

Low-Dose Irinotecan Improves Advanced Lupus Nephritis in Mice Potentially by Changing DNA Relaxation and Anti-Double-Stranded DNA Binding

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Objective. Despite clear advances in the treatment of systemic lupus erythematosus (SLE), many patients still present with refractory lupus nephritis, requiring new treatment strategies for this disease. This study was undertaken to determine whether reduced doses of the topoisomerase I (topo I) inhibitor irinotecan, which is known as a chemotherapeutic agent, suppress SLE in (NZB × NZW)F1 (NZB/NZW) mice, and to evaluate the potential mechanism by which irinotecan influences the course of SLE.

Methods. NZB/NZW mice were treated with low-dose irinotecan beginning at either 24 weeks of age or established glomerulonephritis, defined as proteinuria of grade $\geq 3+$. Binding of anti-double-stranded DNA (anti-dsDNA) antibodies was measured by enzyme-linked immunosorbent assay (ELISA), and DNA relaxation was visualized by gel electrophoresis.

Results. Significantly reduced irinotecan doses improved lupus nephritis and prolonged survival in NZB/NZW mice. The lowest dose successfully used for the treatment of established murine lupus nephritis was

>50 times lower than the dose usually used for chemotherapy in humans. As a mechanism, low-dose irinotecan reduced B cell activity. However, the levels of B cell activity in irinotecan-treated mice were similar to those in BALB/c mice of the same age, suggesting that irinotecan did not induce clear immunosuppression. In addition, incubation of dsDNA with topo I increased binding of murine and human anti-dsDNA antibodies, showing for the first time that relaxed DNA is more susceptible to anti-dsDNA antibody binding. This effect was reversed by addition of the topo I inhibitor camptothecin.

Conclusion. Our findings indicate that topo I inhibition may be a novel and targeted therapy for SLE.

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease mainly affecting women of child-bearing age. It is estimated that in the US up to 275,000 adult women suffer from SLE (1). The disease involves different organs, but immune complex glomerulonephritis is a major cause of morbidity and mortality (2). Due to the use of immunosuppressive drugs, the survival of patients with lupus-associated glomerulonephritis increased from a 5-year survival rate of 44% in the 1950s to a 10-year survival rate of 88% recently (3). Despite these advances in the treatment of SLE, the life expectancy of patients with lupus and renal damage was recently demonstrated to be 23.7 years shorter than that of the general population (4).

Treatment with unselective immunosuppressive drugs remains the central strategy to control lupus nephritis. Treatment consists of induction therapy with cyclophosphamide and prednisolone or mycophenolate mofetil followed by maintenance therapy with azathioprine or mycophenolate mofetil (5,6). Major side effects

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of these treatments include infections and malignancies, and cyclophosphamide may also cause amenorrhea (7,8). Interestingly, despite clear beneficial effects in human disease, none of these agents, alone or in combination, were shown to reverse established proteinuria in mice (9).

New hope for the treatment of lupus came with the development of so-called biologic agents. Recently, belimumab, an antibody directed against BAFF, a B cell survival factor, was approved for the treatment of human SLE. While a phase III trial of belimumab showed statistically significant effects in lupus patients without active glomerulonephritis, belimumab had a number needed to treat of nearly 11 (10). Moreover, a second phase III trial demonstrated moderate clinical effects in patients with SLE only when belimumab was used at higher doses and only at week 52, while there was no statistically significant effect at week 76 despite continuous treatment (11). Other biologic agents, such as rituximab or ocrelizumab, which are antibodies designed to deplete B cells, either failed to improve renal function in a recent phase III trial treating patients with proliferating lupus nephritis (12) or were stopped due to the rate of infections (13). Despite progress in research, the treatment of active lupus nephritis remains an unsolved problem requiring new therapeutic approaches.

Our group previously reported that the topoisomerase I (topo I) inhibitor irinotecan suppressed lupus nephritis and significantly prolonged survival in (NZB \times NZW)F1 (NZB/NZW) mice (14), a model of spontaneous SLE (15,16). This finding was made by serendipity and was entirely new, since enzymatic topo I activity had not previously been implicated in the pathogenesis or treatment of SLE. The function of this ubiquitously expressed protein is the reduction of torsional stress that develops during the replication of DNA. To realize this, topo I binds to supercoiled genomic DNA and induces transient single-stranded DNA (ssDNA) breaks followed by stress-relieving rotation of the nicked DNA strand (DNA relaxation). Then, ssDNA breaks are religated by topo I to reconstitute intact double-stranded DNA (dsDNA) (17).

Inhibitors of topo I bind to the topo I-DNA complex (and not to DNA alone or to topo I alone), which is also called the cleavable complex, thereby stabilizing the cleavable complex and preventing the religation of DNA (18). As a consequence, at least two different scenarios are known for cells treated with an inhibitor of topo I. In dividing cells with enhanced DNA replication, the complex of DNA, topo I, and its inhibitor can collide with DNA replication forks that exist

only during S phase. Collision of the cleavable complex with DNA replication forks results in the generation of irreversible dsDNA breaks followed by the induction of cell death (19,20). This is how inhibitors of topo I induce cell death (apoptosis) in proliferating cancer tissue and explains why the topo I inhibitor irinotecan is approved for the treatment of colorectal malignancies (21). In contrast, in nondividing cells, treatment with topo I inhibitors results in the production of ssDNA, which is believed to reduce cell replication capacity but which is not lethal (22).

Using the topo I inhibitor irinotecan for the treatment of murine lupus nephritis, we proposed the second scenario since our previous data clearly demonstrated an inhibition of apoptosis instead of apoptosis induction in the kidneys of irinotecan-treated lupus-prone mice (14). However, our suggestion to have found a potential new treatment option for SLE was not acknowledged, mainly due to the problems associated with using topo I inhibitor as a chemotherapeutic agent (2,23). In the present study, we aimed to investigate whether irinotecan is still able to suppress lupus nephritis at reduced doses. We further investigated whether immunosuppression, changed DNA sensing processes, or an altered binding of anti-dsDNA antibodies is the leading mechanism in irinotecan-mediated suppression of SLE.

MATERIALS AND METHODS

Patient sera. Blood sera positive for anti-dsDNA antibodies were obtained from lupus patients at Hannover Medical School and at Medizinische Klinik und Poliklinik IV, University of Munich. Sera collection was approved by the institutional review boards, and written informed consent was obtained from all patients.

