

Conjugated linoleic acid enhances glutathione synthesis and attenuates pathological signs in MRL/MpJ-Fas^{lpr} mice¹

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Abstract Conjugated linoleic acid (CLA), a naturally occurring peroxisome proliferator-activated receptor γ (PPAR γ) ligand, exhibits proapoptotic, immunomodulatory, and anti-cancer properties. In this study, we examined the biological effects of CLA administration in the MRL/MpJ-Fas^{lpr} mouse, an animal model of systemic lupus erythematosus (SLE). We found that CLA exerted apparently opposed activities in *in vitro* experiments, depending on its concentration: 100 μ M CLA downregulated IFN γ synthesis and cell proliferation of splenocytes, in association with apoptosis induction and a decrease of intracellular thiols (GSH + GSSG), whereas 25 μ M CLA did not significantly influence cell proliferation but enhanced the expression of γ -glutamylcysteine ligase catalytic subunit (GCLC) and intracellular GSH concentration. Interestingly, the antiproliferative effect at 100 μ M was not inhibited by the PPAR γ antagonist GW9662. *In vivo*, CLA administration drastically reduced SLE signs (splenomegaly, autoantibodies, and cytokine synthesis), a condition paralleled by the enhancement of GCLC expression and intracellular GSH content. Moreover, CLA administration significantly downregulated nuclear factor κ B activity independent of PPAR γ activation and apoptosis induction. **In conclusion, enhanced GSH content and GCLC expression in CLA-treated mice suggest a novel biochemical mechanism underlying its immunomodulatory activity and the beneficial effects on murine SLE signs.**—Bergamo, P., D. Luongo, F. Maurano, G. Mazzarella, R. Stefanile, and M. Rossi. **Conjugated linoleic acid enhances glutathione synthesis and attenuates pathological signs in MRL/MpJ-Fas^{lpr} mice.** *J. Lipid Res.* 2006. 47: 2382–2391.

Supplementary key words autoimmune disease • peroxisome proliferator-activated receptor γ • γ -glutamylcysteine ligase

Oxidative stress has been implicated in the pathogenesis of several degenerative diseases, such as Alzheimer disease, Parkinson disease, systemic lupus erythematosus (SLE), and cancer. In particular, its involvement in autoimmune disease has been supported by the increase of oxidative stress markers (1, 2) and by the preventive effect played by the dietary intake of antioxidants (3). The beneficial effect

of diet enrichment with n-3 PUFAs in autoimmune disease-prone mice has been associated with improved antioxidant defenses (4, 5) and apoptosis modulation (6). Apoptosis is the process that leads to the ordered destruction of cells, avoiding the release of intracellular contents into the extracellular environments, where they have a powerful inflammatory effect. Defective functioning of the cell death program has been recognized to play a critical role in the pathogenesis of autoimmune diseases (7), and a proapoptotic effect has been identified in some antineoplastic and anti-inflammatory agents used as medication (methotrexate and mycophenolate mofetil, respectively) (8, 9). The MRL/MpJ-Fas^{lpr} (MRL/*lpr*) mouse is a prototypical model for human SLE in which the presence of a single gene mutation on the Fas (CD95) gene leads to reduced signaling for apoptosis (10). Actually, the impaired clearance of apoptotic cells favors the self-antigen presentation and the production of multiple autoantibodies, which represents the central immunological disturbance in murine SLE (11). Indeed anti-double-stranded DNA (anti-dsDNA) and anti-tissue transglutaminase (anti-tTG) IgGs (12), splenomegaly, and deregulated production of Th1 and Th2 cytokines (13) have been used as markers to monitor murine SLE disease progression.

Conjugated linoleic acid (CLA) refers to a heterogeneous group of positional and geometric isomers of a conjugated diene of linoleic acid (C18:2n-6). The best sources for CLA in the human diet are ruminant meat and dairy products in which the two predominant isomers are *cis*9,*trans*11 (c9,t11) and *trans*10,*cis*12 (t10,c12) CLA. In rodents, dietary administration of a mixture of CLA isomers was shown to have anticarcinogenic, antidiabetic, and anti-

Abbreviations: anti-dsDNA, anti-double-stranded DNA; anti-tTG, anti-tissue transglutaminase; CLA, conjugated linoleic acid; 15dPGJ₂, 15-deoxy- Δ 12,14-prostaglandin J₂; GCLC, γ -glutamylcysteine ligase catalytic subunit; IL, interleukin; MRL/*lpr*, MRL/MpJ-Fas^{lpr}; NF κ B, nuclear factor κ B; O₂^{•-}, superoxide anion radical; PPAR γ peroxisome proliferator-activated receptor γ ; ROS, reactive oxygen species; SLE, systemic lupus erythematosus.

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inflammatory processes (14), but only limited effects have been observed on autoimmune disease-prone mice (15, 16). The mechanisms whereby CLA could influence immune systems have not been established, although two main mechanisms have been proposed: the first hypothesizes the ability of CLA to alter eicosanoid signaling by modifying cell membrane composition (17); the other assumes its ability to modulate genes through peroxisome proliferator-activated receptors (PPARs) (18). PPAR γ agonists includes some long-chain n-3 and n-6 PUFAs (docosaheptaenoic acid, eicosapentaenoic acid, and CLA) and the prostanoid prostaglandin D₂ dehydration product, 15-deoxy- Δ 12,14-prostaglandin J₂ (15dPGJ₂); their potential use of PPAR γ agonists in the treatment of inflammatory disease was reviewed recently (19). In addition, the ability of PPAR γ ligands (15dPGJ₂ and docosaheptaenoic acid) to upregulate the expression of stress-responding enzymes and/or to enhance cellular redox status by increasing the concentration of intracellular GSH has been demonstrated (20, 21).

The association of CLA-induced pro-oxidant activity with its immunomodulatory and proapoptotic ability was demonstrated previously in Jurkat T-cells (22, 23). In this study, on the basis of the ability of CLA to modify the functioning of different biochemical mechanisms (PPAR γ , apoptosis, and redox status) involved in the pathogenesis of autoimmune disease, its efficacy in attenuated mouse SLE signs was evaluated. In particular, a first set of experiments addressed the question of whether CLA exposure may modulate redox status and cell differentiation of mouse splenocytes from both MRL/*lpr* and congenic control mice (MRL/+). Then, its beneficial effects on typical pathological signs of MRL/*lpr* mice were investigated, and the involvement of biochemical mechanisms was examined. Splenocyte treatment with 100 μ M CLA resulted in enhanced apoptosis and significantly decreased IFN γ and proliferation synthesis. More interestingly, cell exposure to a lower CLA concentration (25 μ M) was accompanied by enhanced cytoprotective defense [increased expression of γ -glutamylcysteine ligase catalytic subunit (GCLC) and increased intracellular GSH concentration]. Significant amelioration of typical pathological signs (splenomegaly, cytokines, and autoantibody synthesis) in MRL/*lpr* by short- and long-term CLA administration was accompanied by nuclear factor κ B (NF κ B) activity suppression and improved cytoprotective defenses independent of apoptosis induction and PPAR γ activation.

