

Reversal of Established Lupus Nephritis and Prolonged Survival of New Zealand Black \times New Zealand White Mice Treated with the Topoisomerase I Inhibitor Irinotecan

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Systemic lupus erythematosus is a chronic autoimmune disorder that predominantly affects women of childbearing age. Lupus-associated glomerulonephritis is a major cause of mortality in these patients. Current treatment protocols for systemic lupus erythematosus include cyclophosphamide, prednisolone, azathioprine, and mycophenolate mofetil. However, in mice none of these agents alone or in combination were shown to reverse established proteinuria. Using New Zealand Black \times New Zealand White F1 mice, we report that administration of the topoisomerase I inhibitor irinotecan from week 13 completely prevented the onset of proteinuria and prolonged survival up to at least 90 wk without detectable side effects. Furthermore, application of irinotecan to mice with established lupus nephritis, as indicated by grade 3+ (≥ 300 mg/dl) and grade 4+ (≥ 2000 mg/dl) proteinuria and, according to a median age of 35 wk, resulted in remission rates of 75% and 55%, respectively. Survival was significantly prolonged with 73 wk (grade 3+ and 4+ combined) versus 40 wk for control animals. Although total IgG and anti-dsDNA Abs in the serum and mesangial IgG deposits in the kidneys were not reduced in irinotecan-treated mice, subendothelial immune deposits were considerably diminished, suggesting a prevention of glomerular basement membrane disruption. This effect was accompanied by increased rates of ssDNA breaks and inhibition of renal cell apoptosis being different to what is known about irinotecan in anticancer therapy. In conclusion, our data provide evidence that irinotecan might represent an entirely new strategy for the treatment of systemic lupus erythematosus. *The Journal of Immunology*, 2010, 184: 2175–2182.

Systemic lupus erythematosus is an autoimmune disease that affects the skin, joints, cardiac and pulmonary systems, CNS, and kidneys. It is estimated that up to 275,000 adult women suffer from lupus erythematosus in the United States (1). The 5-y survival rate of patients with lupus-associated glomerulonephritis increased from 44% in the 1950s to 82% recently (2). Despite clear advances in the treatment of this autoimmune disease, the mean age of death of patients dying from systemic lupus erythematosus is 44 y (3).

The F1 hybrid of New Zealand Black \times New Zealand White (NZB/W) is a well-established mouse model of systemic lupus erythematosus. The disease in these mice resembles human lupus erythematosus with antinuclear Abs, hemolytic anemia, proteinuria, and progressive glomerulonephritis leading to death (4, 5). Generally, there are three types of studies investigating the influence of certain substances on the course of lupus erythematosus in the NZB/W F1 model. The majority of studies are of preventive treatment that started between 8 and 24 wk of age without measurable proteinuria (6–9). Studies examining the

treatment of established disease can be divided into two categories. The first type has a defined start of treatment later than week 24 regardless of the extent of proteinuria that might be also called *late prevention* studies (10–13). The second type of study focusing on established disease initiated treatment from a defined high-grade proteinuria (grade 3+ and 4+ with at least ≥ 300 mg/dl), which requires individual treatment schedules for each mouse. Although this study design most likely reflects the clinical situation with patients with lupus, only a few of them exist (14–16).

Current treatment protocols for human lupus erythematosus consist of an induction therapy with cyclophosphamide and prednisolone followed by a maintenance therapy with cyclophosphamide or azathioprine or mycophenolate mofetil (17–19). In NZB/W F1 mice, the nitrogen mustard alkylating agent cyclophosphamide alone prolonged survival in animals treated from week 36, but only in 1 of 10 animals an amelioration of proteinuria was seen (11). Likewise, azathioprine, a purine synthesis inhibitor, prolonged survival when administered alone to NZB/W F1 from week 39, although no significant effect on proteinuria was seen (12). Moreover, the combination of cyclophosphamide, azathioprine, and methylprednisolone given to animals with advanced disease defined as grade 3+ and 4+ proteinuria failed both to prolong survival and to have an effect on existing levels of proteinuria (14). Although mycophenolate mofetil is used with increased frequency for the treatment of human systemic lupus erythematosus there are no data available regarding the treatment of established disease in mice; only preventive effects have been shown (20, 21).

Our group is working on sensitization for receptor-mediated apoptosis in lung cancer (22). In this context, we injected immunocompetent mice with a species-different cytokine alone and in combination with the topoisomerase I inhibitor irinotecan, an

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Abbreviations used in this paper: CTX, cyclophosphamide; LDH, lactate dehydrogenase; NZB/W, New Zealand Black \times New Zealand White.

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agent that is approved for the treatment of colorectal cancer (23, 24). As a result, all mice that received the cytokine alone died during the second cycle of treatment, most likely explained by the occurrence of cross-species Abs. To our surprise, all mice treated in combination with irinotecan survived. Based on this coincidental observation, we asked whether this finding might be related to the inhibition of some form of hyperreactivity by irinotecan. We therefore decided to investigate whether irinotecan could be protective against increased immunoreactivity observed during autoimmunity.

Materials and Methods

Animals

Six-week-old female NZB/W F1 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were kept in isolated ventilated cages. All experiments were performed with the agreement of the Kantonale Tierversuchskommission (Bern, Switzerland). Directly after arrival, mice were randomized to the respective groups with 6 animals per cage.