Animals. Six-week-old female NZB/NZW mice were obtained from The Jackson Laboratory, and BALB/c mice used as controls were supplied by Charles River. Animals were kept in isolated ventilated cages. All experiments were performed with the approval of the Kantonale Tierversuchskommission (Bern, Switzerland). Immediately after arrival, mice were randomized to the respective groups with 5 animals per cage.

Prevention of SLE in mice treated with low-dose irinotecan. Beginning at 19 weeks of age, all mice ($n = 10$ per group) were monitored for proteinuria and body weight once a week. Beginning at 24 weeks of age, mice were treated intraperitoneally with irinotecan (Campto; Pfizer) 3 times a week at dosages of 3.125 mg/kg, 6.25 mg/kg, 12.5 mg/kg, or 25 mg/kg (i.e., 9.4 mg/kg, 18.8 mg/kg, 37.5 mg/kg, or 75 mg/kg per week, respectively). The control group received saline at a dose of 10 ml/kg body weight. Treatment was repeated every 4 weeks. For this model (the prevention model), the onset of disease was defined as 2 instances of proteinuria of grade 4+

occurring 1 week apart. Mice were killed due to disease severity when they had proteinuria of grade 4+ ($\geq 2,000$ mg/dl) and weight loss of $>25\%$ from the onset of disease. Proteinuria was measured with Albustix (Siemens Healthcare Diagnostics) and quantified as grade 0 (negative), grade 1+ (≥ 30 mg/dl), grade 2+ (≥ 100 mg/dl), grade 3+ (≥ 300 mg/dl), or grade 4+ ($\geq 2,000$ mg/dl) according to the manufacturer's recommendations. The experiment was terminated at week 65. Separate groups of mice ($n = 14$ per group) were killed 1 week and 3 weeks after completion of the second treatment cycle (at week 29 and week 31, respectively). Untreated 8-week-old NZB/NZW mice ($n = 20$), 23-week-old NZB/NZW mice ($n = 14$), and 30-week-old BALB/c mice ($n = 10$) were used as controls.

Treatment of mice with established lupus nephritis with low-dose irinotecan. Treatment of established lupus nephritis was performed as previously described (14) with slight modifications. NZB/NZW mice ($n = 10$ per group) were examined for proteinuria and body weight once a week starting at 16 weeks of age. Disease onset in this model (treatment of established disease) was defined as 2 instances of proteinuria of grade $\geq 3+$, occurring 1 week apart. Treatment with irinotecan was started at the onset of disease, and mice received 1 mg/kg intraperitoneally 3 times a week for 1 week (3 mg/kg per week). The control group received 10 ml/kg saline. After one treatment cycle no further treatment was administered for at least 3 weeks. Relapsing disease was defined as 2 instances of proteinuria of grade $\geq 3+$, occurring 1 week apart. The treatment of relapsing disease was the same as that performed for the initial treatment. Mice were killed when they showed proteinuria of grade 4+ and weight loss of $>25\%$ from the onset of disease.

Isolation of splenocytes. Spleen was isolated, transferred into ice-cold 2% fetal calf serum (FCS)/phosphate buffered saline (PBS), and smashed on a sterile grid with a pestle. Cells were resuspended and debris was allowed to settle out by centrifugation for 2 minutes at 65g at 4°C. Subsequently, splenocytes were isolated by centrifugation at 290g and 4°C for 5 minutes. Cells were resuspended in RPMI 1640, supplemented with 5% FCS, and adjusted to 10^7 cells/ml after checking for viability by trypan blue exclusion.

Flow cytometry. Splenocytes (10^6 per sample) were incubated with Fc receptor–blocking monoclonal antibody (clone 2.4G2; BD Biosciences) for 30 minutes, followed by incubation with fluorochrome-labeled monoclonal antibodies for 30 minutes on ice. Cells were measured on an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star). Anti-CD4 (clone RM4-5), anti-CD8a (clone 5H10), anti-Ly6C/G (clone RB6-8C5), anti-CD69 (clone H1.2F3), and anti-CD45R/B220 (clone RA36B2) were obtained from Caltag Laboratories.

Enzyme-linked immunospot (ELISpot) assay. Serial dilutions of splenocytes (2×10^5 – 1.25×10^4 /well) in RPMI 1640 medium, supplemented with 5% FCS, were added to 96-well Multiscreen HTS Immobilon-P–bottomed plates (Millipore), coated with goat anti-mouse IgG antibody (Fc specific; Sigma). After 4 hours at 37°C, plates were washed and incubated for 1 hour with alkaline phosphatase–conjugated anti-mouse IgG (H+L chain specific; Southern Biotech). Spots were developed with BCIP/nitroblue tetrazolium plus substrate (Sigma) and counted using an ELISpot reader (Autoimmun Diagnostika).

Antibody enzyme-linked immunosorbent assays (ELISAs). Total IgG and anti-dsDNA antibodies were determined as previously described (14).

Quantitative polymerase chain reaction (PCR). Total RNA was extracted from splenocytes using the GenElute Mammalian Total RNA Kit (Sigma). Quantitative PCR was performed as previously described (24) using the TaqMan Assay-on-Demand assay kit Mm00439546_s1 (Applied Biosystems) for murine interferon- β (IFN β).

Preparation of nucleosomal DNA. Nucleosomal DNA was isolated using the EZ Nucleosomal DNA Prep Kit (Zymo Research). Briefly, nuclei from mouse splenocytes were isolated and digested with Atlantis dsDNase. After spin-column purification the quality of the nucleosomal preparation was checked by resolving the DNA on a 2% agarose gel.