MATERIALS AND METHODS

CLA stock solutions

Stock solutions of an isomeric CLA mixture containing 40% c9,t11 and 45% t10,c12 (Sigma, St. Louis, MO), hereafter called CLA, was used. Pure (\geq 98%) c9,t11, t10,c12, and *trans*9,*trans*11 (t9,t11) CLA isomers were purchased from Cayman Chemical (Ann Arbor, MI). CLA and isomer stock solutions were prepared in ethanol to a final concentration of 1 M and stored at -20°C . Fatty acids were diluted just before use in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 5 μ M 2-mercaptoethanol,

and 1% non-essential amino acids (NEAA) (complete RPMI). Cultures exposed to the same amount of ethanol were used as a control.

For in vivo trials, CLA isomeric mixture (containing 38.5% t10,c12, 37.4% c9,t11, 3.4% palmitic acid, 3.8% stearic acid, 15.4% oleic acid, and 1.5% linoleic acid) was purchased from Natural, Inc. (CLA-T). Individual doses consisting of 30 mg of CLA-T in 200 μ l of olive oil were prepared just before administration. Olive oil was given to control animals.

Animal treatment and sample preparation. MRL/*lpr* and congenic control MRL/+ mice purchased from the Jackson Laboratory (Bar Harbor, ME) were bred and maintained under standard conditions of temperature and light in specific pathogen-free conditions at the Istituto di Scienze dell'Alimentazione animal facility. Animals were fed ad libitum with standard mouse chow.

Three to four predisease MRL/*lpr* and congenic control mice (7–8 weeks old) were used as cell sources for each in vitro experiment. Splenocytes from 7–8 (predisease) and 20–22 (diseased) week old MRL/*lpr* and age-matched MRL/+ mice (n = 16) were used to evaluate the age-dependent decrease of intracellular GSH and the antiproliferative effect of CLA.

For in vivo trials, 48 MRL/*lpr* mice were gavaged with CLA-T (13) four times per week up to 21 weeks of age. Four homogeneous groups (sex and body weight; n = 12 animals each) were used to evaluate the effect CLA on MRL/*lpr* mice administered for short (2 weeks) and long (15 weeks) periods with CLA-T or olive oil as a control.

Blood was taken from each mouse, and serum was prepared and stored at -20°C for analysis of autoantibodies. After euthanasia, spleen was aseptically removed and kept on ice in sterile complete RPMI. Spleen weights were expressed as a percentage of total body weight [spleen weight (g) \times 100/body weight (g)]. A portion of spleen was snap-frozen in liquid nitrogen and stored at -80°C for Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) assay.

Splenocyte preparation. Single cell suspensions were prepared according to a published protocol (24). Cells were divided into aliquots at 10^7 cells/ml and used for in vitro incubations (at 37°C in humidified atmosphere of 5% CO₂) or analyzed for intracellular GSH and cytokine levels. Cytoplasmic protein extracts were also prepared (25) and stored at -80°C .

Cell proliferation and toxicity assay. Splenocytes (1×10^5 cells/well) were cultured on 96-well plates in complete RPMI supplemented with 10 μ g/l PMA and 5 μ M ionomycin (PMA/ion). Cells were incubated (37°C , 5% CO₂) for 72 h in the presence of pure isomers or CLA. In some experiments, splenocytes were pretreated for 2 h with 5 μ M GW9662. Eighteen hours before harvesting, cells were pulsed with 1 μ Ci/well [³H]thymidine. At the end of the incubation, cultures were harvested on filters using a semiautomatic cell harvester (Filtermate; Packard, Danvers, MA) and incorporated [³H]thymidine was assessed with a microplate liquid scintillator (Top Count NXTM; Packard). Results were expressed as cpm, and culture incubated without CLA (mixture or pure isomers) was used as a control. The experimental conditions used (mitogen type, concentrations, and incubation times) were based on preliminary in vitro experiments.

Splenocytes (6×10^6 /well), cultured with varying CLA concentrations (0–200 μ M) for 72 h in the presence of PMA/ion, were monitored for cell viability by measuring released lactate dehydrogenase activity with the CytoTox 96[®] detection kit (Promega, Biosciences, Inc.) according to the manufacturer's protocol. Values were expressed as percentages of those obtained by exposure to 10% NP40 (100%). Cells incubated without CLA were used as a control.

Cytokine assay. Splenocytes (8×10^6 per well) were preincubated (3 h) in complete RPMI containing 100 μ M CLA before stimulation with PMA/ion. After 18 h of incubation, IFN γ concentration was quantified in the supernatant by indirect ELISA according to the manufacturer's instructions (R&D Systems, Inc., Minneapolis, MN). IFN γ concentration was expressed as pg/ml, and cells incubated without CLA were used as a control.

At the end of the *in vivo* trial, IFN γ , interleukin-4 (IL-4), and IL-10 mRNA levels were assessed in spleen cells by semiquantitative RT-PCR, as described (26). PCR products were analyzed on a 2% agarose gel stained with VISTRA Green (Amersham International). Quantitative analysis of detected bands was carried out on the STORM 860 system with ImageQuant software (Molecular Dynamics, Inc.). Results were expressed as cytokine/ β -actin mRNA ratio.

Apoptosis assays. Caspase-8 and -3 are key enzymes in the initiation and execution stages of the apoptotic pathway, and their activities were measured in cytoplasmic protein extract using the Fluorometric Assay Kit (Sigma-Aldrich) according to the manufacturer's instructions. Splenocytes (2×10^7 cells/well) were seeded on 12-well plates and incubated for 16 h with 50 ng/ml anti-CD95/Fas/Apo1 monoclonal antibody (clone CH 11; Immunotech) in complete RPMI-supplemented PMA/ion. At the end of the incubation, caspase-3 was measured, and cells incubated without anti-CD95 antibody were used as a control. The time-dependent activities of caspase-3 and -8 were determined on splenic cell aliquots (2×10^7 cells/well) from MRL/+ and MRL/*lpr* exposed to 100 μ M CLA for different time periods. Protease activity was calculated by subtracting the value measured in cells incubated without CLA (spontaneous) for the same time period; after normalization of the protein content (27), its activity was expressed as nmol Amino-4-Methyl Coumarin (AMC)/mg protein. To confirm the proapoptotic ability of CLA *in vitro*, FITC-Annexin (BD Discovery Labware) and DNA fragmentation were evaluated in spleen cells exposed for 4 h to 100 μ M CLA. At the end of the incubation, cells were analyzed by flow cytometry using a FACSCalibur and CellQuest software (BD Biosciences) and by TUNEL assay, respectively. Cell cultures incubated without CLA were used as a control, and cultures exposed to 10 μ M actinomycin D for 6 h were used as a positive control.