Prevention study

From week 12, all mice were monitored for proteinuria and body weight once per week ($n = 10$, control mice; $n = 12$, both groups treated with irinotecan). Treatment with irinotecan (Camptosar) started from week 13 and was conducted i.p. every fourth week, three times per week with doses of 25 or 50 mg/kg (i.e., 75 or 150 mg/kg/wk, respectively). The control group received saline with 10 ml/kg body weight. The beginning of the disease was defined as two instances of proteinuria grade 4+, 1 wk apart. Criteria for sacrifice owing to the severity of the disease were proteinuria grade 4+ (≥ 2000 mg/dl) and weight loss $>25\%$ calculated from the beginning of the disease. Proteinuria was measured with Albustix (Bayer Diagnostics, Deerfield, IL) and quantified as grade 0 (negative), grade 1+ (≥ 30 mg/dl), grade 2+ (≥ 100 mg/dl), grade 3+ (≥ 300 mg/dl) and grade 4+ (≥ 2000 mg/dl) according to manufacturer's recommendations.

Treatment of established disease

Experiments were projected to treat mice with established lupus nephritis defined by high-grade (3+ and 4+) proteinuria. From week 18, proteinuria and body weight were measured twice per week. The beginning of the disease was defined as two instances proteinuria grade 3+ or grade 4+, 3 d apart. Treatment was performed three times per week for 2 wk with 50 mg/kg irinotecan (six applications per treatment cycle). Relapse of the disease was defined as two instances of proteinuria grade 3+ or grade 4+, 3 d apart. The treatment of relapsed disease was performed as the initial treatment. Complete remission was defined as 30 mg/dl or less of proteinuria maintained for at least 3 wk. Criteria for sacrifice owing to the severity of the disease were a proteinuria grade 4+ and a weight loss $>25\%$ calculated from the initial beginning of the disease. Animals that showed significant weight loss without proteinuria were excluded from the study ($n = 10$, control mice; $n = 12$, irinotecan-treated mice [grade 3+]; $n = 11$, irinotecan-treated mice [grade 4+]).

Isolation of splenocytes

The spleen was isolated, transferred into ice-cold 2% FCS/PBS, and smashed on a sterile grid with a pestle. Cells were resuspended, and clumps were allowed to settle out by centrifugation for 1 min at $65 \times g$, 4°C . Subsequently, splenocytes were isolated by centrifugation at $290 \times g$ and 4°C for 5 min. 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

EDTA-blood samples were drawn after euthanasia of the animals by cardiac puncture or retro-orbital bleeding. A complete blood count was obtained using the hematology counter ABX Micros 60 (HORIBA ABX Diagnostics, Kyoto, Japan). Splenocytes or peripheral blood cells (10^6 per sample) were incubated with Fc receptor-blocking mAb (clone 2.4G2, BD Biosciences, San Jose, CA) for 30 min, followed by incubation with fluorochrome-labeled mAbs for 30 min on ice. Erythrocytes were then lysed with ACK buffer (0.15 M NH_4Cl , 10 mM KHCO_3 , 0.1 mM Na_2EDTA , pH 7.3) for 5 min at room temperature. Subsequently, cells were washed and resuspended in 250 μl PBS containing 1% BSA. Staining was measured on an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR). The following Abs were obtained from

Caltag Laboratories (Burlingame, CA): anti-CD4 (clone RM4-5), anti-CD8a (clone 5H10), anti-Ly6C/G (clone RB6-8C5), anti-CD11b (clone M1/70.15), anti-CD69 (clone H1.2F3), and anti-CD45R/B220 (clone RA36B2). Anti-IgD (clone 11-26.c2a) and anti-IgM (clone R6-60.2) were provided by BD Biosciences.

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 Multi-screenHTS Immobilon-P-bottomed plates (Millipore, Bedford, MA), coated with goat anti-mouse IgG (Fc-specific; Sigma-Aldrich, St. Louis, MO) Ab. After 4 h at 37°C plates were washed and incubated for 1 h with alkaline phosphatase-conjugated anti-mouse IgG (H+L chain specific, Southern Biotech, Birmingham, AL). Spots were developed with BCIP/NBT plus substrate (Sigma-Aldrich, St. Louis, MO) and counted with a stereomicroscope (Wild Heerbrugg, Heerbrugg, Switzerland).

ELISAs

Total IgGs. Polystyrene 96-well plates (Maxisorp; Nunc, Naperville, IL) were coated with 5 $\mu\text{g/ml}$ goat anti-mouse IgG (Southern Biotech) and incubated overnight at 4°C . Plates were blocked with PBS containing 1% BSA for 1 h at 37°C . Serum samples were diluted 1:100,000 in PBS and incubated in duplicates for 1 h at 37°C . Bound Abs were detected by incubation for 1 h at 37°C with a goat anti-mouse IgG alkaline phosphatase conjugate (Southern Biotech) and developed with p-nitrophenol phosphate (Sigma-Aldrich). OD was measured at 405 nm with a reference filter at 490 nm. The sample concentrations were calculated by reference to the linear portion of a standard curve of purified mouse IgG (Sigma-Aldrich) run on every plate.

Anti-dsDNA Abs. Calf thymus DNA (Invitrogen, Carlsbad, CA) was passed through a Millex-HA 0.45 mm filter (Millipore) to remove any ssDNA fragments. Maxisorp plates were half coated with 100 $\mu\text{g/ml}$ calf thymus DNA in PBS and half with buffer alone over night at 4°C . Plates were blocked with PBS containing 1% BSA for 1 h at 37°C . Serum samples were diluted 1:500 in PBS and incubated on both DNA coated and uncoated sides of the plate for 1 h at 37°C . Bound Abs were detected as described before for total IgG ELISA.