Effect of topo I and camptothecin on binding of anti-dsDNA antibodies. Modification of DNA using topo I alone or in combination with the topo I inhibitor camptothecin was performed essentially as previously described (22). Fifty micrograms of filtered calf thymus dsDNA, nucleosomal DNA, or AT-rich dsDNA poly(dA–dT) (Sigma) was incubated with different concentrations of recombinant human topo I (Creative Biomart) using 0.9 ng, 2.8 ng, 8.3 ng, 25 ng, 75 ng, or 225 ng per μg DNA in a 1 ml reaction containing 40 mM Tris, pH 7.5, 100 mM KCl, 10 mM MgCl $_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, and 30 $\mu\text{g}/\text{ml}$ bovine serum albumin (BSA; Sigma) for 30 minutes at 37°C. Then, 384-well Nunc MaxiSorp plates were coated with 30 μl per well overnight at 4°C. Plates were blocked with PBS containing 1% casein (Pierce) for 1 hour. Plasma samples from NZB/NZW mice ($n = 10$) or human serum samples ($n = 14$) were diluted 1:500 in PBS and incubated for 1 hour. In some experiments the monoclonal anti-dsDNA antibody HYB331-01 (Abcam) was used at a concentration of 1 $\mu\text{g}/\text{ml}$. Bound antibodies were detected as described above for anti-dsDNA ELISA.

To evaluate the effect of the topo I inhibitor camptothecin, 50 $\mu\text{g}/\text{ml}$ of calf thymus dsDNA was incubated with 75 ng recombinant topo I per μg DNA and the indicated amounts of camptothecin (Sigma) directly on 384-well Nunc MaxiSorp plates for 1 hour at 37°C. Ten percent DMSO was used as a control. Determination of anti-dsDNA binding was assessed as described above.

DNA relaxation assay. Fifty $\mu\text{g}/\text{ml}$ of supercoiled pBR322 plasmid DNA (Inspiralis) was incubated with the indicated concentrations of topo I for 1 hour at 37°C. DNA relaxation was visualized by electrophoresis using a 1% agarose gel.

Statistical analysis. Data are expressed as the mean \pm SEM and were analyzed using either one-way or two-way analysis of variance followed by the Bonferroni post hoc test. Some data were assessed by Kruskal-Wallis test. Survival data were analyzed using the Mantel-Cox log rank test. For all tests the software GraphPad Prism version 6.0 was used. *P* values less than 0.05 were considered significant.

RESULTS

Suppression of lupus nephritis in NZB/NZW mice treated with reduced doses of irinotecan. The original experimental plan for reduced doses of irinote-

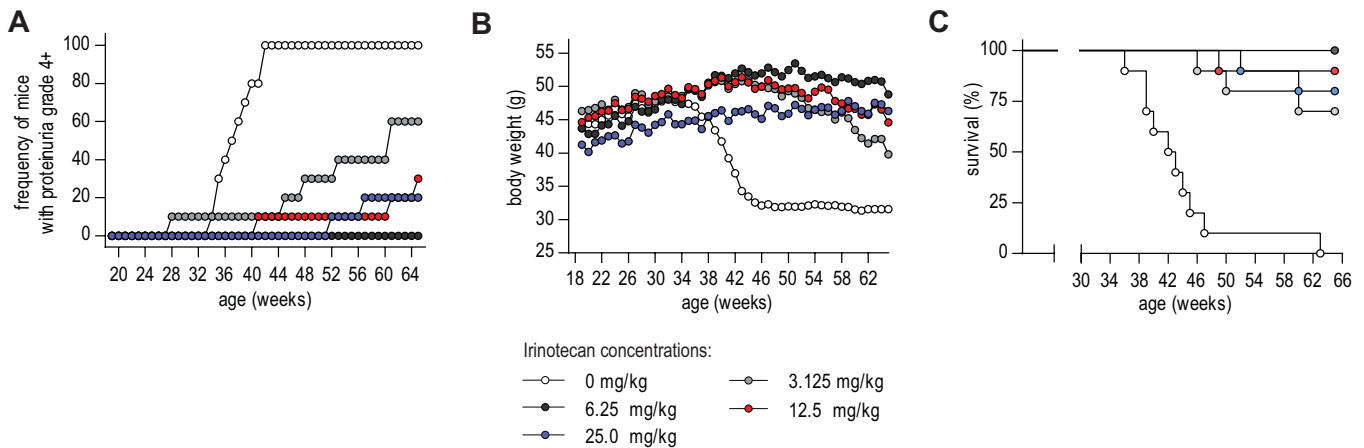


Figure 1. Reduced doses of irinotecan suppress lupus nephritis and prolong survival in (NZB \times NZW)F1 (NZB/NZW) mice. NZB/NZW mice ($n = 10$ per group) were treated 3 times a week beginning at 24 weeks of age with the indicated doses of irinotecan. Treatment was repeated every 4 weeks. **A**, Percentages of saline-treated and irinotecan-treated mice with proteinuria. Proteinuria was measured once a week using Albustix. The onset of disease was defined as 2 different findings of proteinuria of grade 4+ ($\geq 2,000$ mg/dl), obtained 1 week apart. Saline-treated mice showed a significantly higher frequency of grade 4+ proteinuria than all irinotecan-treated groups from 40 through 52 weeks of age. After 53 weeks of age, only doses of ≥ 6.25 mg/kg irinotecan significantly diminished grade 4+ proteinuria. Differences were analyzed by two-way analysis of variance (ANOVA). **B**, Body weight of saline-treated and irinotecan-treated mice. The body weight of all irinotecan-treated groups remained stable until 58 weeks of age and was significantly different from that in saline-treated mice from 42 weeks through 64 weeks of age. Differences were analyzed by two-way ANOVA. **C**, Survival rates of saline-treated and irinotecan-treated mice. Animals were killed due to the severity of the disease. All irinotecan-treated groups had improved survival compared to saline-treated mice. $P < 0.0001$, saline-treated mice versus all other groups, by Mantel-Cox log rank test.

can in lupus-prone mice was designed to yield a dose which is no longer clinically active. This approach would have enabled us to correlate functional effects, such as a potential irinotecan-induced immunosuppression, with clinical data in order to determine the mechanism of irinotecan-mediated suppression of murine SLE. Treatment of NZB/NZW mice with irinotecan was started at 24 weeks of age. At this time point, mice already possess signs of lupus, such as elevated anti-dsDNA antibody titer (25). The highest dosage of irinotecan was 25 mg/kg per injection 3 times a week (e.g., 75 mg/kg per week). This amount was the lowest irinotecan dosage successfully used in previous experiments (14). Moreover, this dosage corresponds to half of the dosage used for chemotherapy in humans (for conversion of dosages from mice to humans see below). Dosages for other groups were divided in half (each consecutive group received half of the dosage received by the previous group). According to this schedule, the lowest irinotecan dosage used for these experiments was 3.125 mg/kg per injection or 9.376 mg/kg per week, corresponding to a dose that is >15 times lower than the dose used for chemotherapy in metastatic cancer.