PPAR γ and GCLC immunoblotting. Cytoplasmic protein extracts, prepared from mouse splenocytes at the end of *in vivo* or *in vitro* treatment, were used to evaluate PPAR γ and GCLC expression. After normalization of protein content (27), aliquots (20 μ g of protein) were fractionated by 10% SDS-PAGE and electroblotted onto ImmobilonTM polyvinylidene difluoride membranes (Millipore). Membranes were incubated (1 h at 37°C) with rabbit polyclonal primary antibody directed against PPAR γ (dilution, 1:5,000; Cayman Chemical) or GCLC (dilution, 1:5,000; LabVision). After immunodetection with goat anti-rabbit biotinylated secondary IgGs and streptavidin-conjugated peroxidase (dilution, 1:2,000; Dako Cytomation), chemiluminescence was performed according to the manufacturer's protocol (ECL; Amersham Biosciences).

Reactive oxygen species production and total GSH measurement. The pro-oxidant effect of CLA was evaluated on splenocytes for short time periods and without the addition of mitogens to avoid additional stress on cells. Superoxide ($O_2^{\cdot-}$) reduction of ferricytochrome *c* was measured as described by Arroyo et al. (28). Total GSH concentration (GSH + GSSG) was determined in splenocytes by measuring the reaction of GSH and DTNB coupled to the recycling of GSSG to GSH by glutathione reductase, as described previously (29). GSSG was measured according to

Davies, Birt, and Schnell (30). Intracellular GSH concentration in mouse splenocytes exposed to different CLA concentrations was expressed as the percentage of that measured in cells incubated without CLA for the same time period. For *in vivo* evaluation of intracellular GSH content, a standard curve was used to calculate the GSH amount and the concentration was normalized to the protein content and expressed as nmol GSH/mg protein/min.

Autoantibodies and NF κ B activation. The titers of anti-dsDNA and anti-tTG IgGs in mouse sera were quantified by ELISA according to published methods (12). The amount of NF κ B p65 was evaluated in nuclear protein extracts of MRL/*lpr* mouse splenocytes at the end of the *in vivo* study using the Trans-AMTM NF κ B p65 enzyme-linked immunosorbent assay (Active Motif) accordingly to the manufacturer's instructions. NF κ B activation was expressed as absorbance at 450 nm.

Statistics. Differences were assessed using Student's *t*-test, and the levels of significance were designated as follows: * $P < 0.001$, ** $P < 0.01$, *** $P < 0.05$.

RESULTS

CLA downregulates cell proliferation and IFN γ secretion *in vitro*

The *in vitro* antiproliferative ability of CLA or of pure isomers in mouse splenocytes from MRL/*lpr* mice was preliminarily investigated. A significant inhibitory effect on cell proliferation was exhibited by CLA and c9,t11 and c10,t12 CLA ($P < 0.001$); by contrast, no change in cell proliferation of cells exposed to the t9,t11 isomer was detected (Fig. 1A). Incubation with different amounts of CLA resulted in a dose-response effect (Fig. 1B); in particular, our data indicate 60% inhibition of cell proliferation produced by 50, 100, and 200 μ M CLA in both MRL/*lpr* and control mice. On the other hand, we found no evidence for CLA cytotoxicity even at the higher concentrations tested (200 μ M) (data not shown). The influence of age/disease activity on the antiproliferative effect of CLA was also evaluated upon the exposure of spleen cells from animals in the range of 7–22 weeks of age. These results demonstrate that the antiproliferative effect of CLA was not affected by the age/disease activity in MRL/*lpr* and control mice (data not shown). Next, to investigate the involvement of PPAR γ , cells were preincubated with a specific PPAR γ antagonist (GW9662) before exposure to 100 μ M CLA. The data show that the CLA-induced antiproliferative effect was not influenced by GW9662, indicating PPAR γ -independent mechanisms (Fig. 1C). Finally, 100 μ M CLA caused an inhibition of IFN γ expression in both MRL/*lpr* and MRL/+ splenocytes (Fig. 1D).

Proapoptotic effect of CLA on murine spleen cells

The defective Fas functioning in MRL/*lpr* mice was preliminarily confirmed by finding different caspase-3 activities in MRL/*lpr* and MRL/+ spleen cells upon anti-Fas IgG treatment (Fig. 2A). Next, the time-dependent apoptosis induction of 100 μ M CLA was evaluated by analyzing the classical effectors of programmed cell death.

