The Role of Interleukin 12 and Nitric Oxide in the Development of Spontaneous Autoimmune Disease in MRL/MP-*lpr/lpr* Mice

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Summary

MRL/MP-*lpr/lpr* (MRL/*lpr*) mice develop a spontaneous autoimmune disease. Serum from these mice contained significantly higher concentrations of nitrite/nitrate than serum from age-matched control MRL/MP-+/+ (MRL/+), BALB/c or CBA/6J mice. Spleen and peritoneal cells from MRL/*lpr* mice also produced significantly more nitric oxide (NO) than those from the control mice when cultured with interferon (IFN) γ and lipopolysaccharide (LPS) in vitro. It is interesting to note that peritoneal cells from MRL/*lpr* mice also produced markedly higher concentrations of interleukin (IL) 12 than those from MRL/+ or BALB/c mice when cultured with the same stimuli. It is striking that cells from MRL/*lpr* mice produced high concentrations of NO when cultured with IL-12 and LPS, whereas only low or background levels of NO were produced by similarly cultured cells from MRL/+ or BALB/c mice. The enhanced NO synthesis induced by IFN-γ/LPS was substantially inhibited by anti-IL-12 antibody. In addition, IL-12–induced NO production can also be markedly inhibited by anti-IFN-γ antibody, but only weakly inhibited by anti–tumor necrosis factor α antibody. The effect of IL-12 on NO production was dependent on the presence of natural killer and possibly T cells. Serum from MRL/*lpr* mice contained significantly higher concentrations of IL-12 compared with those of MRL/+ or BALB/c control mice. Daily injection of recombinant IL-12 led to increased serum levels of IFN-γ and NO metabolites, and accelerated glomerulonephritis in the young MRL/*lpr* mice (but not in the MRL/+ mice) compared with controls injected with phosphate-buffered saline alone. These data, together with previous finding that NO synthase inhibitors can ameliorate autoimmune disease in MRL/*lpr* mice, suggest that the high capacity of such mice to produce IL-12 and their greater responsiveness to IL-12, leading to the production of high concentrations of NO, are important factors in this spontaneous model of autoimmune disease.

MRL/MP-*lpr/lpr* (MRL/*lpr*)[1] mice develop a spontaneous autoimmune disease and have been used extensively as a model for clinical SLE. The disease is characterized by lymphadenopathy, autoantibody production, and inflammatory manifestations such as nephritis, vasculitis, and arthritis (1, 2). The cause of the disease is likely to be multifactorial, including a single gene mutation (*lpr*) of the *fas* apoptosis gene on mouse chromosome 19 (3, 4) and background genes from the MRL strain (1, 4).

Recent studies show that MRL/*lpr* mice excreted significantly higher concentrations of urinary nitrate/nitrite than age-matched normal C3H mice (5). Furthermore, MRL/*lpr* mice showed markedly reduced proteinuria and minimal glomerular proliferation when treated orally with L-N^G monomethyl arginine (L-NMMA), an inhibitor of nitric oxide synthase (NOS) (5). These data therefore strongly suggest that nitric oxide (NO) is an important mediator of the disease manifestation of MRL/*lpr* mice. However, the mechanism(s) for this exaggerated NO synthesis by MRL/*lpr* mice remains obscure.

NO is a critical mediator of a variety of biological functions, including vascular relaxation, platelet aggregation, neurotransmission, tumoricidal and microbicidal activity, and immunosuppression (6–10). It is also implicated in a range of immunopathologies (11–13). NO is derived from the guanidino nitrogen atom(s) (14) and molecular oxygen (15, 16) in a reaction catalyzed by the enzyme NOS. There are three major isoforms of NOS (17): the neuronal form

[1] *Abbreviations used in this paper:* HRP, horseradish peroxidase; L-NMMA, L-N^G monomethyl arginine; MRL/*lpr*, MRL/MP-*lpr/lpr*; NO, nitric oxide; NOS, nitric oxide synthase.

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(nNOS) and the endothelial form (eNOS) produce the amounts of NO required for physiological functions. The cytokine-inducible form (iNOS) is induced by a number of immunological stimuli, such as IFN-γ, TNF-α, and LPS, and catalyzes the high output of NO which can be cytotoxic.

Since the expression and functional activities of IFN-γ and TNF-α in SLE and its animal models are highly variable and controversial (18, 19), we explored the possibility that the exaggerated NO synthesis in MRL/*lpr* mice may be due to enhanced production of other factor(s). We report here that spleen and peritoneal cells from MRL/*lpr* mice produced significantly higher concentrations of IL-12 than those from the control MRL/+, or BALB/c mice when stimulated with LPS and IFN-γ in vitro. IL-12 and LPS synergistically stimulated the spleen and peritoneal cells from MRL/*lpr* mice, but not from the control MRL/+ mice, to produce high levels of NO. Furthermore, young MRL/*lpr* mice developed accelerated glomerulonephritis when injected with rIL-12 compared with control injected with PBS alone. Thus, the enhanced capacity to produce IL-12 and the higher responsiveness to IL-12/LPS to produce NO may play an important role in the pathogenesis of MRL/*lpr* mice.

Materials and Methods

Mice. Female MRL/*lpr* and age- and sex-matched control MRL/MP-+/+, BALB/c, and CBA/6J mice were obtained from Harlan Olac Ltd. (Bicester, UK). Some of the mice were bred in the animal facilities, University of Glasgow, from pairs obtained from Harlan Olac. They were housed in a conventional animal facility.

Cytokines and Reagents. Murine rIFN-γ was a kind gift of Dr. G. Adolf (Bender, Vienna, Austria). Murine rIL-12 and monoclonal (clones C15.6 and C15.1.2) and polyclonal (sheep no. 7) anti-IL-12 antibodies were generously provided by Dr. Stan Wolf (Genetic Institute, Boston, MA). Polyclonal anti-IL-12, anti-IFN-γ, and anti-TNF-α antibodies were raised in rabbits immunized with murine rIL-12, rIFN-γ, or TNF-α, respectively using a standard protocol. Monoclonal anti-CD4 (YTS191) and anti-CD8 (YTS169) were kindly provided by Dr. H. Waldmann (University of Oxford, Oxford, UK). Monoclonal anti-NK antibody (5E6, endotoxin removed) was obtained from PharMingen (San Diego, CA). Monoclonal anti-Thy1.2 (F7D5) was obtained from Olac Ltd. Fresh rabbit serum was used as a source of complement. L-NMMA and D-NMMA were kindly provided by Dr. S. Moncada (Glaxo Wellcome Research Laboratory, Beckenham, UK). LPS (*Salmonella enteritidis*) and Con A were obtained from Sigma (Poole, UK).

Mouse Peritoneal and Spleen Cell Preparation. Peritoneal cells were collected by injecting 5–7 ml of ice-cold PBS into the peritoneal cavity before harvesting and kept on ice before use. Spleen was then removed and a single cell suspension prepared by gently forcing the spleen through a sterile tea strainer into a petri dish in HBSS (Gibco, Paisley, UK) containing 1% FCS. The cells were then washed in serum-free HBSS and viability determined by trypan blue exclusion.

Cell Depletion. Single cell suspensions (10^7 cells/ml) in PBS were incubated on ice for 30 min with anti-CD4, anti-CD8 (hybridoma culture supernatant, 1:1 dilution), anti-NK (5E6, 5 μg/ml), anti-Thy1.2 (ascites, 1:500 dilution) or anti-Thy1.2, plus anti-NK. After 2 washes with ice-cold PBS, the cells were incubated with or without rabbit complement (1:20) in 96-well culture plates for 45 min at 37°C at 2×10^5 cells/well in 100 μl of complete culture medium (RPMI 1640; Gibco) supplemented with 10% heat-inactivated FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 50 μM 2-mercaptoethanol). Cells were then pelleted by centrifugation of the plate and the supernatant was carefully removed. The incubation was repeated with fresh complement and followed by two washes with warm medium. Samples of the residual cells were phenotyped in parallel tubes by flow cytometry (Becton Dickinson & Co.) using FITC- or PE-conjugated antibodies to CD4, CD8 (Becton Dickinson & Co., Oxford, UK) and CD3 (PharMingen).