Histopathology

Kidneys were fixed in 4% paraformaldehyde. Staining for H&E, periodic acid-Schiff reagent, and methenamin silver was performed using standard protocols. IgG deposits were analyzed on cryosections. Sections were fixed in acetone for 10 min and incubated with an Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L chain specific) Ab (Invitrogen). The kidney score of glomerulonephritis was determined in a blinded manner using the ISN/RPS2003 classification. Staining for ssDNA was performed using the mAb F7-26 (Alexis, Laufen, Switzerland) in combination with the Animal Research Kit (Dako, Glostrup, Denmark) according to the manufacturer's instructions.

Apoptosis assay

Caspase-3 activity was determined essentially as described before (25); 100 μg of kidney lysates were incubated with 200 μM DEVD-afc (Alexis), and relative fluorescence was determined over 50 min at 37°C using a SPECTRAMax GEMINI XS (Molecular Devices, Sunnyvale, CA). All experiments were run twice. TUNEL was performed by using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore) following the manufacturer's instructions.

Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) activity in plasma samples was determined spectrophotometrically as described previously (26). Sample values were calculated from a standard curve generated with pyruvate.

Quantification of dsDNA at the ratio of total DNA

DNA from mouse kidney tissue was isolated using a genomic DNA extraction kit (Qiagen, Valencia, CA). RNA was digested with RNase A (Qiagen) at a final concentration of 20 $\mu\text{g/ml}$. Total DNA content was measured at 260 nm and adjusted to 100 ng/ml. DsDNA was then quantified using the Quant-iT PicoGreen reagent (Invitrogen) according to the manufacturer's instructions.

Statistical analysis

For statistical analysis, data were subjected to one- or two-way ANOVA using GraphPad Prism (GraphPad Software, San Diego, CA). Differences among experimental groups were determined by Bonferroni posthoc test. Differences were considered statistically significant at $p < 0.05$.

Results

Prevention of lupus-associated glomerulonephritis by irinotecan

We first analyzed whether irinotecan influences the onset of spontaneous lupus erythematosus in NZB/W F1 mice. Treatment of mice starting from week 13, with administration of irinotecan repeated every fourth week, completely inhibited proteinuria. Whereas 100% of control mice possessed at least grade 2+ (≥ 100 mg/dl) proteinuria from week 42, none of the mice treated with irinotecan showed signs of lupus nephritis (Fig. 1A). At week 50/51, half of the animals from the irinotecan-treated groups were sacrificed to preserve blood and tissue samples for further analysis. At this time point, all saline-treated control mice were already dead or had been sacrificed “due to the severity of the disease,” which was defined as grade 4+ proteinuria and weight loss $>25\%$ calculated from the beginning of the disease. The experiment was terminated at week 90. At this time point, there were no deaths in the group treated with 50 mg/kg irinotecan (150 mg/kg per wk). In the group treated with 25 mg/kg irinotecan (75 mg/kg per wk), 4 of 6 animals were alive yielding a median survival of 90 wk for both irinotecan-treated groups compared with 36 wk for saline-treated controls (Fig. 1B).

Importantly, we observed no toxic side effects of irinotecan treatment associated with the doses used. Although saline-treated animals demonstrated a significant weight loss beginning at week 35, caused by the onset and rapid progression of the lupus disease,

irinotecan-treated mice possessed a normal behavior with a regular increase in body weight over time (Fig. 1C). To further analyze possible side effects of irinotecan treatment, we measured serum LDH activity, which is a commonly used marker for drug-related toxicity. As a result, no changes in LDH activity of irinotecan-treated mice compared with saline-treated controls were determined (data not shown). Moreover, no tumors were observed in irinotecan-treated animals sacrificed at week 50/51, an effect that was described for the long-term treatment with azathioprine (12).

Histologic and immunologic effects of long-term treatment with irinotecan

Histologic examination of irinotecan-treated mice demonstrated nearly complete repression of glomerulonephritis. Whereas kidney sections of control mice showed signs of severe glomerulonephritis, animals treated with irinotecan at week 50/51 possessed almost normal renal parenchyma with occasional mesangial hypercellularity in some animals (Fig. 2A, 2C). Staining for IgG revealed mesangial immune deposits in both saline- and irinotecan-treated animals, demonstrating a decrease in animals treated with 50 mg/kg irinotecan but no significant change in animals treated with 25 mg/kg irinotecan. In contrast, subendothelial IgG deposits were abundant in saline-treated control mice, but nearly undetectable in the kidneys of both irinotecan-treated groups (Fig. 2B, 2D, 2E), suggesting that irinotecan prevents the disruption of glomerular basement membranes.

We next asked whether the observed prevention of subendothelial immune deposits in irinotecan-treated mice was associated with an impaired function of immune cells. At first, different cell populations from peripheral blood—obtained by retro-orbital puncture at weeks 18, 20, and 22—were determined. Analysis of B220, CD4, CD8, CD11b, and Gr-1-positive cells showed no significant differences between saline-treated and irinotecan-treated groups, except for some increase of CD8 lymphocytes at week 20 and some higher Gr-1 positive cells at week 22 in the group treated with 25 mg/kg irinotecan (data not shown). In addition, we analyzed spleen cells and sera from saline-treated animals that were sacrificed “due to the severity of the disease” and from irinotecan-treated animals terminated at week 50/51. Spleen cell populations demonstrated a decrease of approximately one third of the total number of B cells as well as lower B and T cell activity in the irinotecan-treated groups. B cell activity was determined by ELISpot and the percentage of IgM^{low}IgD^{low} B220⁺ cells; T cell activity was expressed as the percentage of CD69⁺ CD4 cells. However, despite decreased B cell activity, the level of both, total serum IgG and anti-dsDNA Abs remained unchanged (Table I).