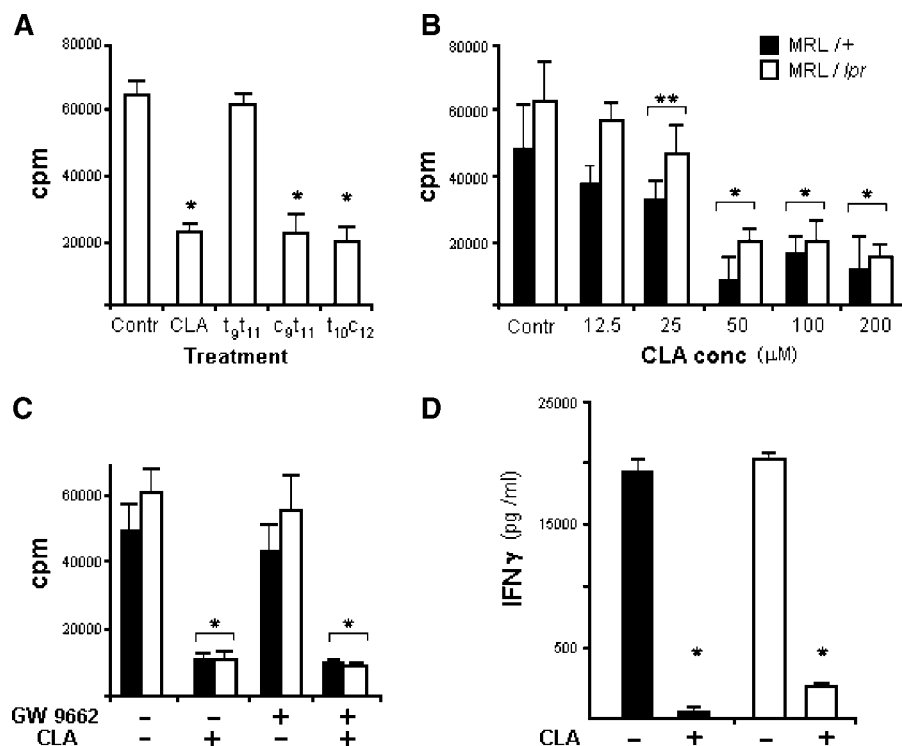


Fig. 1. Conjugated linoleic acid (CLA) downregulates cell proliferation and cytokine synthesis in MRL/MpJ-Fas^{lpr} (MRL/*lpr*) and MRL/+ splenocytes. **A:** Ability of 100 μM CLA or pure isomers to modulate cell proliferation. t₉t₁₁, *trans*₉,*trans*₁₁ CLA; c₉t₁₁, *cis*₉,*trans*₁₁ CLA; t₁₀c₁₂, *trans*₁₀,*cis*₁₂ CLA. **B:** Dose-dependent effect of CLA on the proliferation of MRL/*lpr* and MRL/+ splenocytes. **C:** Preincubation (3 h) with a specific peroxisome proliferator-activated receptor γ (PPARγ) antagonist (GW9662) was used to evaluate PPARγ involvement in CLA-induced antiproliferative activity. **D:** IFNγ release in the culture medium was measured in MRL/*lpr* and MRL/+ splenocytes preincubated (3 h) with 100 μM CLA. Cultures were then stimulated with 10 μg/l PMA and 5 μM ionomycin for 18 h, and IFNγ concentration was measured by ELISA and expressed as pg/ml. Results are given as means ± SD of duplicate analyses from three separate experiments. Cells incubated without CLA were used as controls. Significant differences from control cells are indicated (* *P* < 0.005, ** *P* < 0.01).

As expected, caspase-3 activity was increased significantly in MRL/*lpr* splenocytes after 1–2 h of incubation (Fig. 2B) compared with MRL/+ cells, and the number of Annexin V- and dUTP-positive cells was also increased in MRL/*lpr* cells exposed to 100 μM CLA (3 h) compared with untreated culture (Fig. 2C, D). By contrast, caspase-8 activation was not induced by CLA (data not shown). Collectively, these results support the notion that the proapoptotic ability of CLA is independent of Fas involvement and is mainly responsible for its antiproliferative effect.

CLA at low dose enhances cellular GSH and GCLC expression *ex vivo*

As reactive oxygen species (ROS) involvement in CLA-induced apoptosis was shown previously, O₂^{•-} yield was measured in spleen cells incubated with 100 μM CLA. MRL/*lpr* and MRL/+ CLA-treated cells yielded significantly higher O₂^{•-} concentrations than untreated cells (Fig. 3A). Moreover, because another PPARγ ligand, 15dPGJ₂, was shown to induce GSH production via GCLC activation (23), we next determined the concentration- and time-dependent induction of GSH synthesis by CLA in MRL/*lpr* splenocytes. Interestingly, exposure to 25 μM CLA for 3 h produced

a significant intracellular GSH increase, compared with untreated cells (*P* < 0.01). On the contrary, a marked time-dependent decrease of cellular GSH concentration resulted from incubation with 100 μM CLA (Fig. 3B), whereas the GSH/GSSG ratio was not altered (data not shown).

Noteworthy, increased PPARγ expression also occurred in MRL/*lpr* cells incubated with 100 μM CLA (Fig. 3C). A 4-fold increase of GCLC expression resulted upon 3 h of incubation with 25 μM CLA, whereas no difference in PPARγ expression was seen (data not shown). The proapoptotic ability of CLA at 25 μM was not completely abolished, as indicated by the cleavage of GCLC to a 60 kDa fragment, visualized after 3 h of incubation (Fig. 3C). Together, these findings suggest that CLA, depending on its concentration, may exhibit different abilities. Indeed, 100 μM CLA enhanced PPARγ protein expression and exhibited prooxidant properties; on the other hand, antioxidant activities may be triggered by lower CLA concentrations (25 μM).

Spleen weight is reduced by CLA administration

Next, the effects *in vivo* of short- and long-term CLA treatment were assessed. As splenomegaly is a typical sign of murine SLE, we determined the effect of the treatment on