Cell Culture. Spleen (2×10^5 viable cells/well) or resident peritoneal cells ($1.5–3 \times 10^5$ cells/well, varied between different experiments) in 200 μl were cultured in full medium in 96-well plates (Nunc, Roskilde, Denmark) at 37°C and 5% CO_2 for up to 6 d. To stimulate for NO synthesis, graded doses of IL-12 and LPS were titrated and optimal doses determined. IFN-γ was used at 50 U/ml unless indicated otherwise. To stimulate for IL-12 production, graded doses of LPS were titrated with 50 U/ml of IFN-γ. In the antibody neutralization experiments, cells were preincubated with specific antibodies to murine IL-12 (sheep no. 7 or rabbit anti-IL-12), IFN-γ, or TNF-α for 30 min at 37°C before the addition of stimulators. Concentrations of antibody used were supraoptimal for neutralizing the amounts of cytokines likely to be produced as determined in preliminary experiments.

Cytokine Assays. IL-12 concentration was determined by an ELISA method using a combination of two rat monoclonal antibodies (C15.1.2 and C15.6, Genetic Institute) to mouse IL-12 (p40 chain) as capture antibodies, and a sheep anti–mouse IL-12 antibody (sheep no. 7) or a rabbit anti–mouse IL-12 (Rab.74.6) as detecting antibody. ELISA in 96-well plates (Immulon 4; Dynatech, Billingshurst, UK) was developed with a biotin-conjugated donkey anti–sheep IgG antibody (Sigma) followed by StrepAvidin-horseradish peroxidase (HRP) or a HRP-conjugated donkey anti–rabbit IgG (SAPU, Carluke, UK) accordingly, TMB HRP substrate (KPL Laboratories, Gaithersburg, MD); optical density was read on a Dynatech MR5000 ELISA reader at 630 nm. Recombinant murine IL-12 (Genetic Institute) was used as standard. Normal donkey serum (2%) was used as blocker. IL-12 production was also determined by Western blot. Peritoneal cells from four 13-wk-old MRL/*lpr* mice were pooled and cultured at 2.5×10^5 cells/ml in 25-cm^2 flasks in the presence or absence of IFN-γ (50 U/ml) and LPS (1 μg/ml). Culture supernatant was harvested at 6, 12, 24, and 48 h, 3-ml samples were immune precipitated with rat monoclonal anti-IL-12 antibodies (clones C15.1.2, and C15.6, both against the p40 chain of IL-12), and the immune complexes were captured by protein A–Sepharose beads. The precipitate was then resolved on 10% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Herts, UK). After blocking with Tris-buffered saline containing 0.1% Tween 20 and 2% BSA, the membrane was incubated sequentially with anti-IL-12 antibody (sheep no. 7), biotin-conjugated donkey anti–sheep IgG, and HRP-conjugated avidin, and protein bands visualized by the enhanced chemiluminescence (ECL) system (Amersham Biosciences, Amersham, Bucks, UK). Recombinant murine IL-12 was run in parallel with the test samples.

The IFN-γ concentration was also determined by ELISA using a rat monoclonal antibody (R46AT) and a rabbit anti–mouse IFN-γ antibody. The assay was developed with an alkaline phos-

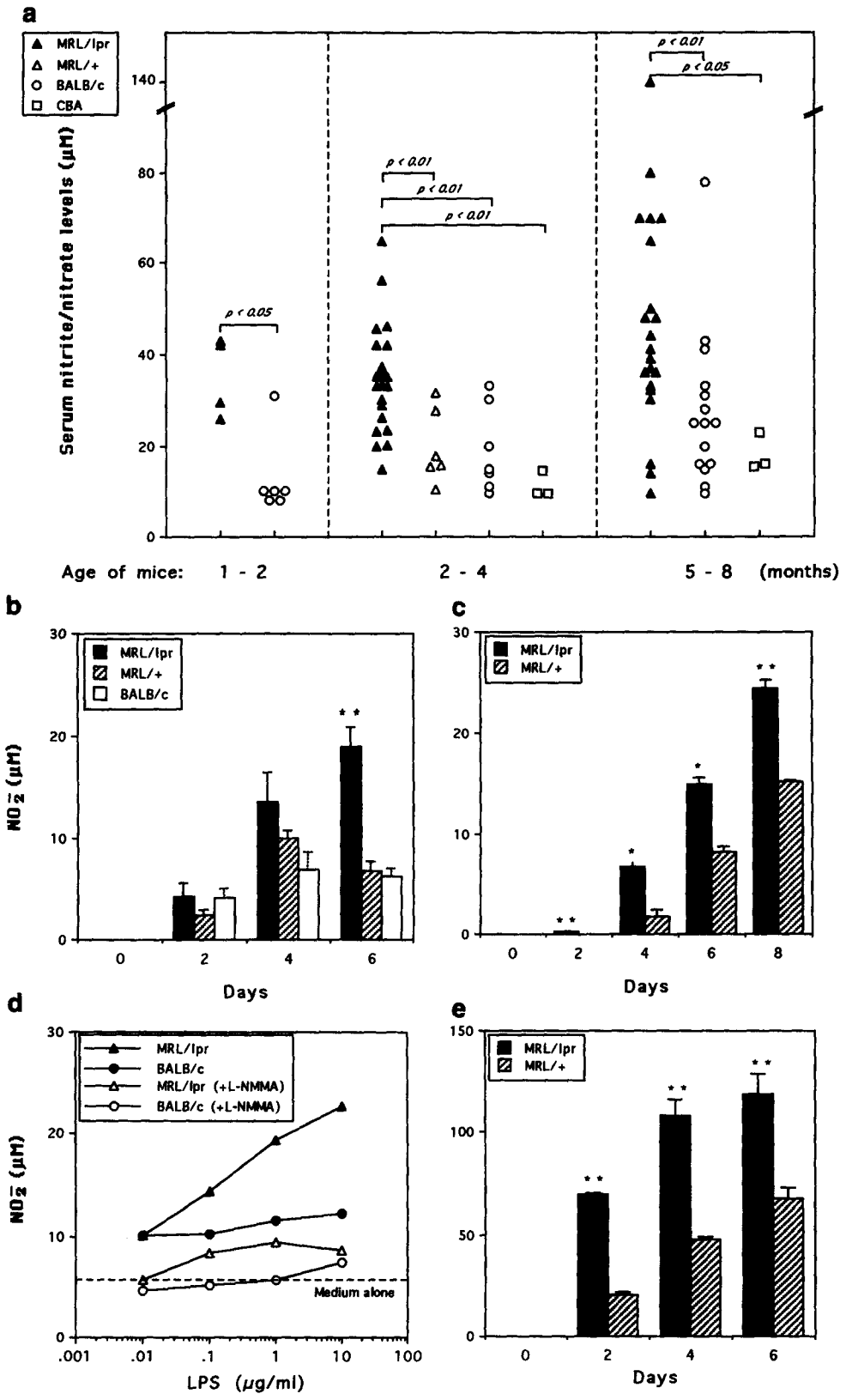


Figure 1. Enhanced NO synthesis in MRL/*lpr* mice in vivo and in vitro. (*a*) Serum nitrite/nitrate levels in MRL/*lpr* lupus strain (n = 47) and three control strains (n = 40) of mice at different ages. Total nitrite and nitrate concentration in serum was determined by the nitrate reductase method which converted nitrate into nitrite before measurement (see Materials and Methods). LPS/IFN-γ–induced NO production by splenic (*b–d*) or peritoneal (*e*) cells from young (6-wk; *b* and *d*) and old (25-wk; *c* and *e*) MRL/*lpr*, MRL/+, or BALB/c mice. Spleen or peritoneal cells from three mice per group were pooled and stimulated in 96-well culture plates with IFN-γ (50 U/ml) and either 1 μg/ml (*b*, *c*, and *e*) or graded doses (*d*) of LPS. In some cultures (*d*) L-NMMA (500 μM) was added. Culture supernatants were collected at daily intervals and nitrite levels were measured by the Greiss method. Data shown are time course (*b*, *c*, and *e*), or (*d*) at day 6 as mean and SD of triplicate cultures. (*P <0.05, **P <0.01). (*d*, *dotted line*) Nitrite level in unstimulated cultures. Consistent results were obtained in more than 10 repeated experiments.

phatase–conjugated goat anti–rabbit IgG antibody (Sigma) followed by *p*-nitrophenyl phosphate. Optical density was read on a Dynatech MR700 ELISA reader at 410 nm. Recombinant murine IFN-γ was used as standard. In some experiments, an ELISPOT assay was also used to enumerate the number of IFN-γ–secreting cells. This was carried out as described previously (20).