Surprisingly, even the lowest irinotecan dose demonstrated a suppression of lupus nephritis, as shown

by a significant reduction in proteinuria for all irinotecan-treated groups from 40 weeks to 52 weeks of age. After 53 weeks of age only doses of ≥ 6.25 mg/kg irinotecan significantly diminished the grade of proteinuria (Figure 1A). The body weight of saline-treated control mice started to decline after 35 weeks of age, which was attributed to severely diseased animals. In contrast, the body weight of all irinotecan-treated groups remained stable until 58 weeks of age and was significantly different from saline-treated mice from 42 weeks until 64 weeks of age (Figure 1B). Mice were killed due to the severity of the disease, which was defined as proteinuria of grade 4+ ($\geq 2,000$ mg/dl) and a weight loss of $>25\%$ calculated from the onset of disease. Subsequent analysis of survival data revealed a significantly prolonged survival for all irinotecan-treated groups compared to saline-treated control mice (Figure 1C).

Low-dose irinotecan does not induce immunosuppression. Besides the clinical effects of low-dose irinotecan on lupus nephritis in NZB/NZW mice, we were interested in whether these doses still mediate immunosuppression. Previous experiments by our group had demonstrated that although irinotecan at higher doses caused a transient down-regulation of B cell activity, the levels of anti-dsDNA antibodies were not

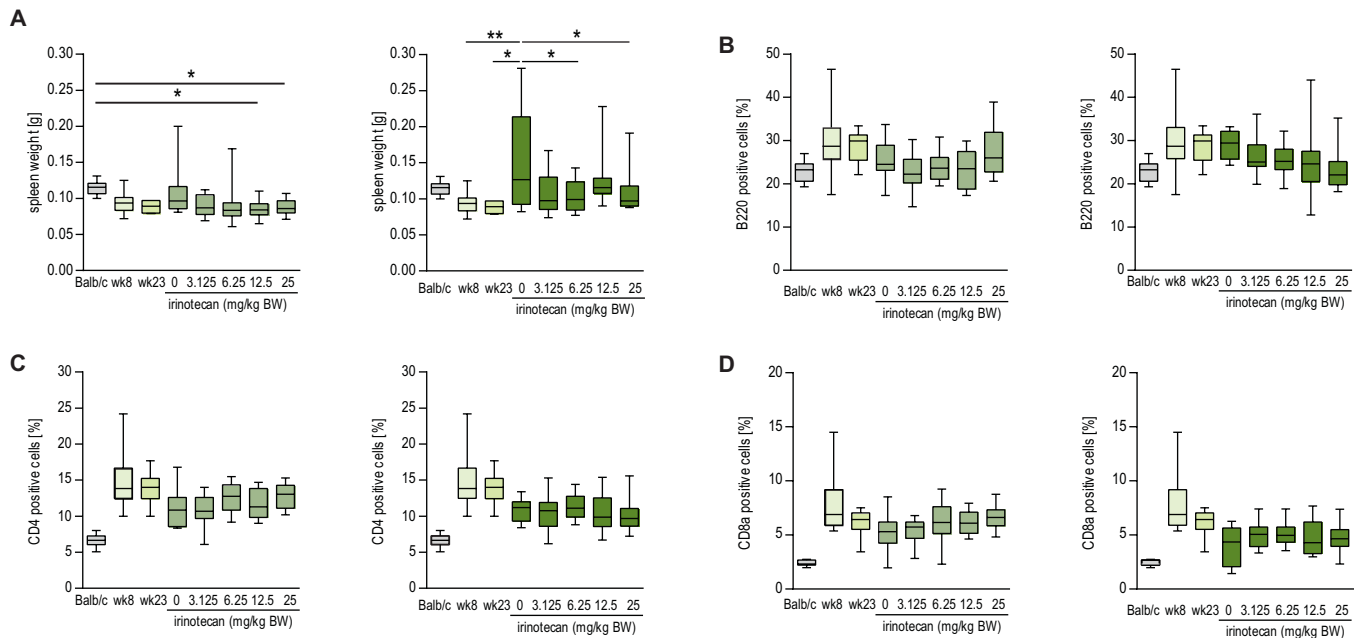


Figure 2. Treatment with low-dose irinotecan is not accompanied by a reduction in T and B lymphocytes. (NZB × NZW)F1 (NZB/NZW) mice were treated 3 times a week beginning at 24 weeks of age with the indicated doses of irinotecan or with saline. Treatment was repeated every 4 weeks. Mice were killed 1 or 3 weeks after the second treatment cycle (at 29 weeks or 31 weeks of age). **A**, Spleen weight, determined immediately postmortem, in 30-week-old BALB/c mice (n = 10), 8-week-old NZB/NZW mice (n = 20), and 23-week-old NZB/NZW mice (n = 14), and in 29-week-old (left) or 31-week-old (right) NZB/NZW mice treated with the indicated amounts of irinotecan (n = 14 per group). **B–D**, Percentage of B cells (**B**), CD4+ cells (**C**), and CD8+ cells (**D**) in 30-week-old BALB/c mice (n = 10), 8-week-old NZB/NZW mice (n = 20), and 23-week-old NZB/NZW mice (n = 14), and in 29-week-old (left) or 31-week-old (right) NZB/NZW mice treated with the indicated amounts of irinotecan (n = 14 per group), determined by flow cytometric analysis. Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the smallest and largest values. * = *P* < 0.05; ** = *P* < 0.01, by one-way analysis of variance. BW = body weight. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.38665/abstract>.

decreased (14). Lupus-prone mice were killed 1 and 3 weeks after the second cycle of treatment with irinotecan (e.g., at 29 and 31 weeks of age). Untreated 8-week-old and 23-week-old NZB/NZW mice and 30-week-old BALB/c mice were used as controls. Determination of spleen weight revealed no significant differences between saline-treated and irinotecan-treated mice at 29 weeks of age. Furthermore, only NZB/NZW mice that received 12.5 mg/kg or 25 mg/kg irinotecan exhibited a lower spleen weight than age-matched BALB/c mice (Figure 2A). At 31 weeks of age, there were significant reductions in spleen weight in mice treated with 6.25 mg/kg and those treated with 25 mg/kg irinotecan compared to the saline-treated group, but there were no differences between saline-treated mice and the other irinotecan-treated groups (Figure 2A). Moreover, no differences were seen in the number of B cell splenocyte populations at week 29 or at week 31 (Figure 2B). The number of CD4+ and CD8+ T cells in saline-treated

mice did not differ from that in any of the groups of irinotecan-treated animals at 29 weeks or 31 weeks of age (Figures 2C and D).