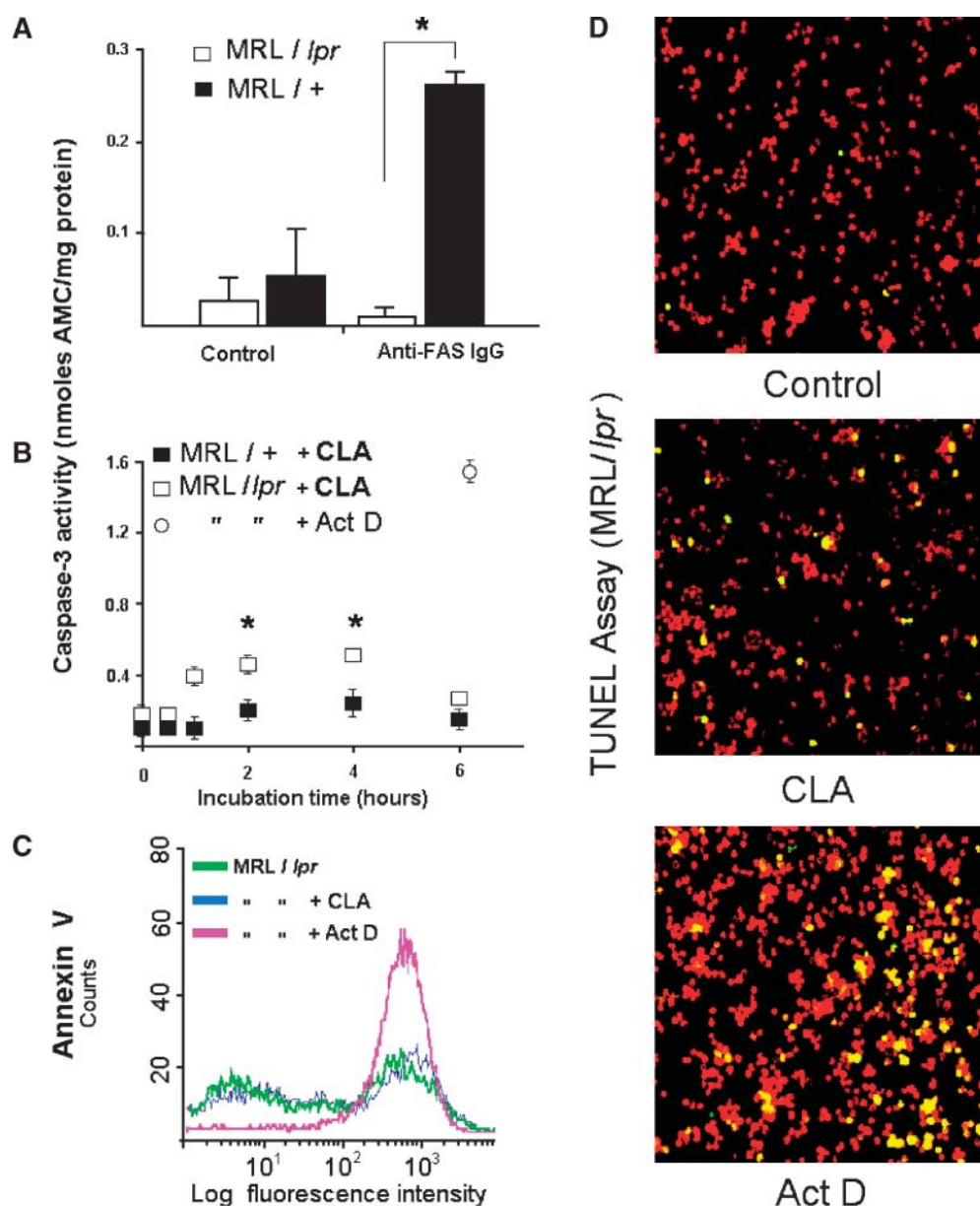


Fig. 2. CLA induces apoptosis in MRL/*lpr* and MRL/+ mice independent of FAS activation. **A:** Differential ability of anti-Fas IgGs to induce caspase-3 activation in MRL/+ and MRL/*lpr* splenocytes. **B:** Time course of caspase-3 in MRL/+ and MRL/*lpr* splenocytes incubated with 100 μ M CLA for up to 6 h. Spontaneous caspase-3 activity was subtracted from that induced by CLA and after normalization of the protein content; caspase activity is expressed as nmol Amino-4-Methyl Coumarin (AMC)/mg protein. Results are given as means \pm SD of triplicate analyses from three separate experiments. Significant differences from MRL/+ cells are indicated (* $P < 0.005$). **C, D:** Ability of CLA to induce apoptosis in MRL/*lpr* splenocytes was also determined by Annexin V (**C**) and TUNEL assay (**D**). Cells incubated without CLA were used as controls, and those cultured with 10 μ M actinomycin D (Act D) were used as positive controls. Typical results from three independent experiments are shown.

spleen weight in MRL/*lpr* mice. Animals receiving CLA-T for 2 weeks exhibited body weights that were indistinguishable from those of controls (38.3 ± 2.84 and 38.8 ± 6.2 , respectively; $P = 0.99$), whereas a significant decrease resulted upon long-term administration of CLA-T compared with controls (37.6 ± 2.8 and 41.4 ± 3.4 , respectively; $P = 0.032$). Consequently, we decided to express spleen weight as a percentage of total body weight. A significant reduction of

spleno-megaly resulted from both short- and long-term treatments with CLA-T (1.26 ± 0.44 and 1.23 ± 0.30 , respectively) compared with vehicle-treated animals (1.52 ± 0.36 and 1.55 ± 0.44 ; $P < 0.05$) (**Fig. 4A**). Apoptosis involvement in the reduction of spleen weight was next evaluated in isolated cells. Interestingly, the number of apoptotic cells, as measured by caspase-3 activity (**Fig. 4B**) and TUNEL (data not shown), was not substantially affected by CLA treatment.