Assays for NO Production. Total nitrate and nitrite concentration in serum was determined by the conversion of nitrate into

nitrite as described previously (21). Briefly, serum samples (30 μl) were incubated with an equal volume of reaction buffer containing nicotinamide adenine dinucleotide phosphate (1 mg/ml), flavin adenine dinucleotide (8.3 mg/ml), KH_2PO_4 (0.1 M), and nitrate reductase (0.7 mg/ml; Sigma), added immediately before use. Conversion was carried out at 37°C for 2 h in a 96-well ELISA plate (Immulon 2; Dynatech). Total nitrite content was then measured in a chemiluminescence NO analyzer (Dabisi model 2107; Quantitech Ltd., Milton Keynes, UK) according to the manufacturer's instruction. Nitrate standard was run in parallel with test samples. The assay was performed in triplicate and had a detection limit of 5 μM. Nitrite concentration in culture supernatants was determined in triplicate by the Greiss reaction (22), using $NaNO_2$ as standard with a detection limit of 1 μM.

Detection of Serum Autoantibodies by ELISA. This was carried out as described previously (23) using single (ss) or double (ds) stranded calf thymus DNA (Sigma) as target antigens. Pooled serum from 20-wk-old MRL/*lpr* mice of known high titer of anti-DNA antibodies was used as standard serum. One titration unit was arbitrarily defined as the amount of antibody present in a fixed dilution of the standard serum (1/10,000 for anti-ssDNA and 1/1,000 for anti-dsDNA antibodies).

Renal Histology. Mouse kidney tissues were fixed in formalin and embedded in paraffin; 5-μm sections were stained with periodic acid-Schiff. For histological examination by light microscopy, sections were randomly labeled and examined blind twice by two investigators. The severity of kidney pathology was assessed by the extent of enlargement of glomeruli and mesangial cell proliferation, tuft-to-capsule adhesions, protein casts in tubules, interstitial cellular infiltration, and vasculitis.

Statistical Analysis. Statistical significance (*p* value) was calculated by the Mann Whitney test (Minitab software program; Minitab Inc., State College, PA).

Results

MRL/lpr Mice Produced Higher Concentrations of NO Metabolites than Normal Mice. Serum from MRL/*lpr*, MRL/+, BALB/c, and CBA mice of various ages were analyzed for NO metabolites by converting nitrate to nitrite and then determining the total nitrite content. Serum from MRL/*lpr* mice consistently contained significantly higher concentrations of nitrate and nitrite than those from age- and sex-matched MRL/+, BALB/c, or CBA/6J mice (Fig. 1 *a*). There was no significant difference between the concentrations of NO metabolites produced by MRL/+, BALB/c, or CBA mice. These results therefore confirm previous findings (5) that MRL/*lpr* mice produce exaggerated levels of NO in vivo.

To analyze the mechanism(s) for the exaggerated production of NO by the MRL/*lpr* mice, spleen (Fig. 1, *b–d*) or peritoneal (Fig. 1 *e*) cells from MRL/*lpr*, MRL/+, or BALB/c mice were cultured with IFN-γ and LPS in vitro for up to 8 d, and the concentrations of nitrite in the culture supernatants determined. Cells from MRL/*lpr* mice consistently produced significantly higher levels of NO than similarly cultured cells from age-matched young (6-wk-old, Fig. 1, *b* and *d*) or old (24-wk-old, Fig. 1, *c* and *e*) MRL/+, or BALB/c mice. The production of NO was LPS dose dependent and was inhibitable by L-NMMA (Fig. 1 *d*). IFN-γ alone or LPS alone induced only a minimum level of NO synthesis by spleen cells (see Fig. 3, *b* and *c*). LPS alone did, however, induce significant levels of NO production by *lpr* peritoneal cells (see Fig. 3 *e*).

Peritoneal and Spleen Cells from MRL/lpr Mice Produce High Concentrations of IL-12. We next investigated the produc-

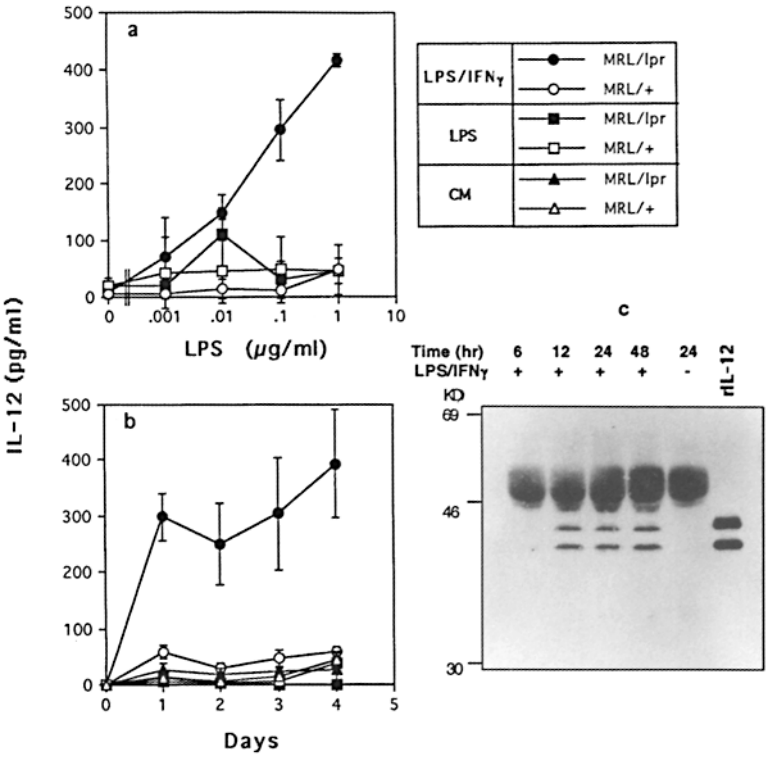


Figure 2. Enhanced IL-12 production by peritoneal cells from MRL/*lpr* mice. Pooled peritoneal cells from three mice per group of MRL/*lpr* or MRL/+ strains (2–3-mo-old) were cultured in the presence or absence of IFN-γ (50 U/ml) and LPS. IL-12 production in the cell cultures (triplicates) was determined by ELISA: (*a*) LPS dose–responses at day 4; (*b*) time course with 1 μg/ml LPS. The data shown were representative of three repeated experiments. (*c*) Western blot analysis of IL-12 expression showed inducible IL-12 p40 chain (time course) identical to that of the rIL-12 control. The two distinct p40 bands may be due to different degrees of glycosylation (24).