Further investigation of the activity of T and B cells revealed a significant reduction in activated T cells (expressed as CD4+CD69+ cells) only in mice treated with 12.5 mg/kg irinotecan, and not in any other irinotecan-treated groups, at week 29 (Figure 3A). At week 31 a decline in CD4+CD69+ cells was observed only in the groups treated with 6.25 mg/kg and 25 mg/kg irinotecan (Figure 3A). Notably, all irinotecan-treated mice showed a higher number of CD4+CD69+ cells than age-matched BALB/c mice or 8-week-old NZB/NZW mice. B cell activation, which was determined by the number of IgG-secreting splenocytes, was lower only in the group treated with the highest dose of irinotecan at week 29 (Figure 3B), consistent with previously published data (14). No differences between the saline-treated group and any of the irinotecan-treated groups

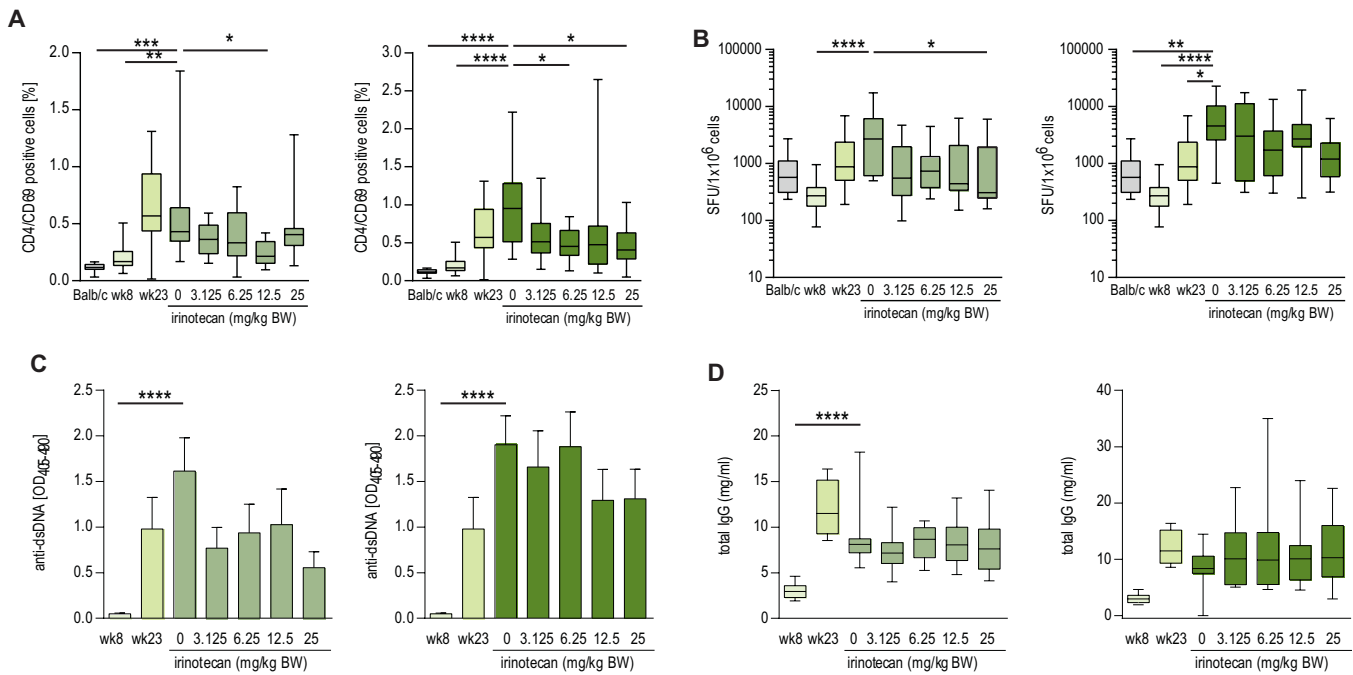


Figure 3. Low-dose irinotecan corrects lupus-associated overstimulation of the immune system but does not induce immunosuppression. (NZB × NZW)F1 (NZB/NZW) mice were treated 3 times a week beginning at 24 weeks of age with the indicated doses of irinotecan or with saline. Treatment was repeated every 4 weeks. Mice were killed 1 or 3 weeks after the second treatment cycle (at 29 weeks or 31 weeks of age). **A** and **B**, T cell activation, determined by flow cytometry measuring CD4+CD69+ cells (**A**) and B cell activity, determined by enzyme-linked immunospot assay assessing the number of IgG-secreting cells (**B**) in 30-week-old BALB/c mice ($n = 10$), 8-week-old NZB/NZW mice ($n = 20$), and 23-week-old NZB/NZW mice ($n = 14$), and in 29-week-old (left) or 31-week-old (right) NZB/NZW mice treated with the indicated amounts of irinotecan ($n = 14$ per group). **C** and **D**, Anti-double-stranded DNA (anti-dsDNA) antibodies in the blood plasma, measured by enzyme-linked immunosorbent assay (ELISA) (**C**) and total IgG in the blood plasma, measured by ELISA (**D**) in 8-week-old NZB/NZW mice ($n = 20$) and 23-week-old NZB/NZW mice ($n = 14$), and in 29-week-old (left) or 31-week-old (right) NZB/NZW mice treated with the indicated amounts of irinotecan ($n = 14$ per group). Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the smallest and largest values. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$, by one-way analysis of variance. BW = body weight; SFU = spot-forming unit. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.38665/abstract>.

were seen at week 31 (Figure 3B). Moreover, B cell activation was not lower than that in age-matched BALB/c mice in any of the irinotecan-treated groups at either week 29 or week 31. Based on lymphocyte numbers as well as B and T cell activity assays, there was no evidence of significant immunosuppression caused by irinotecan. This was confirmed by measurement of anti-dsDNA antibodies and total IgG in the plasma of lupus-prone mice, which showed no differences between the saline-treated and irinotecan-treated groups (Figures 3C and D).