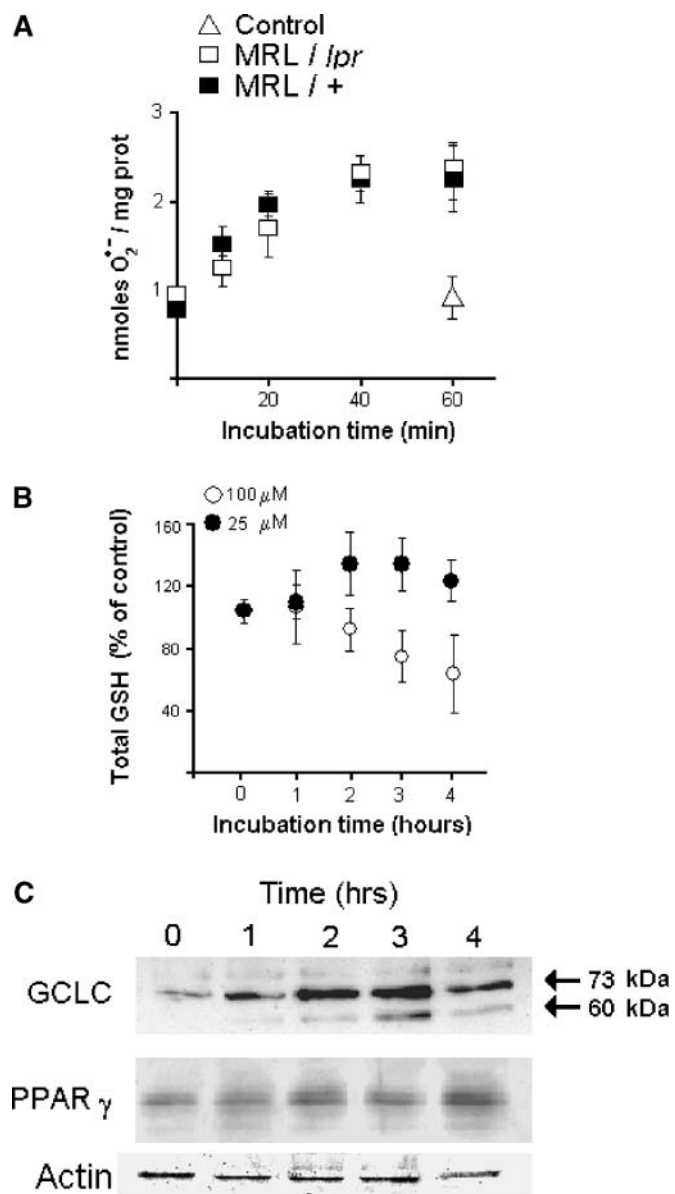


Fig. 3. CLA increases γ -glutamylcysteine ligase catalytic subunit (GCLC) and PPAR γ expression in splenic cells. **A:** Superoxide anion radical ($O_2^{\bullet-}$) production was measured at different time intervals in MRL/*lpr* and MRL/+ splenocytes incubated with 100 μ M CLA, and its concentration was expressed as nmol/mg protein. Cells incubated for 60 min without CLA were used as controls. **B:** Splenocytes from MRL/*lpr* mice were incubated in the presence of 25 or 100 μ M CLA, and total cytoplasmic GSH concentration was measured at different time intervals. Cells incubated without CLA were used as controls. Results are given as means \pm SD of triplicate analyses from two separate experiments. Significant differences from control cells are indicated ($P < 0.005$). **C:** Western blot analysis was used to evaluate the expression of GCLC and PPAR γ protein in splenocytes from MRL/*lpr* mice exposed to 100 μ M CLA for different time periods. Coomassie blue staining of the actin band is shown as a control for protein loading in each lane.

CLA downregulates autoimmune signs

Autoantibody production is another hallmark of murine SLE. Interestingly, both short- and long-term treatment with CLA-T produced a significant decrease of anti-dsDNA and

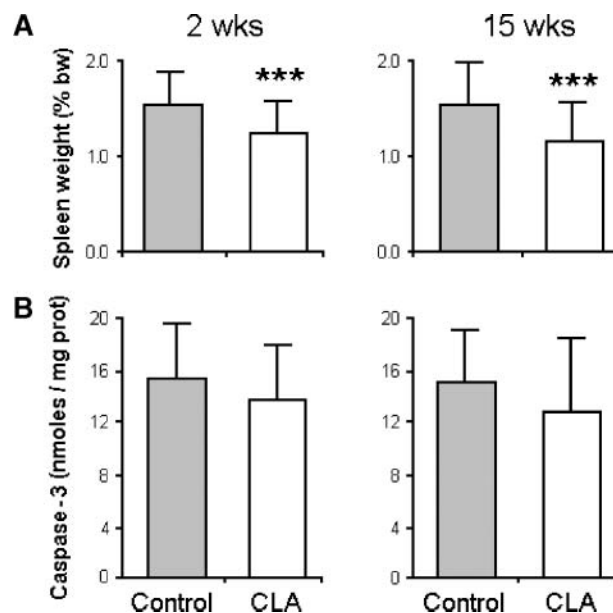


Fig. 4. CLA decreases splenomegaly in MRL/*lpr* mice independent of its proapoptotic activity. **A:** Spleens of MRL/*lpr* mice receiving short-term (2 weeks) or long-term (15 weeks) CLA administration were measured, and weight was expressed as a percentage of total body weight. Values are means \pm SD. Significant differences from control cells are indicated (***) $P < 0.05$). **B:** Caspase-3 was measured on splenocytes isolated from MRL/*lpr* mice at the end of the in vivo trials. Mice receiving olive oil were used as controls. The results are given as means \pm SD of triplicate analyses from two separate experiments.

anti-tTG IgG titers (**Fig. 5A**). The cytokine profile, analyzed by semiquantitative RT-PCR, showed reduction of IL-4, IL-10, and IFN- γ mRNA levels after short-term treatment with CLA-T compared with controls (**Fig. 5B**). Densitometric analysis of the fluorescent bands, after normalization to β -actin mRNA levels, indicated a statistically significant downregulation of all examined cytokine transcripts. In particular, a 2-fold reduction of IFN γ and IL-10 was detected in CLA-T-treated mice (**Fig. 5B**). Thus, the in vivo efficacy of both short- and long-term CLA treatment in downregulating autoantibody production and cytokine levels in MRL/*lpr* mice was shown.

CLA-induced increase of cellular GSH and GCLC expression is associated with reduced NF κ B activation

As an inefficient antioxidant defense system has been linked to the progression of autoimmune diseases, the age-dependent alteration of GSH concentration was evaluated. As expected, a significantly lower GSH concentration was found in spleen cells from diseased (20–22 weeks old) than from predisease (7–8 weeks old) MRL/*lpr* mice (9.1 ± 0.2 vs. 13.6 ± 0.8 nmol total GSH/mg protein/min, respectively; $P < 0.005$), whereas no difference was measured between the different MRL/+ groups (13.8 ± 0.5 nmol total GSH/mg protein/min) (**Fig. 6A**). The ability of dietary CLA to enhance cellular GSH concentration was next evaluated. In **Fig. 6B**, significantly higher concentrations of cellular total GSH in splenocytes after both short-term (12.8 ± 0.6 nmol total GSH/mg protein/min) and long-term (10.9 ± 0.9 nmol total GSH/mg