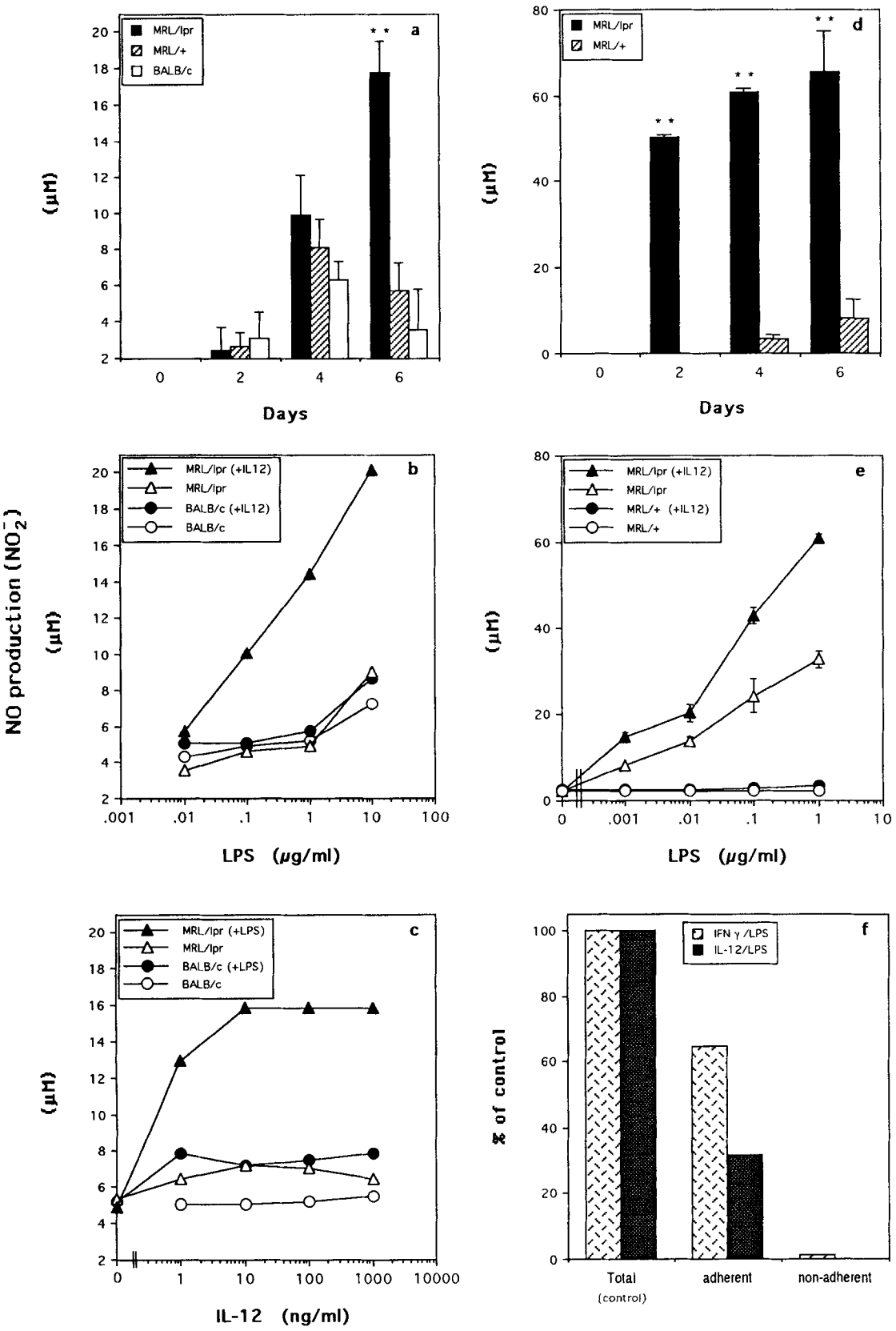


Figure 3. Spleen and peritoneal cells from MRL/*lpr* mice produced high concentrations of NO in response to rIL-12 and LPS. Pooled splenic (*a–c*) or peritoneal (*d–f*) cells from MRL/*lpr*, MRL/+, and BALB/c mice (3-mo-old, three mice per group) were stimulated in 96-well culture plates with or without fixed or different doses of rIL-12 and LPS. Culture supernatants were collected at daily intervals and nitrite levels measured by the Greiss

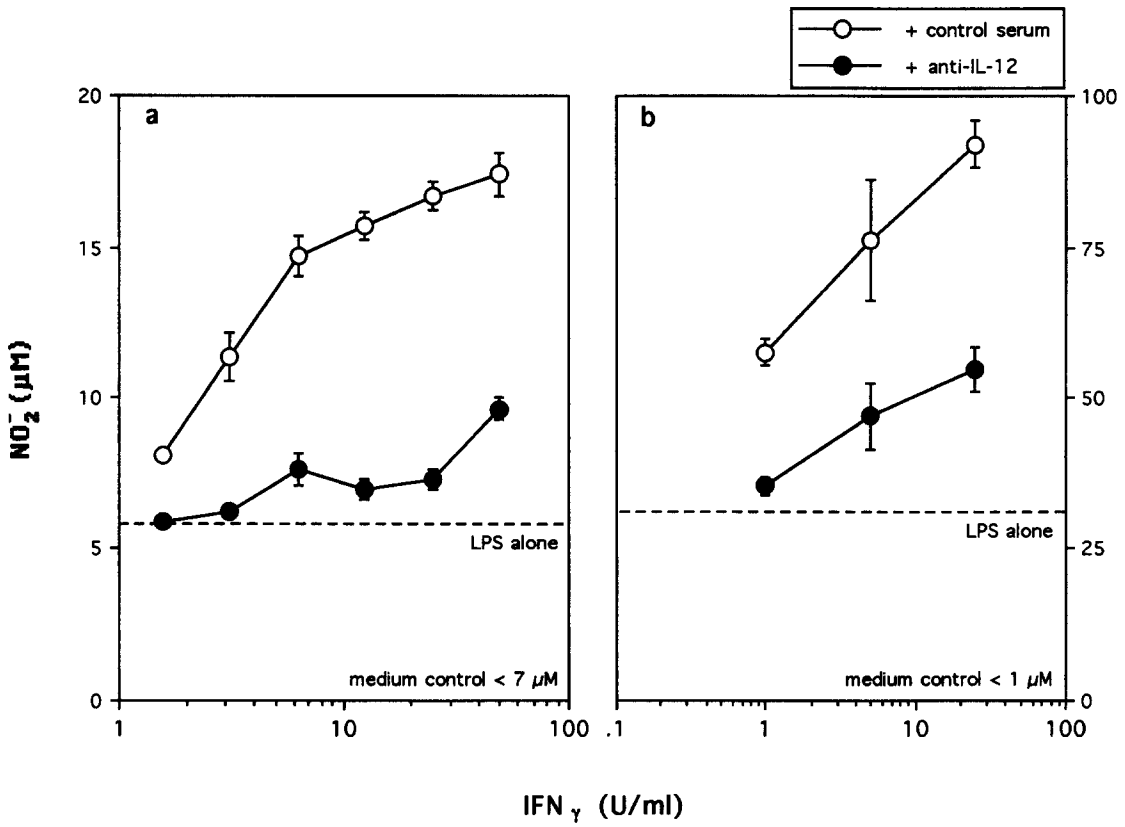


Figure 4. Effects of anti-IL-12 antibodies on IFN-γ/LPS–induced NO production by splenic and peritoneal cells from MRL/*lpr* mice. Pooled spleen (*a*) or peritoneal (*b*) cells from three MRL/*lpr* mice (3-mo-old) were stimulated for 6 or 4 d, respectively, with graded doses of IFN-γ and 1 μg/ml LPS in the presence of a rabbit anti-IL-12 antiserum (Rab.74.6) or preimmune control serum (1:100). Nitrite concentrations were expressed as mean and SD of triplicate cultures. (*Dotted lines*) Nitrite levels in cultures with LPS alone. Results from two repeated experiments were similar. (Note different scale for *a* and *b*).

tion of IL-12, a powerful immune stimulatory cytokine, by the MRL/*lpr* mice. Peritoneal cells from normal BALB/c or MRL/+ mice produced only a low level of IL-12 when cultured with IFN-γ and LPS in vitro. By contrast, peritoneal cells from age-matched *MRL/lpr* mice produced up to 10-fold more IL-12 when cultured under identical conditions (Fig. 2). IL-12 production by the cells of *MRL/lpr* mice was LPS dose- and time-dependent, reaching a plateau level after 12 h (Fig. 2, *b* and *c*). LPS or IFN-γ alone induced minimum amounts of IL-12 synthesis. Similar results were obtained with spleen cells, except that the levels of IL-12 produced were lower (data not shown).