Lack of involvement of DNA sensing in irinotecan-mediated suppression of lupus nephritis. According to our previous work (14) and the data presented here, immunosuppression does not seem to play an important role in irinotecan-mediated suppression of lupus disease. Hence, we looked for alternative mecha-

nisms. As described above, topo I and its inhibitors are known for modification of genomic DNA with induction of ssDNA or dsDNA breaks depending on the rate of cell division. Therefore, we speculated that treatment with irinotecan produced ssDNA breaks potentially in circulating DNA. Since DNA sensing pathways require dsDNA exclusively (26), the induction of ssDNA might affect DNA sensing-mediated systemic inflammation. Because type I IFN production is characteristic of DNA sensing-induced systemic inflammation (27), IFN β levels in the splenocytes of NZB/NZW mice treated with irinotecan were measured. No differences in IFN β levels were found between saline-treated and irinotecan-treated mice at either week 29 or week 31, suggesting that inhibition of DNA sensing is not involved in irinotecan-mediated suppression of SLE (results are available online at <http://www.luniri.com>).

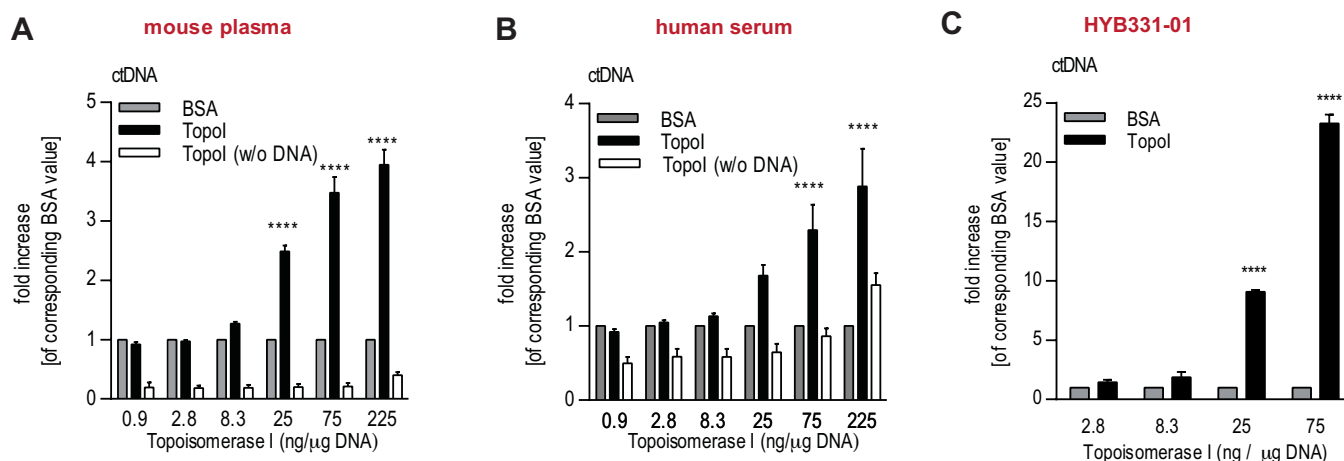


Figure 4. Topoisomerase I (topo I) induces increased binding of anti-double-stranded DNA (anti-dsDNA) antibodies to calf thymus dsDNA (ctDNA). Fifty micrograms per milliliter of DNA was treated with the indicated concentrations of recombinant topo I for 30 minutes at 37°C and then incubated on MaxiSorp plates at 4°C overnight. Bound anti-dsDNA antibodies from **A**, plasma from lupus-prone mice ($n = 10$) or from **B**, sera from lupus patients ($n = 14$), or **C**, using the monoclonal antibody HYB331-01 were determined by enzyme-linked immunosorbent assay. Values are expressed as the fold increase compared to the respective control treated with the same concentration of bovine serum albumin (BSA). Results are representative of 2 or 3 independent experiments for polyclonal sera. Bars show the mean \pm SEM of 3 independent experiments, performed in duplicate for HYB331-01. **** = $P < 0.0001$ versus BSA, by two-way analysis of variance. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.38665/abstract>.

Enhancement of binding of anti-dsDNA antibodies by topo I-induced DNA relaxation. We next postulated that DNA modified by topo I and its inhibitor might change the binding of anti-dsDNA antibodies to DNA (26). We therefore incubated different types of dsDNA with recombinant topo I alone or in combination with the topo I inhibitor camptothecin and measured binding of anti-dsDNA antibodies. Unexpectedly, topo I alone increased binding of anti-dsDNA antibodies from the plasma of lupus-prone NZB/NZW mice and from the sera of patients with SLE to calf thymus DNA (Figures 4A and B). Similar effects were shown for nucleosomal DNA and for AT-rich DNA (results are available online at <http://www.luniri.com>). The concentration of topo I required to mediate an augmentation of dsDNA binding was 10–40 times less than the amount of DNA that is comparable to what has been published for DNA relaxation assays using recombinant topo I (28). When omitting dsDNA in these assays, some increased binding was seen only when using the highest concentration of topo I (Figures 4A and B), excluding a contribution of anti-topo I antibodies reported to be present in lupus patients (29,30). Moreover, when the monoclonal anti-dsDNA antibody HYB331-01 was applied in similar ELISA settings, a >20-fold increase in binding of topo I-treated calf thymus DNA was observed (Figure 4C) compared to an ~3-fold increase obtained using polyclonal mouse or human sera.

Since topo I alone mediates DNA relaxation (31), we further determined whether increased anti-dsDNA binding correlates with the amount of relaxed DNA. Supercoiled pBR322 plasmid DNA was incubated with increasing concentrations of recombinant topo I, and DNA relaxation was visualized by gel electrophoresis (Figure 5A). In addition, modified pBR322 plasmid DNA was used for the measurement of anti-dsDNA binding (Figure 5B), demonstrating a correlation between DNA relaxation and enhanced binding of anti-dsDNA antibodies. Upon addition of camptothecin, an *in vitro* active topo I inhibitor (irinotecan itself is an inactive prodrug which has to be converted *in vivo* into its active form), the effect of topo I on dsDNA binding was reversed. Camptothecin at a concentration of $\geq 2.5 \mu\text{M}$ significantly decreased the binding of anti-dsDNA antibodies from both mice and humans to calf thymus DNA (Figures 5C and D). Inhibitors of topo I might therefore suppress murine, or potentially human, lupus by diminishing the binding of anti-dsDNA antibodies and by decreasing proinflammatory immune complexes.