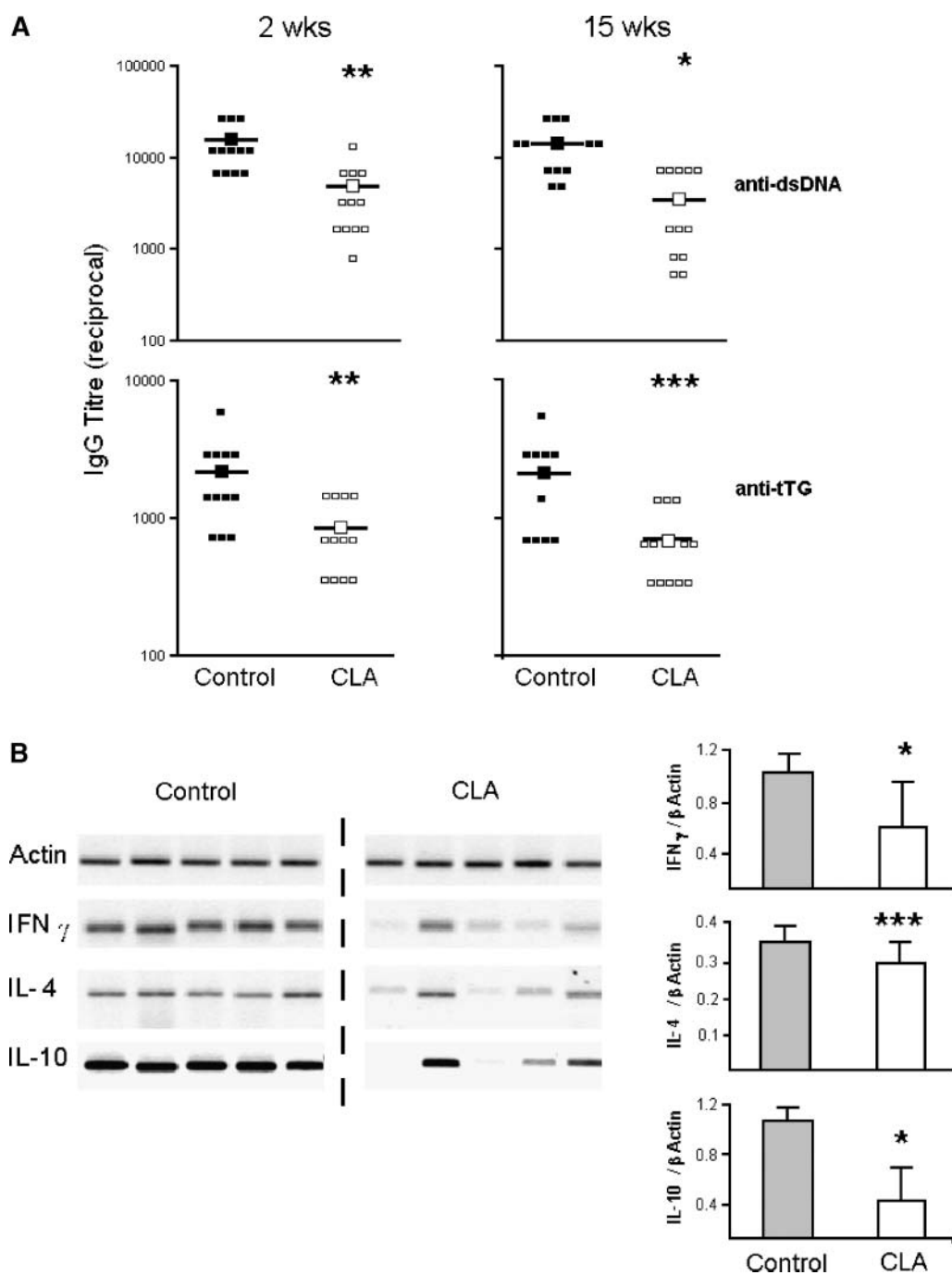


Fig. 5. CLA downregulates the disease-associated autoantibody and cytokine synthesis in MRL/*lpr* mice. **A:** The titers of anti-double-stranded DNA (anti-dsDNA) and anti-tissue transglutaminase (anti-tTG) IgGs were evaluated by ELISA in MRL/*lpr* mice receiving a short (2 weeks) or long (15 weeks) CLA administration. Horizontal bars denote means. **B:** IFN γ , interleukin-4 (IL-4), and IL-10 transcript levels in splenocytes from individual CLA-treated ($n = 5$) and control ($n = 5$) mice. After densitometric scanning, the level of expression was calculated as a ratio of cytokine to β -actin mRNA. Results are given as means \pm SD. Significant differences from control cells are indicated (* $P < 0.005$, ** $P < 0.01$, *** $P < 0.05$).

protein/min) CLA treatment than in control animals (7.0 ± 0.4 and 7.4 ± 0.7 nmol total GSH/mg protein/min, respectively; $P < 0.001$) are seen. Notably, the significant increase in GSH concentration mirrored the 2-fold increase of GCLC synthesis, whereas only minor differences in PPAR γ expression were shown by Western blot

analysis (Fig. 6C). Finally, as anti-inflammatory effects of PPAR γ ligands have been associated with NF κ B inhibition and the subsequent blockade of inflammatory gene expression (e.g., cytokines), the involvement of this nuclear transcription factor in the CLA-mediated anti-inflammatory effect was investigated. A significantly lower NF κ B ac-

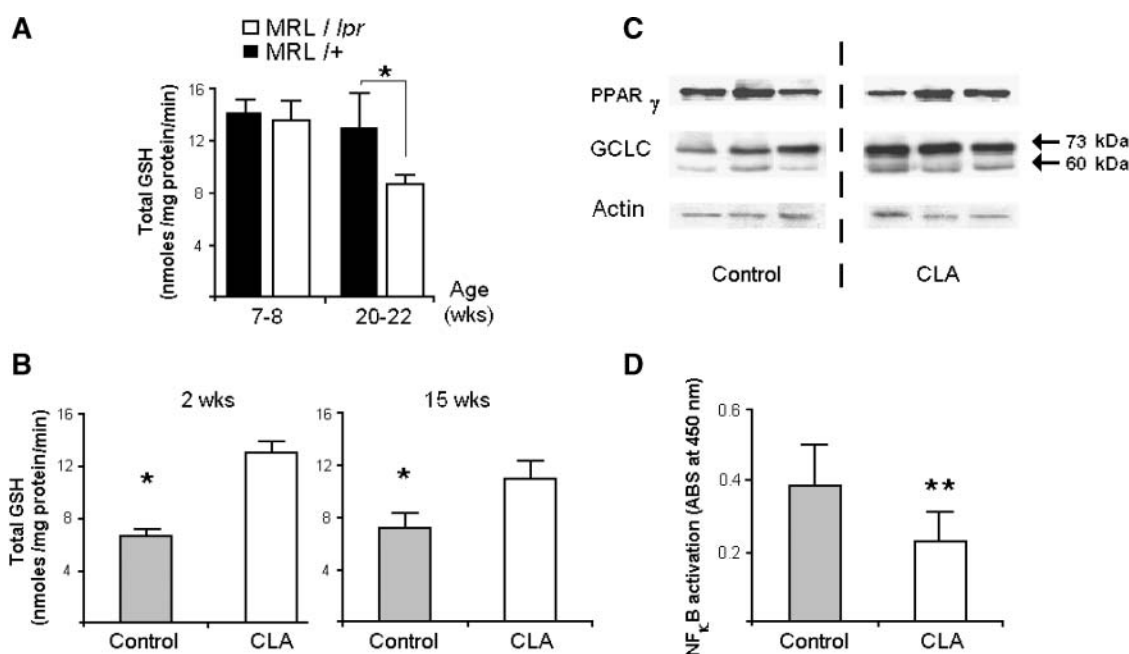


Fig. 6. Increased total GSH, GCLC expression, and reduced nuclear factor κ B (NF κ B) activation in splenocytes in CLA-treated mice compared with control animals. **A:** Intracellular GSH measured in splenocytes from 7–8 week old (predisease) and 20–22 week old (diseased) MRL/lpr mice was compared with that in age-matched MRL/+ mice ($n = 16$). **B:** At the end of the in vivo trial, intracellular GSH concentration was measured in splenocytes from MRL/lpr mice receiving short-term (2 weeks) or long-term (15 weeks) CLA administration. Samples were analyzed at least in duplicate, and the results are presented as means \pm SD. Significant differences from control cells are indicated (* $P < 0.005$). **C:** GCLC and PPAR γ protein contents in spleen cells from CLA-T-treated and control mice were analyzed by Western blotting. Typical results obtained from three CLA-treated and three control animals are shown. Coomassie blue staining of the actin band is shown as a control for protein loading in each lane. **D:** NF κ B activation was measured in splenocyte nuclear extracts by NF κ B p65 enzyme-linked immunosorbent assay. NF κ B activation is expressed as absorbance (ABS) at 450 nm ($n = 24$). Samples were analyzed at least in duplicate, and the results are presented as means \pm SD. Significant differences from control cells are indicated (** $P < 0.01$).

tivation resulted after CLA-T treatment ($P < 0.01$) (Fig. 6D), and this effect was not associated with decreased PPAR γ expression (Fig. 6C).