Induction of ssDNA and prevention of renal cell apoptosis by irinotecan

Given similar levels of anti-dsDNA Abs in saline- and irinotecan-treated mice, we hypothesized that the above demonstrated prevention of glomerular basement membrane disruption in irinotecan-treated mice, as shown by the avoidance of subendothelial immune deposits, was instead caused by changes on the level of kidney cells, thus preventing the fatal action of anti-dsDNA Abs. Because it is known that topoisomerase I inhibitors avoid the religation of ssDNA breaks, which are produced by topoisomerase I (27), we first determined the amount of ssDNA on kidney sections using the mAb F7-26, which was shown to bind to ssDNA but not to dsDNA (28). As expected, we found a marked increase of ssDNA in irinotecan-treated mice (Fig. 3A–C). Providing an equal amount of total DNA, it suggested that the amount of dsDNA was reduced compared with saline-treated animals. We therefore quantified dsDNA at the ratio of total

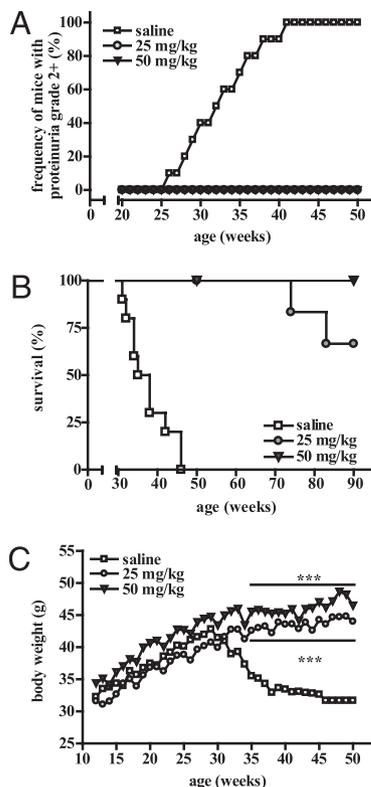


FIGURE 1. Prevention of murine lupus erythematosus by inhibition of topoisomerase I. NZB/W F1 mice were treated from week 13 with 25 mg/kg or 50 mg/kg irinotecan three times per week every fourth week ($n = 10$, control mice; $n = 12$, both groups treated with irinotecan). **A**, Frequency of mice with proteinuria. Each point reflects the frequency of mice with grade 2+ (≥ 100 mg/dl) proteinuria at the indicated time points. **B**, Survival of irinotecan-treated groups was significantly better compared with saline-treated groups. $p < 0.0001$; Kaplan-Meier log-rank test. At week 50/51, half of the groups treated with irinotecan were sacrificed for further analysis. **C**, Normal development of body weight in irinotecan-treated mice versus decline of body weight in saline-treated controls owing to the onset of the lupus disease. $***p < 0.001$; two-way ANOVA.