Amelioration of established lupus nephritis and prolonged survival of mice treated with further reduced doses of irinotecan. To further investigate the efficacy of irinotecan in the treatment of lupus, we performed experiments using yet another reduced dose of irinotecan to treat established lupus nephritis. Irinotecan was

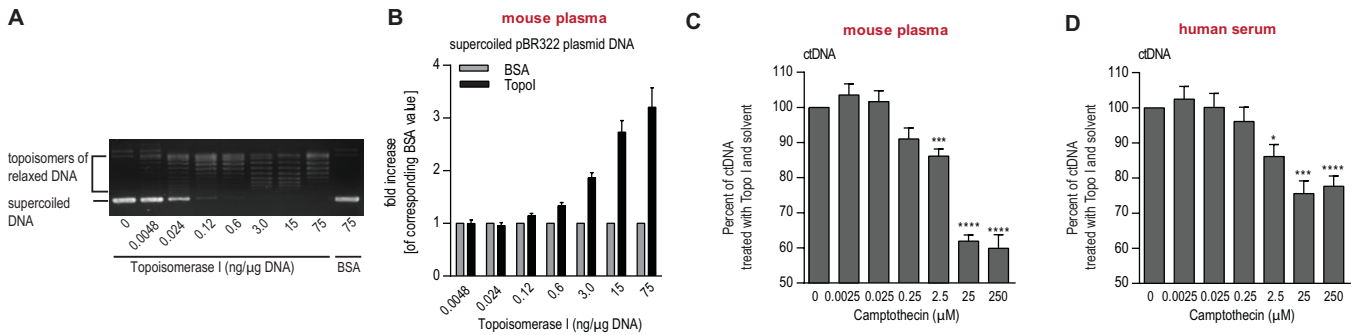


Figure 5. DNA relaxation correlates with enhanced binding of anti-double-stranded DNA (anti-dsDNA) antibodies and inhibition of this effect by addition of the topoisomerase I (topo I) inhibitor camptothecin. **A**, DNA relaxation visualized by electrophoresis using a 1% agarose gel. Fifty micrograms per milliliter of supercoiled pBR322 plasmid DNA was incubated with the indicated concentrations of topo I for 1 hour at 37°C. **B**, Binding of murine anti-dsDNA antibodies to supercoiled DNA treated with topo I, determined by enzyme-linked immunosorbent assay. Values are expressed as the fold increase compared to the value for the respective control treated with the same concentration of bovine serum albumin (BSA). **C and D**, Inhibition of enhanced binding of anti-dsDNA antibodies to topo I-modified calf thymus DNA (ctDNA) after addition of the topo I inhibitor camptothecin. Fifty micrograms per milliliter of DNA was incubated with 75 ng/μg topo I, the indicated concentrations of camptothecin, and 10% DMSO for 1 hour at 37°C. After incubation with mouse plasma (**C**) or human sera (**D**), bound antibodies were measured. Values are expressed as the fold increase compared to the value for the respective control treated with the same concentrations of BSA and camptothecin. In **B–D**, bars show the mean ± SEM. Results are representative of 2 independent experiments. * = $P < 0.05$; *** = $P < 0.001$; **** = $P < 0.0001$ versus no camptothecin, by one-way analysis of variance. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.38665/abstract>.

administered to NZB/NZW mice beginning at the onset of disease, which was defined as proteinuria of grade $\geq 3+$ measured twice, 1 week apart. Irinotecan 1 mg/kg was given 3 times a week. After 1 treatment cycle, both saline-treated and irinotecan-treated mice received no further treatment for at least 3 weeks. Relapsing disease was diagnosed and treated as initial disease. This treat-

ment schedule demonstrated a reversal of established proteinuria in irinotecan-treated mice at the second and third week after the initiation of treatment (Figure 6A). While the body weight of saline-treated control mice declined beginning at week 29, the body weight of irinotecan-treated mice was stable until 38 weeks of age. A significant difference in body weight between the 2

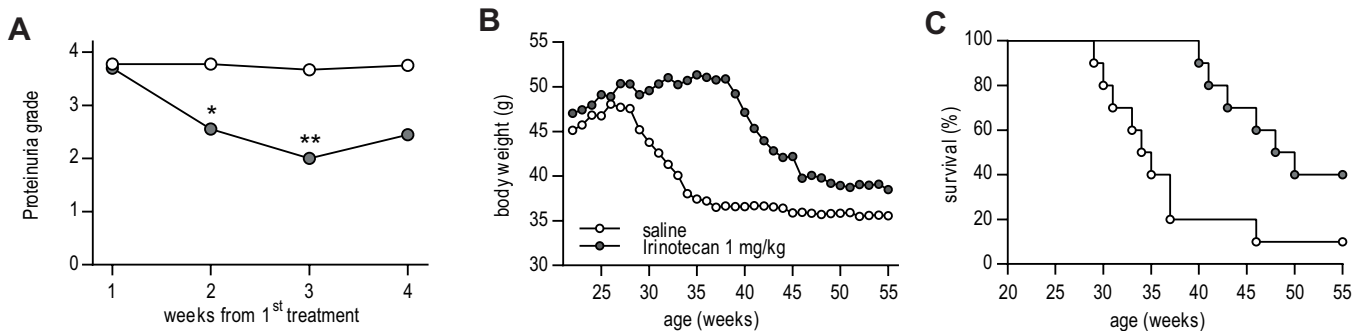


Figure 6. Reversal of established lupus nephritis in (NZB × NZW)F1 mice that received an irinotecan dose >50 times lower than the dose used for chemotherapy. Mice were treated beginning when proteinuria was established, which was defined as 2 different findings of grade $\geq 3+$ (300 mg/dl) obtained 1 week apart. Irinotecan at a dose of 1 mg/kg was administered 3 times a week. Further treatments were given according to the grade of proteinuria as described for the initial treatment initiation. Treatment cycles had to be at least 3 weeks apart. **A**, Reversal of established proteinuria determined after the first treatment cycle. Values are the mean ($n = 9$ for saline-treated mice and $n = 10$ for irinotecan-treated mice). * = $P < 0.05$; ** = $P < 0.01$ versus saline-treated mice, by two-way analysis of variance (ANOVA). **B**, Body weight of irinotecan-treated mice and saline-treated mice. Values are the mean ($n = 10$ mice per group). $P < 0.05$, saline-treated mice versus irinotecan-treated mice from 34 to 39 weeks of age, by two-way ANOVA. **C**, Survival of both saline-treated mice and irinotecan-treated mice. Animals were killed when disease became severe, which was defined as proteinuria of grade 4+ (2,000 mg/dl) and a weight loss of $>25\%$ calculated from the onset of the disease ($n = 10$ mice per group). $P = 0.0064$, saline-treated mice versus irinotecan-treated mice, by Mantel-Cox log rank test.