Spleen and Peritoneal Cells from MRL/lpr Mice Produce High Levels of NO When Stimulated with IL-12 and LPS. Subsequent experiments were therefore carried out to investigate the possible link between IL-12 and NO synthesis by MRL/*lpr* mice. Spleen cells from MRL/*lpr* mice produced markedly higher concentrations of NO than those from age-matched BALB/c or MRL/+ mice when cultured with IL-12 and LPS (Fig. 3, *a–c*). Nitrite was detectable in the culture supernatant after 2 d and continued to increase up to day 6 (Fig. 3 *a*). NO production was both IL-12 and LPS dependent (Fig. 3, *b* and *c*). High concentrations of nitrite were also detected in cultures of peritoneal cells from *MRL/lpr* mice (Fig. 3, *d* and *e*). NO production in the present system is dependent on the adherent cell population (>90% macrophages) and barely detectable in the nonadherent cell population. However, removal of nonadherent cells significantly reduced (by 70–82%) the IL-12–induced NO production (Fig. 3 *f*). This suggests that the IL-12–driven NO synthesis was via its effect on nonadherent cells.

Since IFN-γ and IL-12 are known to induce each other's synthesis, we then determined whether the enhanced production of NO by spleen and peritoneal cells from *MRL/lpr* mice activated by IFN-γ and LPS was IL-12

method. (*a* and *d*) Kinetics of NO production with 1 μg/ml LPS plus 10 ng/ml rIL-12. (*b* and *e*) show LPS dose–responses with 10 ng/ml IL-12 at days 6 and 4, respectively. (*c*) IL-12 dose–response with 1 μg/ml LPS at day 6. (*f*) Induction of NO production by IL-12/LPS or IFN-γ/LPS by adherent and nonadherent peritoneal cells from MRL/*lpr* mice. Nonadherent cells were separated from adherent cells by plastic adhesion and cultured in separate wells with fixed doses of LPS (1 μg/ml) and IL-12 (10 ng/ml) or IFN-γ (50 U/ml). Data shown are nitrite concentrations as percentage of the total unseparated cells in the control cultures (mean of triplicates). ($^*P < 0.05$, $^{**}P < 0.01$).

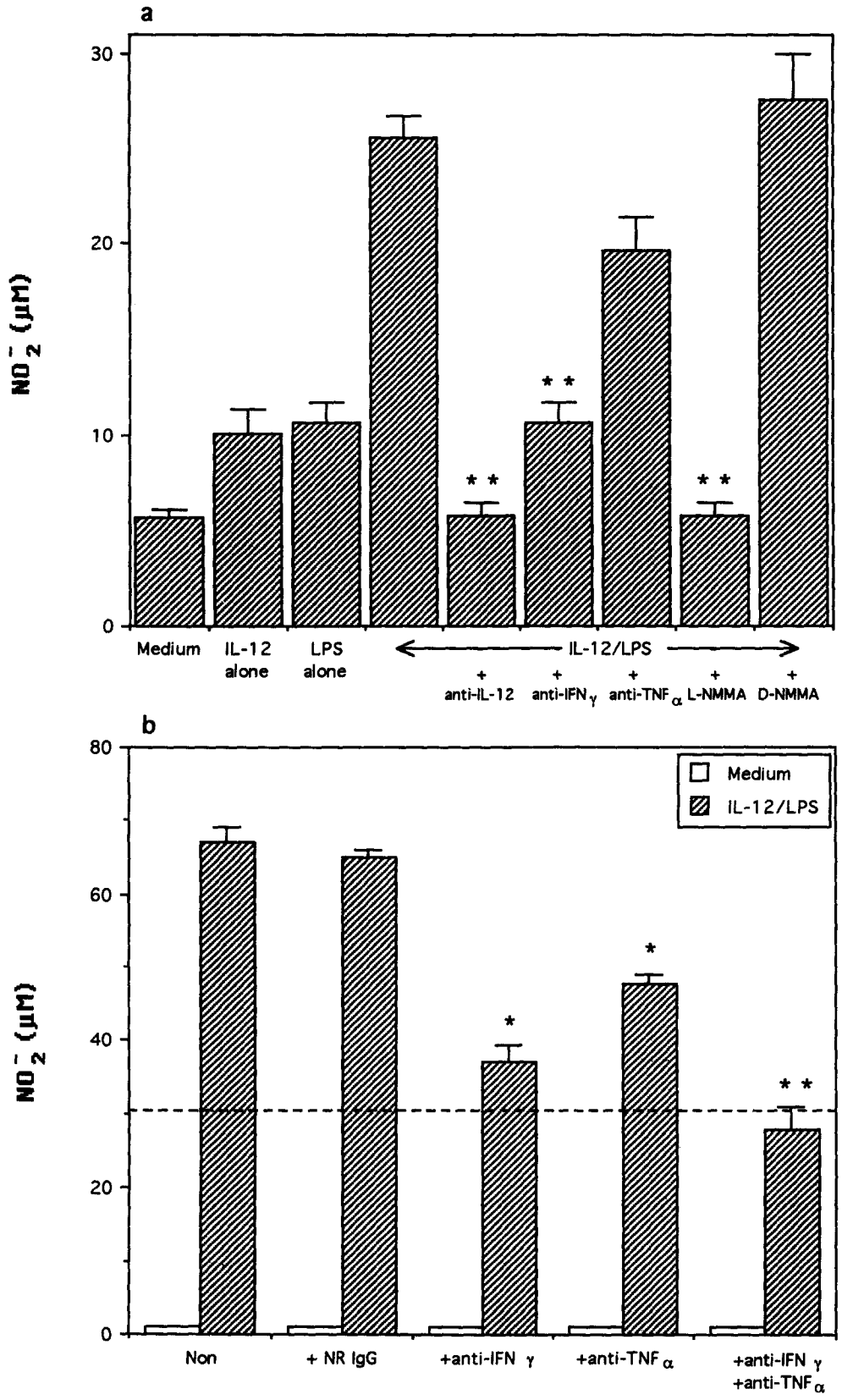


Figure 5. Inhibition of IL-12/LPS–induced NO production by antibodies to IL-12, IFN-γ, and TNF-α, and by L-NMMA. Pooled spleen (from two mice, *a*) or peritoneal (from five mice, *b*) cells from 2–3-mo-old MRL/*lpr* mice were stimulated for 6 or 4 d, respectively, with rIL-12 (10 ng/ml) and LPS (1 μg/ml) with or without addition of polyclonal antibodies (50 μg/ml) to mouse IL-12, IFN-γ, TNF-α, IFN-γ and TNF-α, or control normal rabbit IgG (*NR IgG*). In some spleen cell cultures, a NOS inhibitor, L-NMMA or its inert enantiomer control D-NMMA (500 μM) were added. Data shown are mean and SD of triplicate cultures ($^*P < 0.05$, $^{**}P < 0.01$). Similar results were obtained from four experiments. (*Dotted line, b*) Nitrite level in the cultures of peritoneal cells with LPS alone.