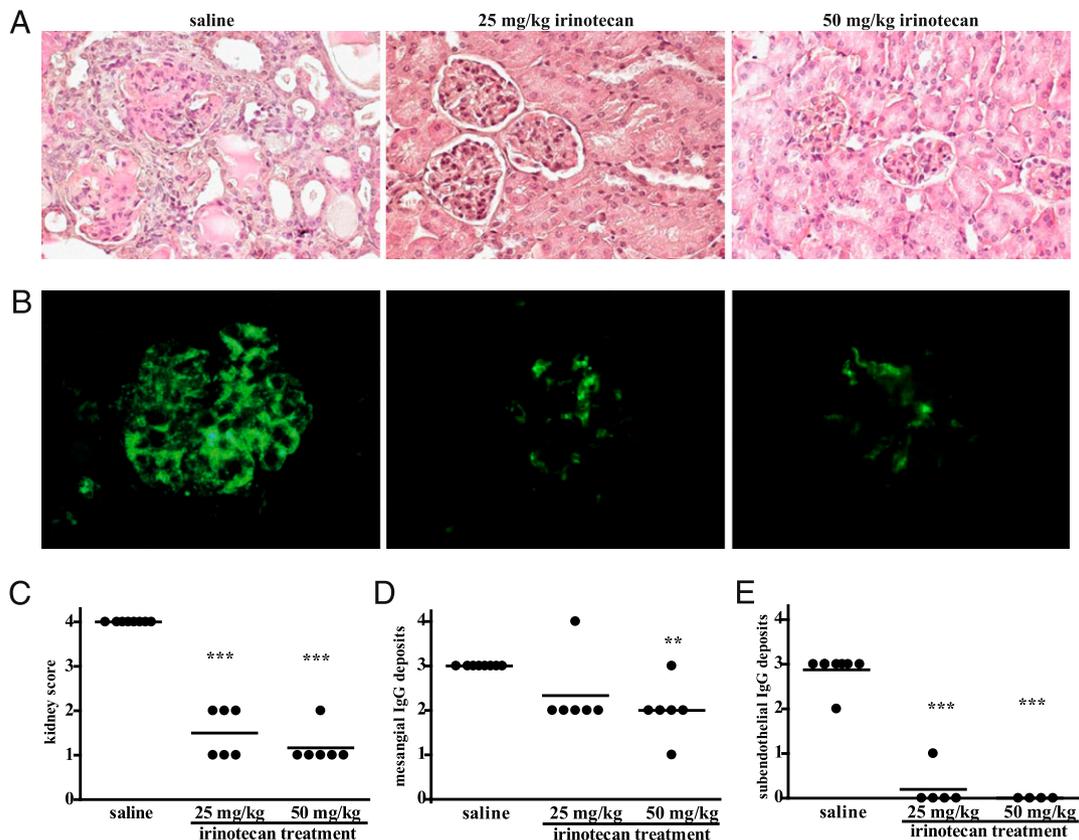


FIGURE 2. Irinotecan protects NZB/W F1 from the onset of lupus-associated glomerulonephritis. **A** and **C**, Representative kidney sections stained with H&E from a control mouse with end-stage lupus nephritis and from 50-wk-old mice that had been treated with either 25 mg/kg or 50 mg/kg irinotecan. The kidney score was assessed in a blinded manner according to the ISN/RPS2003 classification using paraffin sections stained with H&E, methenamine silver, and periodic acid-Schiff reagent as well as frozen sections stained for IgG. **B**, **D**, and **E**, Demonstration of IgG deposits in the kidneys of lupus-prone mice. The score for mesangial and subendothelial immune deposits was determined by the pathologist in a blinded manner. Original magnifications $\times 40$ (**A**) and $\times 63$ (**B**).

DNA of kidney tissue using the PicoGreen assay and found a reduction in mice treated with 50 mg/kg irinotecan, but not in mice treated with 25 mg/kg irinotecan (Fig. 3G). This finding implied that 50 mg/kg irinotecan produced enough ssDNA breaks to generate a significant amount of ssDNA, whereas 25 mg/kg irinotecan produced only ssDNA breaks while leaving intact the double-helix of DNA. Because irinotecan already showed its lupus-protective effects at a dose of 25 mg/kg, we concluded from these data that the generation of ssDNA breaks, but not a reduced amount of dsDNA, might be crucial for the prevention of anti-dsDNA Ab-mediated effects.

In this context it was shown recently that lupus-derived auto-antibodies can directly induce apoptosis (29). Adapted to our conditions, we therefore postulated that if the fatal action of anti-dsDNA Abs was impaired, the induction of apoptosis was prevented as a result. To prove this hypothesis, we measured the extent of apoptosis by TUNEL staining of kidney sections and by determination of caspase-3 activity in kidney lysates of NZB/W F1 mice. TUNEL, which detects the amount of apoptosis-induced double-stranded breaks (30, 31), demonstrated a strong signal in saline-treated mice that were sacrificed at end-stage lupus nephritis. In contrast, irinotecan-treated animals sacrificed at

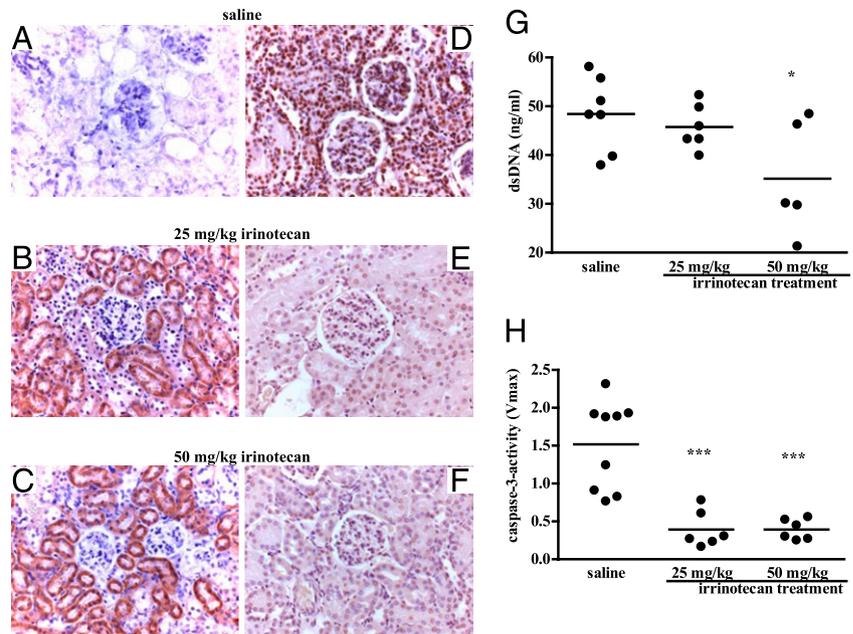
Table I. Immunology of NZB/W F1 mice treated from week 13 with irinotecan compared with saline-treated controls

