. The TNF-induced immunotoxicity correlated with an increased sensitivity of virus-elicited lymphocytes to TNF-mediated killing. The results clearly demonstrate a mechanism for IL-12-mediated immunotoxicity. To our knowledge, this is the first report of a TNF-mediated inhibition of specific protective immune responses. The results have major implications concerning potential detrimental consequences of in vivo TNF induction, by any pathway, for expansion of protective CD8⁺ T cell responses to viral infections.

The characterization of the profound immunotoxicities against CD8⁺ T cells and anti-viral defenses as TNF-mediated toxicities is unique. The studies are an extension of our earlier work demonstrating IL-12 induction of these immunotoxicities (9), i.e., they show that the IL-12-elicited effects are dependent upon endogenous TNF (Figs. 7, *c* and *d*). The earlier report presented experiments examining IL-12 effects on in vivo CD8⁺ T cell numbers in both infected and uninfected mice. Those studies demonstrated that the reduction in CD8⁺ T cell numbers was only apparent in virus-infected animals. Although the differential in vivo sensitivities may be in part a consequence of the higher levels of TNF induced by IL-12 in LCMV-infected mice, the culture studies presented here clearly show (a) that in vivo elicited splenic populations from infected mice are 3 log more sensitive than those from uninfected mice to killing mediated by exogenously added TNF (Fig. 8), and (b) that this differential sensitivity is apparent in CD8⁺ T cell populations (Table 2). Taken together, the results identify a previously unappreciated toxic effect of TNF, i.e., killing of specific T cell populations undergoing activation and proliferation in response to stimulation during viral infection.

The culture studies also indicate that IL-12 is not required for but does enhance the immunotoxicity induced by added TNF (Fig. 8 and Table 2). IL-12 can promote sensitivity of cells to TNF-mediated effects. IL-12 has been shown to induce expression of TNF receptors (2, 39, 40). Furthermore, IL-12 induces IFN- γ expression (41) and this factor also increases sensitivity to and cell priming for TNF-mediated tox-

icity (42, 43). IL-2 is an additional cytokine which may independently contribute to the increased TNF sensitivity of activated T cells. This factor also induces TNF receptor expression (39). Thus, IL-12 administration and/or endogenous IL-2 production may promote TNF hyper-responsiveness of activated T cells.

The IL-12-induced corticosterone levels reported here were as high as 30 $\mu\text{g}/\text{dl}$ in sera from day 7 LCMV-infected mice (Fig. 5). These levels were in striking contrast to the baseline levels in either uninfected or day 7 infected control-treated mice, and the modestly induced levels in IL-12-treated uninfected mice. The IL-12-induced corticosterone responses are particularly remarkable for two reasons. First, the assay samples were collected under low stress conditions at the nadir of the mouse diurnal cycle. Thus, the high levels were reached without induction through these pathways. Second, the dramatically elevated corticosterone levels could be observed a full 24 h after the last IL-12 treatment. This is in contrast to studies demonstrating induction of corticosterone in response to other cytokines. Bolus doses of TNF (15, 16), interleukin-1 (IL-1) (16, 44–46), and interleukin-6 (IL-6) (16) have all been shown to induce corticosterone. In contrast to the IL-12-induced responses, however, corticosterone responses to TNF, IL-1, and IL-6 are all short-lived and return to baseline levels by 2–6 h after treatment. The relatively long half-life of IL-12 and the resulting induction of TNF (Figs. 3 and 4) (9) are factors which could promote the long-lived corticosterone response induced by IL-12.

Treatments with antibodies neutralizing TNF blocked the peak IL-12-induced corticosterone responses measured here (Fig. 6). Thus, the TNF induced by IL-12 elicited corticosterone induction. These results provide insights about cytokine/glucocorticoid response cascades induced in other conditions such as endotoxic shock induced by gram-negative bacteria (14, 47, 48). Studies of experimental endotoxic shock models have demonstrated the acute induction of TNF, IL-1, and IL-6 (14, 48), and the induction of a glucocorticoid response to each of these cytokines administered alone (15, 16, 44–46). It has been suggested that these pathways are in place to induce glucocorticoid-mediated down-regulation of detrimental immune responses during endotoxic shock (47). Studies neutralizing TNF have shown that in vivo elicited TNF plays a pivotal role in the induction of IL-1 and IL-6 (14). However, the pivotal role of TNF in the induction of the glucocorticoid response has not been previously reported. The studies presented here demonstrate that, if TNF is inhibited, corticosterone induction is also blocked.

Both neutralization of TNF (Fig. 7 *b*) and blocking of the type II corticosteroid receptor with the antagonist RU486 (Fig. 9 *a*) reversed the thymic atrophy observed in IL-12-treated infected mice. Taken together with the demonstrated role for TNF in corticosterone induction (Fig. 6), the studies indicate that thymic atrophy is secondary to endogenous glucocorticoid induction and downstream activation of the intracellular receptor. This is consistent with the known steroid sensitivity of thymocytes (17, 49) and previously reported thymic atrophy resulting from repeated treatments with other cytokines in-

ducing elevated levels of endogenous corticosterone (46).