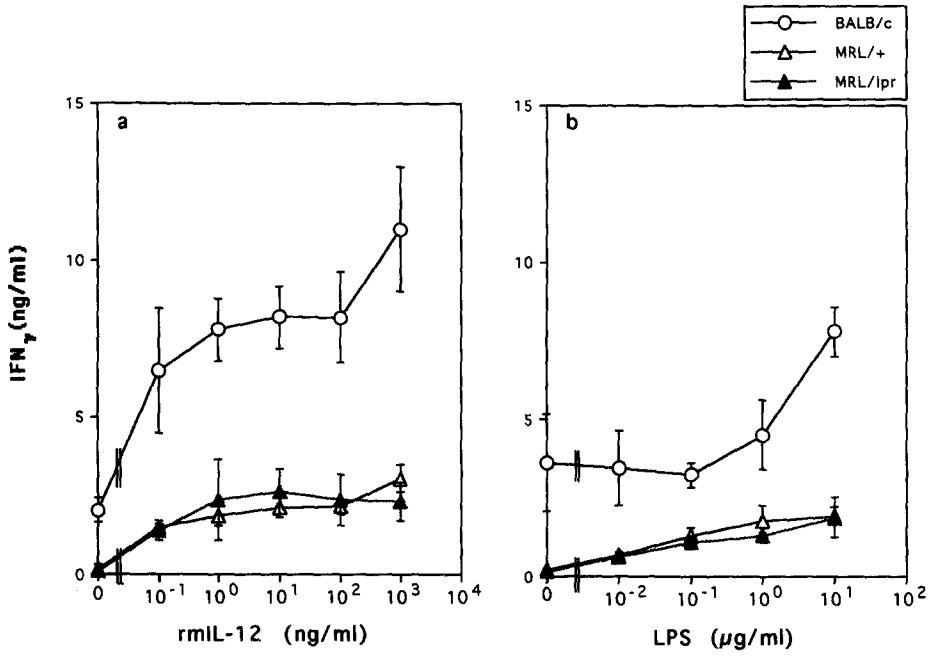


Figure 6. IL-12/LPS–induced IFN-γ production in normal and lupus mice. Pooled spleen cells from age- and sex-matched MRL/*lpr*, MRL/+, or BALB/c mice (n = 3) were stimulated with IL-12 and LPS for 3 d and IFN-γ production was determined by ELISA. (*a*) Dose–response studies of IL-12 with 1 μg/ml LPS; (*b*) dose–response studies of LPS with 10 ng/ml IL-12. IFN-γ levels in the unstimulated cultures were <35 pg/ml. Data shown are mean and SD of triplicates cultures.

dependent. Spleen and peritoneal cells were stimulated with IFN-γ and LPS as above in the presence of a rabbit anti-IL-12 antiserum. The production of NO by these cells was markedly inhibited by the antiserum but not by the control preimmune serum (Fig. 4). The inhibition was incomplete. This was because IFN-γ and LPS can be expected to directly activate macrophages to produce NO. The ability of an anti-IL-12 antibody to inhibit NO synthesis also indicated that the IL-12 detected by ELISA in the culture supernatants of cells activated with IFN-γ/LPS (e.g., Fig. 2) was not due to the IL-12 p40 homodimer.

IL-12/LPS–induced NO Production Involves IFN-γ and TNF-α. Production of NO by spleen and peritoneal cells from MRL/*lpr* mice activated with IL-12 and LPS can be completely abrogated by anti-IL-12 antibody, markedly inhibited by anti-IFN-γ antibody, and was marginally affected by anti-TNF-α antibody, but was further inhibited by the combination of anti-IFN-γ and anti-TNF-α antibodies (Fig. 5), suggesting that IL-12/LPS–induced NO synthesis may be via IFN-γ and TNF-α. However, there was no direct correlation between NO synthesis and the level of IFN-γ produced in cultures of spleen cells from MRL/*lpr*, MRL/+, or BALB/c mice when stimulated with IL-12 and LPS under identical conditions (Fig. 6, as compared to Fig. 3, *b* and *c*). Thus, whereas cells from MRL/*lpr* mice produced a high concentration of NO and those from the

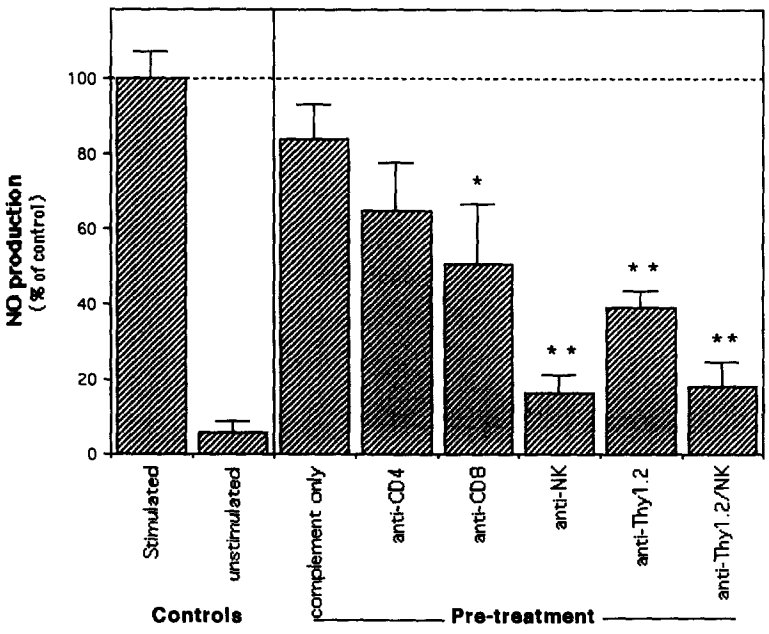


Figure 7. IL-12/LPS–induced NO synthesis involves NK and T cells. Pooled peritoneal cells from two MRL/*lpr* mice (5-mo-old) were pretreated with antibodies against CD4, CD8, NK cell marker 5E6, Thy1.2, or NK 5E6 plus Thy1.2 and then followed by incubation with rabbit serum as the source of complement. The cells were then stimulated for 4 d with rIL-12 (10 ng/ml) and LPS (1 μg/ml), or cultured in medium only as unstimulated control. Data are from one of two similar experiments and are expressed as percentage of NO concentration in the control cultures of stimulated cells without pretreatment (mean ± SD, n = 3, $^*P < 0.05$, $^{**}P < 0.01$).

MRL/+ and BALB/c mice produced only a minimum amount of NO, cells from the BALB/c mice produced markedly higher concentrations of IFN-γ than those produced by cells from MRL/*lpr* mice which was indistinguishable from those of MRL/+ mice. Spleen cells from BALB/c mice also produced markedly higher concentrations of IFN-γ and higher numbers of IFN-γ–secreting cells than those from MRL/*lpr* mice when cultured with the T cell mitogen, Con A (2.5 μg/ml), over an extended period (up to 120 h) as detected by ELISA and by ELISPOT (data not shown).

IL-12–induced NO Synthesis Involves NK and T Cells. To determine the cell types involved in the enhanced NO synthesis, cell-depletion experiments were carried out in vitro using cytolytic antibodies and complement. Depletion of Thy1.2$^+$ cells partially reduced NO production, whereas depletion of NK cells almost completely abrogated the production of NO by peritoneal cells from MRL/*lpr* mice stimulated with IL-12 and LPS (Fig. 7). Depletion of CD4$^+$ or CD8$^+$ cells alone had only a modest effect. Since some NK cells also express CD8 and Thy1.2 antigens (25), it is likely that IL-12–induced NO synthesis involves mainly NK cells in addition to the adherent population.

Evidence for the Enhanced IL-12 Synthesis in MRL/lpr Mice In Vivo. To confirm the *in vitro* observations of enhanced IL-12 activity in the lupus model, experiments were carried out to measure serum levels of IL-12, IFN-γ, and TNF-α in mice of different age and compared with those of sex- and age-matched MRL/+ and BALB/c control mice. Fig. 8 shows that serum IL-12 levels were markedly higher in the *lpr* mice, especially in the old mice with clinical disease compared with controls (12-fold in 5–8-mo-old mice). This was in parallel with the elevated levels of nitrite/nitrate in the serum (Fig. 1 *a*). Treatment of young (1–2-mo-old) mice with LPS for as little as 2 h resulted in significantly higher serum IL-12 levels in MRL/*lpr* mice compared with similarly treated control MRL/+ mice (Fig. 8 *c*). Serum IFN-γ and TNF-α were found to be low, variable, and comparable (data not shown).

rIL-12 Accelerates Autoimmune Disease in MRL/lpr Mice. To investigate directly the role of IL-12 in the induction of autoimmune disease, young (3-wk-old) MRL/*lpr* mice were given daily intraperitoneal injections of rIL-12 (300 ng/mouse/day) or a similar volume of PBS for 9 wk. Mice were then killed and the histopathology of the kidney examined. Gross morphology of the kidneys from the IL-12–treated mice had a pale waxy surface and were firmer on sectioning, whereas those from the control PBS-injected mice appeared normal. Histological examination revealed enlarged glomeruli with significant glomerular and mesangial hypercellularity in the IL-12–treated group. In particular, most of the IL-12–treated group showed severe damage to the glomeruli, with thickening of the Bowman's capsule basement membrane and tuft-to-capsule adhesions and protein casts (Fig. 9 *c*) which were largely absent or scanty in the PBS-treated group (Fig. 9 *a*). In contrast, pyelonephritis with extensive vasculitis and infiltration of mononuclear cells at the kidney medullary region (Fig. 9 *b*) was prominent in all the mice in the PBS-treated group. These changes were minimal in the IL-12–treated mice (Fig. 9 *d*).