	Saline	25 mg/kg Irinotecan	50 mg/kg Irinotecan
Spleen cell populations			
B220 ⁺ cells (%)	32.89 \pm 3.31	22.17 \pm 2.32*	20.95 \pm 3.89***
IgM ^{low} IgD ^{low} B cells (%)	9.79 \pm 3.72	14.25 \pm 4.65	11.13 \pm 3.59
IgG-producing B cells (ELISPOTs per 10 ⁶ cells)	5497 \pm 3813	1787 \pm 1703**	479 \pm 602**
CD4 ⁺ /CD8 ⁺ ratio	4.79 \pm 2.2	2.34 \pm 0.43**	2.09 \pm 0.25**
CD69 ⁺ CD4 cells (%)	6.01 \pm 1.56	4.03 \pm 1.13**	3.13 \pm 0.77***
CD11b ⁺ cells (%)	1.73 \pm 0.48	2.64 \pm 1.47	2.24 \pm 0.32
Neutrophils from peripheral blood (10 ³ /mm ³)	0.40 \pm 0.37	0.42 \pm 0.21	0.34 \pm 0.17
IgG anti-ds DNA (A ₄₀₅₋₄₉₀ nm)	2.01 \pm 0.67	2.31 \pm 0.50	1.29 \pm 0.63
Total IgG (mg/ml)	9.87 \pm 4.82	13.99 \pm 5.39	12.72 \pm 6.77

Saline-treated control mice were sacrificed when they had reached proteinuria grade 4+ and weight loss >25% calculated from the beginning of the disease ($n = 6$). The mean age of saline-treated animals was 39 wk. Irinotecan-treated animals were sacrificed at week 50/51 ($n = 6$, both groups).

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

FIGURE 3. Induction of ssDNA and impaired renal cell apoptosis in irinotecan-treated mice. *A–C*, ssDNA was determined on kidney sections using the mAb F7-26. *D–F*, Apoptosis-induced dsDNA breaks were assessed by TUNEL staining. *G*, Quantification of dsDNA at a ratio of total DNA. Genomic DNA was isolated from kidneys; total DNA was measured at 260 nm and adjusted to 100 ng/ml. dsDNA was determined using the Pico-Green assay. * $p < 0.05$; one-way ANOVA. *H*, Caspase-3 activity was measured in kidney lysates using DEVD-afc as a substrate (original magnification $\times 63$). *** $p < 0.001$; one-way ANOVA.



week 50/51 showed only a weak signal for dsDNA (Fig. 3*D*, 3*E*). Furthermore, we found a marked increase of caspase-3 activity in saline-treated mice compared with irinotecan-treated mice (Fig. 3*H*). Thus, both TUNEL staining and caspase-3 activity clearly demonstrated that irinotecan inhibited renal cell apoptosis.

Treatment of established lupus nephritis with irinotecan reverses proteinuria and prolongs survival

We further asked whether irinotecan can influence the course of established lupus erythematosus in NZB/W F1 mice. Therefore, we started treatment from proteinuria grade 3+ (≥ 300 mg/dl) and grade 4+ (≥ 2000 mg/dl) according to a median age of 35 wk. The control group received similar volumes of saline (10 ml/kg) starting from grade 3+ proteinuria. Criteria for sacrifice “due to the severity of the disease” were again a grade 4+ proteinuria and weight loss $>25\%$ calculated from the beginning of the disease. Treatment with 50 mg/kg irinotecan three times per week for 2 wk resulted in a decline of existing levels of proteinuria within 2 wk after initiation of the treatment. In contrast, none of the saline-treated animals displayed improvement in their lupus nephritis (Fig. 4*A*). After completion of the first treatment with irinotecan, 75% of animals with grade 3+ and 55% with grade 4+ proteinuria achieved remission, which was defined as 30 mg/dl (grade 1+) or less of proteinuria maintained for at least 3 wk. Median time to relapse was 9 wk for animals treated from grade 3+ and 12 wk for the group treated from grade 4+. Treatment of relapsing disease was performed as the initial treatment. Mice initiated from grade 3+ proteinuria received 5.8 ± 3.6 complete treatment cycles, mice treated from grade 4+ proteinuria underwent 4.4 ± 2.2 treatment cycles (mean \pm SD). Median survival was significantly prolonged with 73 wk (grade 3+ and grade 4+ combined) versus 40 wk for saline-treated animals (Fig. 4*B*). Remarkably, 5 of 12 animals treated from grade 3+ proteinuria were still alive at week 90 when the experiment was terminated.

Effects of short-term treatment with irinotecan

To further analyze the effects of treatment with irinotecan in NZB/W F1 mice with established disease, additional groups of animals were initiated: two groups treated with irinotecan from grade 3+ proteinuria were sacrificed 1 or 4 wk after completion of the treatment. The respective control group was sacrificed 1 wk after completion of

saline applications. Analysis of spleen cell populations revealed lower B cell activity in irinotecan-treated mice compared with saline-treated mice, whereas T cell activity as well as serum anti-dsDNA Abs and total IgG were not decreased (Table II). Determination of immune deposits in the kidneys showed no difference in the accumulation of mesangial IgG, but a significant diminution of sub-endothelial IgG deposits in the irinotecan-treated group sacrificed 1 wk after completion of the treatment (Fig. 5*A–C*). The