The IL-12-induced physiological toxicities and elevation of endogenous TNF and glucocorticoids seen in this and the preceding studies (9) are strikingly similar to those induced by lipopolysaccharide in experimental models of endotoxic shock (14, 25, 48, 50). Interesting new evidence indicates that endogenous IL-12 is part of the cytokine cascade elicited during endotoxic shock: (a) a factor with the characteristics of IL-12 is induced in vivo by endotoxin (51, 52); (b) administration of IL-12 increases lethality in murine models of endotoxic shock induced by the gram-negative bacteria *Escherichia coli* (7); and (c) IL-12 is expressed in the cytokine cascade at times preceding TNF expression in certain models of endotoxic shock (53). In contrast to toxicities in response to endotoxin, however, the toxicities reported here after IL-12 administration require additional in vivo conditions provided by the viral infection. The magnitude of the TNF response induced by 500 ng/d of IL-12 in uninfected mice is clearly sub-lethal and precipitates generally mild physiological changes, whereas administration of the same dose to LCMV-infected mice gives rise to TNF levels sufficient to induce dramatic physiological and pathological changes. The IL-12-treated infected mice have toxicity in a variety of tissues and undergo profound wasting (Fig. 1) and thymic atrophy (Table 1, Fig. 2). Indeed, LCMV-infected mice given high enough doses of IL-12 (500–1,000 ng/d) for long enough periods of time (~8–9 d) will die (data not shown). Thus, administration of IL-12 in the context of the response to this virus drives no apparent or marginally apparent toxic effects to extremely detrimental effects. These results are particularly significant because the doses eliciting toxicity in the context of a viral infection are similar to IL-12 doses eliciting protective effects in mice infected with intracellular parasites (4, 6) or bacteria (7) and in tumor-bearing mice (8).

The conditions responsible for the synergism between exogenously added IL-12 and LCMV infection are not completely understood. We have previously shown that IL-2 is induced during LCMV infections of normal mice (10, 11), and IL-2 has been shown to synergize with IL-12 in vitro to promote cytotoxic effector cell activity (3, 54), proliferation of activated T cells (55, 56), and IFN- γ production (41). Although the mechanism responsible for known synergism between IL-12 and IL-2 may be multifactorial, it results in part from reciprocal induction of receptors, i.e., IL-12 induces the p55 IL-2R (57) and IL-2 induces the IL-12R (58). The studies presented here with IL-2-deficient mice clearly indicate that endogenous expression of this factor is required for the synergism between infection and IL-12 leading to elevated TNF expression, circulating corticosterone, and the accompanying physiological toxicities (Table 3). These results, along with the demonstrated increase in frequency of cells expressing TNF in IL-12-treated infected (Fig. 4 *d*) as compared to uninfected (Fig. 4 *c*) mice, indicate that the synergism is a direct consequence of the ongoing immune response.

The highly significant reversals of toxicities seen with either anti-TNF antibody or RU486 treatments were greater than 60 but not 100%. The level of reversal achieved was

proportional to the in vivo efficacy achieved with each of the treatments, i.e., anti-TNF neutralized ~50–60% of the circulating TNF and RU486 blocked the type II glucocorticoid receptors by ~66% (see Materials and Methods). Thus, although the toxicities remaining after treatment may be mediated by other unidentified factors, it is more likely that they are mediated by residual free TNF or glucocorticoid receptors. As we were unable to mediate any additional blocking by delivering higher concentrations of anti-TNF or RU486, the conditions used appeared to be as optimal as possible for in vivo blocking.

To sum up the results and their implications, the studies presented here demonstrate profound and detrimental consequences resulting from IL-12 and IL-12-induced TNF during viral infections. They suggest that, during viral infections, any conditions which promote elevated endogenous IL-12 and/or TNF expression could be detrimental to protective CD8⁺ T cell responses. Thus, infections with endotoxin-positive bacteria could block anti-viral CD8⁺ T cell responses and anti-viral states by inducing elevated IL-12 (53) and TNF (12, 13). The results also suggest that, if IL-12 plays

an enhancing role in CD8⁺ T cell responses, to promote these protective responses without inducing inhibitory TNF, the levels of endogenously produced IL-12 will have to be very low. The data predict that the physiological changes characterized with endotoxic shock may result from either synergism between bacterial and viral infections and/or viral infections which induce significant levels of IL-12. These predictions may provide insights into the Dengue hemorrhagic fever/Dengue shock syndrome (DHF/DSS) pathology sometimes seen during secondary infections with Dengue virus (59). Individuals with DHF/DSS have elevated levels of serum TNF (60), IL-2 (61), and IFN- γ (61). Interestingly, Dengue virus stimulation is reported to induce a "helper cytokine" sharing certain biochemical characteristics with IL-12 (62, 63). Thus, the results presented here may help to explain a broad range of complications reported during other viral infections and/or other conditions of viral infection. Finally, the studies demonstrate that administration of cytokines, which promote protective responses in one context, can be detrimental to the host in the context of particular endogenous immune responses.

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