Spleen cells from the rIL-12–treated mice showed a significantly higher percentage of CD3$^+$ (51.5 vs 44.5%, P = 0.02, n = 5), CD8$^+$ (9.2 vs 5.3%, P = 0.02) and double negative (22.6 vs 17.3%, P = 0.02) T cells than those from untreated mice. There was, however, no significant difference in the spleen weight or the percentage of CD4$^+$ T cells in the spleen cell populations between the two groups. There was also no significant difference in the anti-ss or ds DNA antibody (total antibody as well as IgM and IgG isotype) concentrations in the serum between treated and untreated mice (data not shown). However, serum IFN-γ (16.5 ± 3.8 vs 5.2 ± 1.4 pg/ml, P = 0.0189) and nitrite/nitrate (54.2 ± 2.6 vs 29.6 ± 3.0 μM, P = 0.0304) were elevated in the IL-12–treated mice compared with those of PBS-treated mice.

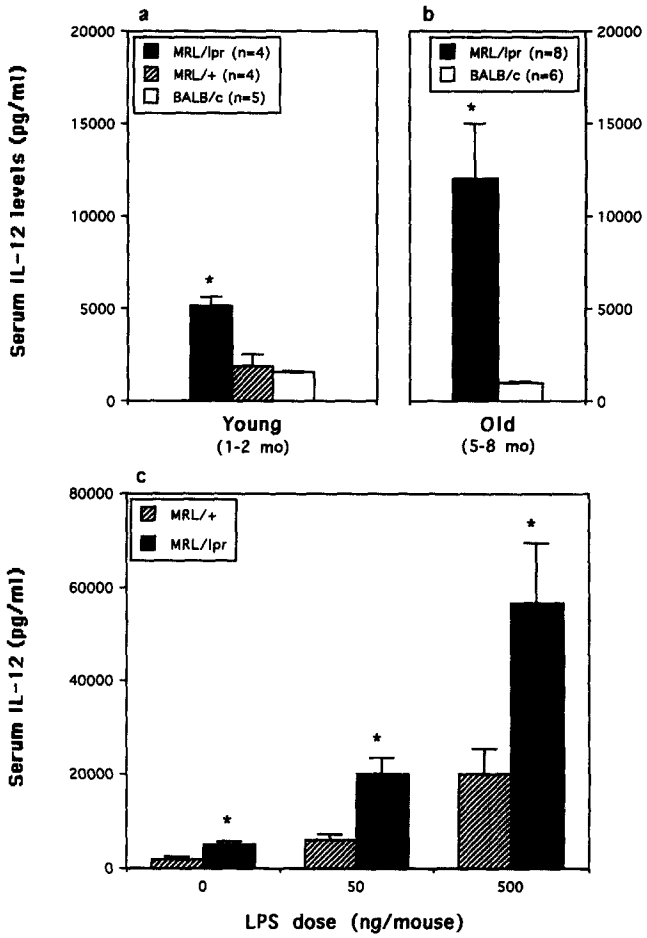
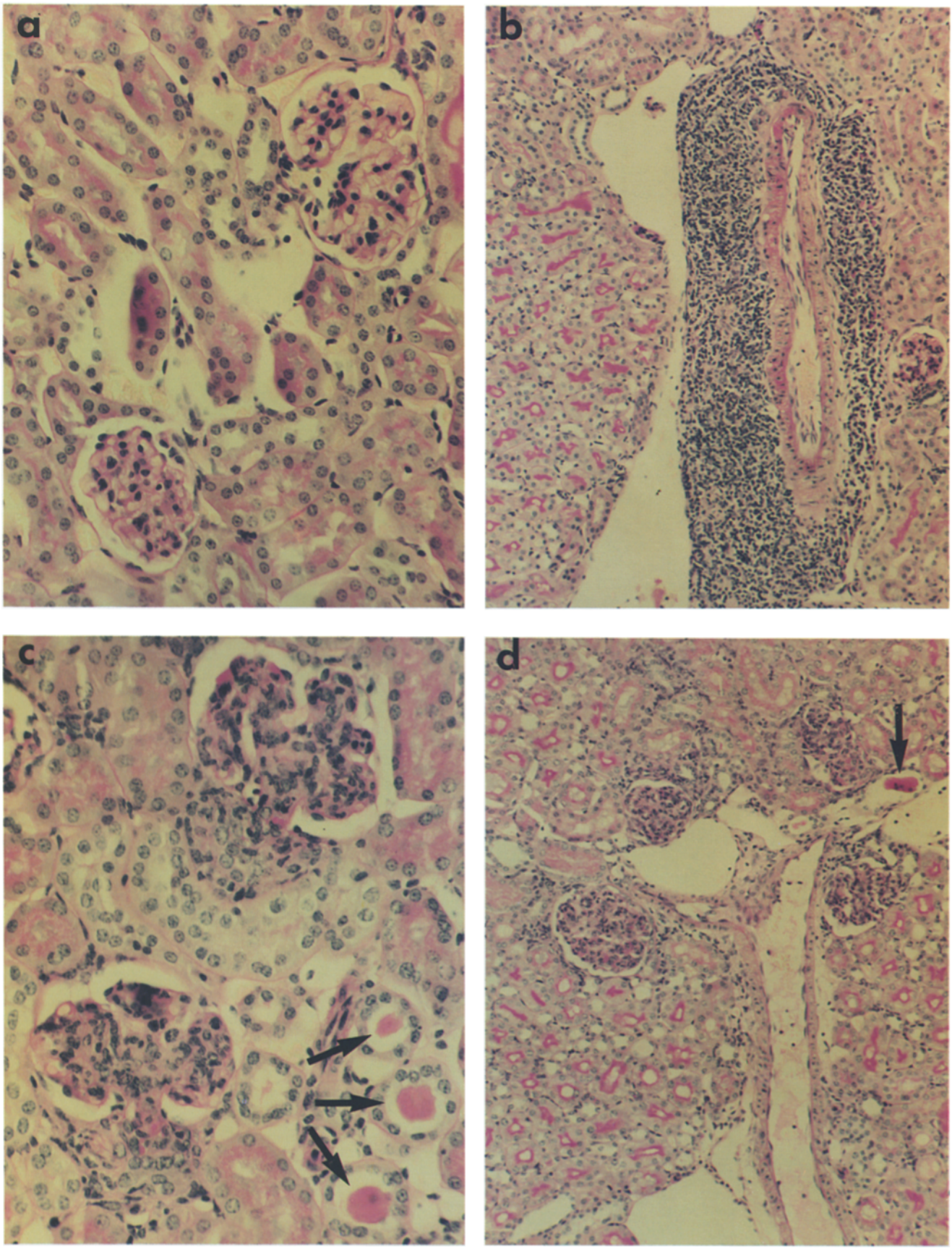


Figure 8. Elevated serum IL-12 levels in untreated and LPS-treated MRL/*lpr* mice. Serum IL-12 levels in untreated (*a*) young (1–2 mo) and (*b*) old (5–8 mo), and in (*c*) LPS-treated young (1–2 mo) MRL/*lpr* mice. Control mice were sex- and age-matched MRL/+ or BALB/c mice. For the LPS treatment, a total of six groups of mice (four per group) were injected (i.v. tail vein) with 50 or 500 ng per mouse of LPS in 0.1 ml PBS or PBS alone as indicated. Serum samples were collected 2 h after the treatment and IL-12 levels were determined. All samples were assayed individually by ELISA; the statistical significance of differences between lupus and the control strains of the same age groups is indicated (*P <0.05).