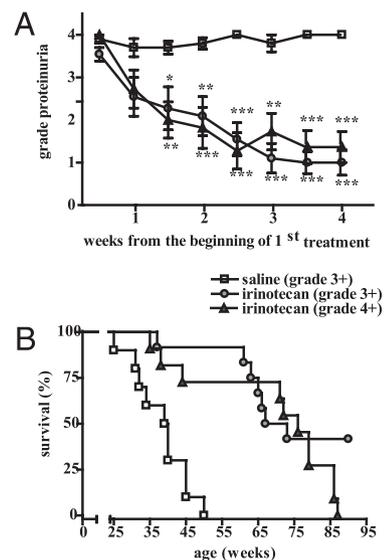


FIGURE 4. Recovery from established murine lupus erythematosus after treatment with irinotecan. NZB/W F1 mice with grade 3+ proteinuria (≥ 300 mg/dl) were treated three times per week for 2 wk with either 50 mg/kg irinotecan or saline. A third group was treated with irinotecan beginning from grade 4+ proteinuria (≥ 2000 mg/dl; $n = 10$, control mice; $n = 12$, irinotecan-treated mice [grade 3+]; $n = 11$, irinotecan-treated mice [grade 4+]). Relapsing disease was handled similar to the initial treatment. *A*, Irinotecan induced a decline of proteinuria within 2 wk after initiation of the treatment. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; two-way ANOVA. Data represent mean values \pm SEM. *B*, Kaplan-Meier survival curves showed significantly increased survival of both irinotecan-treated groups compared with saline-treated controls. $p < 0.0001$; log-rank test.

Table II. Immunology of NZB/W F1 mice with established lupus nephritis treated with irinotecan compared with saline-treated controls.

	Treatment from Proteinuria Grade 3+ (Time to Sacrifice after Completion of the Treatment)		
	1 wk after Saline	1 wk after Irinotecan	4 wk after Irinotecan
Spleen cell populations			
B220 ⁺ cells (%)	32.19 ± 7.34	24.04 ± 5.20	31.78 ± 6.81
IgM ^{low} IgD ^{low} B cells (%)	20.59 ± 5.94	13.67 ± 2.52*	23.68 ± 3.77
IgG-producing B cells (ELISPOTs per 10 ⁶ cells)	12011 ± 5952	1387 ± 1120**	11093 ± 1577
CD4 ⁺ /CD8 ⁺ ratio	5.91 ± 2.98	3.92 ± 1.34	6.16 ± 1.88
CD69 ⁺ CD4 cells (%)	5.79 ± 3.28	4.63 ± 1.52	9.66 ± 1.95*
Neutrophils from peripheral blood (10 ³ /mm ³)	0.33 ± 0.12	0.65 ± 0.72	0.33 ± 0.15
IgG anti-ds DNA (A _{405-490 nm})	2.17 ± 0.63	1.68 ± 0.90	2.37 ± 0.50
Total IgG (mg/ml)	11.72 ± 8.22	8.62 ± 5.62	25.78 ± 10.60*

Two groups of NZB/W F1 mice were treated from grade 3+ proteinuria with irinotecan (50 mg/kg, a total of six applications within 2 wk) or saline (10 ml/kg), and sacrificed 1 wk after completion of the treatment. A third group of irinotecan-treated mice was sacrificed 4 wk after the last application.

* $p < 0.05$; ** $p < 0.001$.

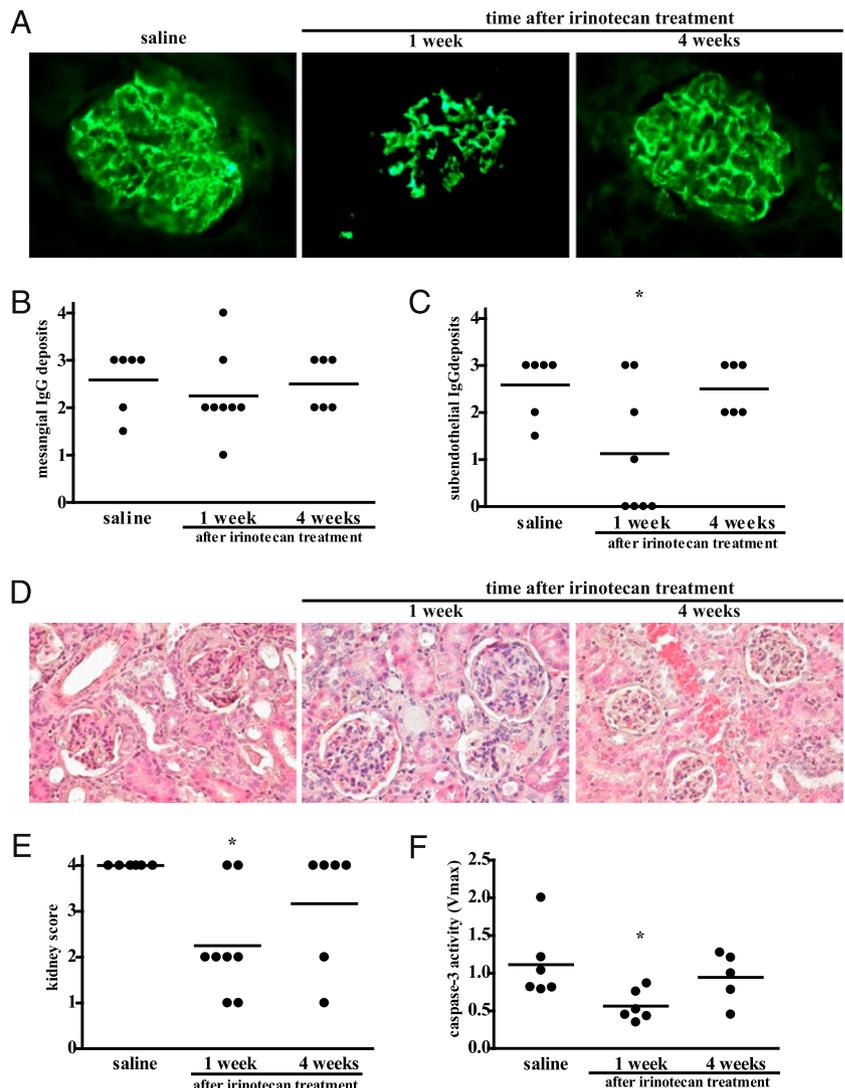
glomerulonephritis score blindly assessed from kidney sections was significantly improved in irinotecan-treated animals sacrificed after 1 wk, but it worsened 3 wk later (Fig. 5D–F), explaining the observed time to relapse of 9–12 weeks (Table III). Correlating with the kidney score and subendothelial IgG deposits, caspase-3 activity in kidney lysates was significantly diminished in irinotecan-treated animals sacrificed 1 wk after treatment and reverted to the level of