groups was observed between 34 and 39 weeks of age (Figure 6B). In addition to the reversal of established proteinuria, this minimal dose of irinotecan significantly prolonged the survival of NZB/NZW mice with lupus ($P = 0.0064$) (Figure 6C).

DISCUSSION

Our data show that lupus in NZB/NZW mice can be treated with remarkably reduced doses of the topo I inhibitor irinotecan. The minimal dose used in our experiments for the treatment of established lupus nephritis was >50 times lower than the dose normally used for chemotherapy in humans. The calculation was as follows. In Europe, for chemotherapy in humans a dose of 350 mg/m² is given every third week (21,32). In lupus-prone mice a dose of 3 mg/kg per week was administered for the treatment of established disease. Depending on the grade of proteinuria, this treatment could be repeated every 4 weeks. The dose of 3 mg/kg in mice corresponds to 9 mg/m² in humans (for conversion see <http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm078932.pdf>). Over a period of 12 weeks, humans received a total irinotecan dose of 1,400 mg/m², while the experimental plan for NZB/NZW mice allowed a total dose of 27 mg/m² during the same period of time. It is also worth mentioning that the dose of irinotecan used in anticancer experiments in mice varies from 100 mg/kg to 200 mg/kg per week (33–35). These numbers indicate that the molecular pathways targeted by irinotecan for chemotherapy and for the suppression of SLE are not similar. Whereas our previous data suggested a mild and transient immunosuppression using high-dose irinotecan (14), the results of the present study of low-dose irinotecan showed an amelioration of lupus-associated B cell hyper-responsiveness, but provided no evidence of immunosuppression. Instead, our findings indicate that irinotecan-mediated effects on lupus may occur through modification of DNA.

The idea to target DNA is not new, since circulating DNA is believed to be a major pathogen in SLE (36). One approach has been the use of recombinant DNase I to destroy circulating DNA. The results of these experiments were conflicting (37,38), perhaps reflecting the recently reported protection of circulating DNA from extracellular degradation by certain peptides produced as neutrophil extracellular traps in patients with SLE (39). If extracellular DNA is difficult to eliminate, an alternative possibility is to manipulate the DNA in order to make it less antigenic. As a consequence,

modified circulating DNA can transform the binding of extracellular DNA to renal structures, as has been shown previously for the glucosaminoglycan heparin (40). Alternatively, modified DNA might also reduce the binding of anti-dsDNA antibodies to the DNA and so lead to fewer immune complexes.

We hypothesize, based on the data presented here, that irinotecan reverses the increased binding of DNA to anti-dsDNA antibodies that is mediated by topo I. The question is how topo I facilitates enhanced binding of anti-dsDNA antibodies to the DNA. Preferential binding of anti-dsDNA antibodies to a protein-DNA complex is unlikely because binding of topo I to the DNA is transient (41). Alternatively, the induction of ssDNA breaks as the underlying mechanism for enhanced anti-dsDNA binding is also not very likely, since without its inhibitor topo I cleaves and subsequently rejoins dsDNA, leaving intact strands. A third possibility is a change in DNA conformation, which is supported by our data showing that anti-dsDNA antibodies increase their binding to dsDNA after topo I has catalyzed relaxation of the DNA. This suggests that anti-dsDNA antibodies prefer binding to relaxed DNA. Accordingly, enhanced binding of anti-dsDNA antibodies to topo I-modified dsDNA was prevented by administering the topo I inhibitor camptothecin. It is known that topo I inhibitors inhibit topo I-mediated DNA relaxation (42,43). Consequently, we believe that inhibition of DNA relaxation could explain how irinotecan influences the course of SLE. Interestingly, inhibition of DNA relaxation by topo I inhibitors was reported to preferentially affect negatively supercoiled DNA and to omit positive supercoils (44). Whether this has an impact on irinotecan-mediated suppression of SLE remains unknown.

The next question is where topo I induces relaxation of circulating DNA. Assuming that this effect indeed occurs in vivo, it may be that DNA is modified intracellularly and then released by apoptotic cells, since it is believed to be the mechanism for the generation of circulating DNA in SLE (45). Alternatively, modification of DNA might arise in the blood, presuming the presence of extracellular topo I. While topo I contains several nuclear localization signals at the N-terminus and seems to be localized exclusively in intracellular DNA-containing structures, e.g., the nucleus and mitochondria (46), there is some evidence that topo I can access the extracellular space under certain circumstances. Otherwise, how anti-topo I antibodies arise in SLE or other autoimmune diseases would be not explainable (29,47).

Based on the data provided here we are planning a first clinical trial in which patients with active lupus nephritis refractory to conventional medication will be treated with low-dose irinotecan. If low-dose irinotecan shows the same efficacy during the trial as we have shown here in lupus-prone mice, undesired side effects known from conventional immunosuppressive therapy (48) might disappear. However, in the context of side effects, there may be concerns as to whether irinotecan can be given to patients with impaired renal function. The answer is favorable, since detoxification and elimination of the active metabolite of irinotecan, SN-38, is facilitated by glucuronidation by uridine diphosphate-glucuronosyltransferase 1A in the liver (49). Therefore, irinotecan can be used in patients with renal failure without the risk of increased side effects (50).

In conclusion, given our findings in lupus-prone mice treated with low-dose irinotecan and given that topo I is a highly conserved enzyme showing 96% homology between mice and humans (51), low-dose irinotecan may be a valuable therapeutic option for patients with lupus nephritis and potentially with other SLE manifestations.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Frese had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Frese-Schaper, Keil, Huynh-Do, Schmid, Frese.

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