a
b
c
d

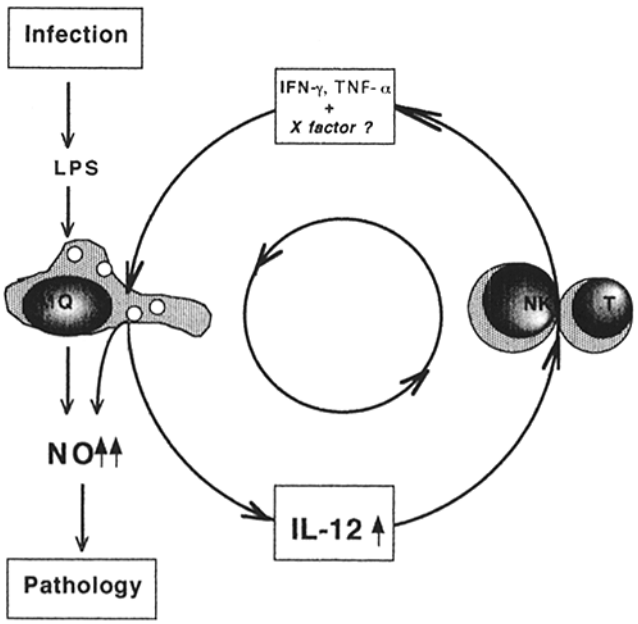


Figure 10. Schematic representation of the possible mechanism for the induction of renal pathology (glomerulonephritis) in MRL/*lpr* mice by enhanced IL-12 production and NO synthesis (for details see text).

It is interesting to note that treatment of MRL/+ mice with IL-12 did not induce any detectable renal pathology (glomerulonephritis or proteinuria, data not shown), suggesting that MRL/*lpr* mice are genetically predisposed to the effect of IL-12. However, treatment of MRL/*lpr* mice (starting age: 2 mo) with a sheep anti–mouse IL-12 antibody (sheep no. 7, 100 μg/mouse i.p., weekly for 7 wk) led to a initial reduction of proteinuria followed by a rebound (data not shown) possibly due to increased formation of immune complexes resulting from repeated injection of foreign proteins.

Discussion

Data presented here demonstrate that serum from MRL/*lpr* mice contained significantly higher concentrations of IL-12 with or without treatment with LPS in vivo compared with those of similarly treated control MRL/+ mice. In addition, spleen and peritoneal cells from MRL/*lpr* mice produced significantly higher concentrations of IL-12 than MRL/+ or BALB/c mice in response to activation by IFN-γ and LPS in vitro. Furthermore, cells from MRL/*lpr* mice were more responsive to IL-12 and LPS, producing higher concentrations of NO than those from the control MRL/+ mice. Finally, daily injection of rIL-12 led to accelerated glomerulonephritis in the MRL/*lpr* mice but not in the MRL/+ mice.

These results suggest a causal relationship between enhanced capacity to produce IL-12 and the spontaneous autoimmune disease in this model of SLE as depicted schematically in Fig. 10. An earlier report (5) demonstrated that NO is a critical mediator of the autoimmune disease in MRL/*lpr* mice. It has also been documented that many autoimmune animal models including the lupus MRL strain of mice do not develop autoimmune disease when kept in a germ-free environment (26, 27), consistent with our finding that LPS and IFN-γ are required for the activation of macrophages to produce high concentrations of NO and IL-12, which is produced by monocyte/macrophages (28). In contrast to normal MRL/+ or BALB/c mice, the production of NO in the MRL/*lpr* mice is further exaggerated by the high concentration of IL-12 produced by activated macrophages. IL-12 activates NK and T cells to produce IFN-γ and perhaps other yet unidentified factor(s) which, together with LPS, further enhance NO synthesis. This cycle of amplification produces exaggerated levels of NO leading to the pathology. This is consistent with the delayed onset of NO synthesis in the cultures activated with IL-12 (Fig. 3, requiring 4–6 d for optimal production of NO). The activation of NK and T cells by IL-12 for the production of IFN-γ has been well documented (29–31). However, in the present system, there was a lack of direct correlation between enhanced NO synthesis and IFN-γ production by MRL/*lpr* and control MRL/+ and BALB/c mice. Nevertheless, IFN-γ and TNF-α were required for IL-12–driven NO synthesis. It is therefore likely that an additional factor(s) produced by IL-12–activated NK or T cells is required to synergize with IFN-γ for the production of high concentrations of NO. NK cell activity is known to be altered in MRL/lpr mice. However, this was based on their lytic activity rather than their activity to produce IFN-γ.

IL-12 is essential for the differentiation of the Th1 subset of T cells (32–34). It is also a powerful adjuvant for the induction of protective immunity against diseases such as cutaneous leishmaniasis (35) in which Th1 cells are the main protective mechanism (for reviews see references 36, 37). Our results indicate that excessive production of IL-12 or the administration of rIL-12 can cause autoimmune disease in susceptible mice, demonstrating the negative side of the therapeutic use of IL-12. This is consistent with a number of recent reports showing that administration of IL-12 induced: (*a*) earlier onset of insulin-dependent diabetes mellitus in female NOD mice (38); (*b*) more severe and prolonged

Figure 9. Effects of IL-12 treatment of MRL/*lpr* mice on renal pathology. Photomicrographs of kidney sections from MRL/*lpr* mice (3-mo-old) showing two essentially normal glomeruli in the kidney cortex of a PBS-treated control mouse (*a*); and severe glomerulonephritis in the cortical region of a IL-12–treated mouse, including structural damage to two enlarged glomeruli with hypercellularity and adhesion (*c*), as well as protein casts in the tubules (*c* and *d*, *arrows*). In contrast, histological examination of the medullary region shows severe pyelonephritis in the PBS-treated mouse (*b*) featuring extensive mononuclear cell infiltration in the perivascular interstitium, which is, however, markedly reduced in the IL-12–treated mouse (*d*). Periodic Acid-Schiff stain, ×250 (*a* and *c*), ×100 (*b* and *d*).

disease in adoptively transferred experimental allergic encephalomyelitis (39); and (*c*) destructive collagen-induced arthritis (40). Our study here demonstrates that the pathogenic effect of IL-12 in the lupus model is likely to be due to the increased production of NO. This finding not only advances our knowledge of the pathogenesis of this lupus model (and by extension to SLE), it also suggests two potential means of therapeutic intervention of the progression of this disease: neutralization of IL-12 or inhibition of iNOS.

Glomerulonephritis is a severe complication of the renal involvement which is the major cause of pathology and death in SLE (18, 41). Although it is generally believed that the renal pathology is due to autoantibody production, immune complex deposition and complement activation, slow infections have been shown to play an important role in triggering these autoimmune responses in many models of autoimmune disorders (26). We observed here that IL-12–treated mice had clearly reduced pyelonephritis which is known to be commonly induced by infections (42). Our results suggest that IL-12 might have strengthened the host's defense against infection in these mice which are otherwise immunodeficient (43–45). Thus, treatment of the autoimmune disease aiming at neutralization of IL-12 may weaken the host immune response, leading to uncontrolled infection. These results therefore demonstrated that IL-12 is beneficial in controlling infections. However, excessive production of IL-12, as in the lupus mice, will lead to severe immunopathology.

MRL/*lpr* mice differ from the MRL/+ mice in the impairment of transcription of the gene encoding Fas antigen by insertion of a transposable element into the second intron of the gene (46). However, *lpr* is not a null mutation and the inhibition of Fas expression is incomplete (47). The relationship between the impaired *fas* gene expression and enhanced IL-12 and NO production by the MRL/*lpr* mice is at present unclear, but amenable to experimental investigation.

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