DISCUSSION

The major finding of this study is CLA's ability to alleviate the autoimmune signs of SLE in MRL/lpr mice; indeed, a significant reduction of all markers used to monitor murine SLE disease progression was observed for the first time. In particular, our results suggest that part of the beneficial effect of CLA on murine SLE occurs through the modulation of the redox status (enhanced intracellular GSH concentration via the upregulated expression of the cytoprotective GCLC protein) independent of apoptosis or PPAR γ activation.

PPARs are fatty acid receptors widely expressed in the cells of the immune system that regulate the expression of genes involved in energy homeostasis and immune function; the ability of synthetic PPAR γ ligand to modulate cytokine synthesis in autoimmune disease was demonstrated recently (31, 32). Cytokines have been hypothesized to play a major role in the immunopathogenesis of both human and murine SLE (33); in particular, the upregulation of IFN γ synthesis was linked to disease progression (34), whereas the overexpression of


IL-10 has been shown to be functional in Th1 suppression (35) and considerable evidence has been produced supporting the central role of IL-10 in autoantibody production (36). The lack of PPAR γ involvement in the CLA-mediated antiproliferative effect on mouse splenocytes, as demonstrated by the experiment with a specific inhibitor, was in overall agreement with literature data showing that other specific ligands for this receptor down-regulate cell proliferation via this mechanism (37). The biochemical mechanism responsible for the immunomodulatory effect in vitro was not investigated, but owing to the lack of association between the significant decrease of typical pathological signs (splenomegaly, autoantibodies, and enhanced Th1 and Th2 cytokine expression) and the unchanged PPAR γ protein expression in spleen cells, PPAR γ involvement in the attenuation of SLE in MRL/lpr mice can be excluded.

ROS are pleiotropic modulators of cellular functions and have been demonstrated to be both physiological and pathological cellular messengers (38). Their ability to cause apoptosis and necrosis is well established, and although these processes are mediated through distinct pathways, both are dependent upon ROS intensity. The antiproliferative and proapoptotic effects of CLA isomers on several cancer cell lines have been reviewed (17). As the link between CLA-induced alteration of intracellular oxidative status and proapoptotic effect was demonstrated previously

by us and others (23, 39), this relationship was investigated in mouse splenocytes. Results from our in vitro experiments resembled those reported previously in Jurkat T-cells: proapoptotic, antiproliferative, and immunomodulatory activities resulted from splenocyte exposure to 100 μ M CLA. Variable effects on mouse splenocyte proliferation have been obtained; in this context, our in vitro data, showing the antiproliferative effect of CLA, are apparently in disagreement with literature data reporting its enhancing or null effect on cell proliferation (40, 41). Unfortunately, because different mouse strains and experimental conditions were used (mitogens and proliferative analysis upon feeding trials), it is difficult to compare those data with ours. Data from in vivo experiments showing the unimportant apoptosis differences among the different experimental groups clearly indicate that the role of apoptosis recovery in CLA-induced amelioration of murine SLE signs can also be excluded.

Redox status is important in the regulation of the immune system (39), and GSH homeostasis plays a key role in the treatment of diseases in which cytokines are major participants in their pathophysiology (42). Our findings showing age- and disease-dependent decreases of intracellular GSH concentration in MRL/*lpr* mice are consistent with literature data indicating decreased blood sulfhydryl or GSH concentrations in T-cells (2) of patients with SLE (1). Moreover, the reduced GSH content in diseased MRL/*lpr* splenocytes, compared with MRL/+ cells (Fig. 6A), is in good agreement with literature data suggesting a defective antioxidant system in MRL/*lpr* mice (43). GCLC represents the rate-limiting enzyme in GSH synthesis; it is composed of catalytic and modifier subunits, and transcriptional activation of cognate genes occurs through an antioxidant response element that plays an essential role in regulating the cellular responses to oxidative stress (44). In this study, CLA's ability to elicit cytoprotective effects (enhanced cellular GSH and GCLC expression) at lower doses was shown for the first time, and this is in agreement with literature data showing the antiapoptotic effect of GCLC (45). In addition, our in vivo results showing CLA-induced upregulation of GSH via the enhanced expression of GCLC in vivo are consistent with recent findings showing the ability of other PPAR γ ligands to upregulate the expression of stress-responding enzymes and/or to enhance cellular redox status (23, 46, 47). NF κ B activity has a critical role for T-cell maturation and in normal immune and inflammatory responses, and it is a pivotal transcription factor in the regulation of several cytokine genes, including IFN γ and IL-10 (48). Interestingly, the modulation of NF κ B activity (49) and its binding activity to a number of target genes in T-cells have proven to be modulated by intracellular redox status in a wide variety of cell types (50). Inhibitory effects of CLA on NF κ B activity have been demonstrated for colon, macrophage, and dendritic cells (51–53), whereas opposite results have been obtained from human adipocytes (54). Results from our in vivo experiments further support the protective effect exhibited by CLA on the intracellular redox status in vitro (increased GSH via GCLC enhancement), and the downregulatory effect on NF κ B activity

occurring via this mechanism may result in a downstream reduction of both Th1 and Th2 cytokines in CLA-treated MRL/*lpr* mice.

Together, our experiments indicate that the modulatory effects of CLA on GCLC expression and GSH content in vivo mirror the protective effect elicited by exposure to 25 μ M in vitro and that this activity, rather than the recovery of apoptotic signaling, may be responsible for the beneficial effects on murine SLE signs. Investigations aimed at greater understanding of the underlying biochemical mechanisms are in progress; further studies are needed to determine the suitability of CLA treatment for autoimmune pathologies in humans. 

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