saline-treated mice 4 wk after treatment (Fig. 5F), suggesting that impaired renal cell apoptosis is critically involved in irinotecan-mediated reversal of lupus-associated glomerulonephritis.

Discussion

Our data obtained in a mouse model of spontaneous lupus erythematosus suggest that the topoisomerase I inhibitor irinotecan

FIGURE 5. Histologic changes and extent of apoptosis in NZB/W F1 mice treated with established disease. To judge the severity of the disease, kidney sections and lysates of mice (sacrificed 1 wk after completion of saline and 1 and 4 wk after completion of irinotecan treatment) were assessed. A–C, Demonstration of IgG deposits in kidneys of lupus-prone mice. The score for mesangial and subendothelial IgG was assessed in a blinded manner. D and E, The kidney score was determined in a blinded manner using the ISN/RPS2003 classification. F, Caspase-3 activity was measured in kidney lysates using DEVD-afc as a substrate. Original magnifications $\times 63$ (A) and $\times 40$ (D). * $p < 0.05$; one-way ANOVA.



provides a new treatment option for this disease. Table III summarizes our findings in relation to what has been published regarding the treatment of lupus erythematosus in NZB/W F1 mice. To our knowledge, only the use of CTLA4Ig in combination with cyclophosphamide (CTX) (15, 32) and administration of C-reactive protein (16) have demonstrated a reversal of defined high grade proteinuria with ≥ 300 mg/dl. However, a median survival of 73 wk in NZB/W F1-treated mice with established disease as seen in our experiments has not been described before. Of note, none of the agents currently used for the treatment of patients with lupus, including CTX, prednisolone, azathioprine, and mycophenolate mofetil, were shown to ameliorate existing levels of proteinuria in NZB/W F1. Because tolerated doses and side effects of irinotecan are well known (as described previously (33)), the most important side effects in patients receiving irinotecan are a reversible, not cumulative neutropenia of short duration in 23–44% and diarrhea in 34%), clinical trials with patients that are refractory to conventional treatment regimens might approve its use for the treatment of systemic lupus erythematosus.

The crucial question is how the topoisomerase I inhibitor irinotecan affected the course of lupus erythematosus. There are two conceivable possibilities and one could be a simple immunosuppression, because irinotecan is known from clinical anticancer trials to induce myelosuppression, namely neutropenia in some patients. Our data do not support this hypothesis. Although we found a lesser number and reduced activity of B cells, the level of total IgG and anti-dsDNA Abs as well as T cell activity were not decreased in irinotecan-treated mice. The discrepancy of decreased B cell activity and unchanged both total IgG and anti-dsDNA Ab titer can be explained by an only temporary B cell suppression insufficient to reduce Ab levels. Thus, during treatment of established lupus disease we observed an impaired B cell activity 1 wk after completion of the treatment with irinotecan, but normalized values 3 wk later (Table II). Instead of a simple immunosuppression, we favor an alternative mechanism that is closely related to the action of topoisomerase I at the level of genomic DNA. Topoisomerase I is an enzyme responsible for reducing torsional stress that develops during the replication of DNA. To realize this, topoisomerase I induce transient DNA single-strand breaks followed by stress-relieving rotation of the DNA. Next, topoisomerase I religates the single-strand breaks to reconstitute intact DNA. Inhibitors of topoisomerase I bind to the topoisomerase I-DNA complex, also named the *cleavable complex*, thereby preventing religation of DNA and resulting in the production of ssDNA (27). The accelerated production of DNA single-stranded breaks is not lethal but reduces the replication

capacity of the cell. However, if topoisomerase I inhibitors bind to cells in S-phase with enhanced DNA replication existing in cancer tissue, the complex of inhibitor, topoisomerase I, and DNA can collide with DNA replication forks, generating irreversible DNA–double-strand breaks followed by the induction of cell death (34). Applied to the conditions of the experiments presented in this study, we detected ssDNA breaks but not dsDNA breaks in the kidneys of irinotecan-treated mice, likely because the situation in lupus nephritis is different from rapidly dividing cells in cancer tissue. Providing anti-dsDNA Abs as an accepted pathogenic factor in NZB/W F1 and considering similar levels of these Abs in our experiments, we hypothesized that augmented ssDNA breaks caused less binding of anti-dsDNA Abs to genomic DNA, conceivably through changes in the sequence motif for binding or by changes in DNA conformation. Impaired binding of anti-dsDNA Abs in irinotecan-treated mice might be similar to what has been shown for mutated anti-dsDNA Abs. When formerly pathogenic anti-dsDNA Abs were mutated in a way to no longer bind to dsDNA, the mutants were not able to induce proteinuria in mice (35). However, it is known that anti-dsDNA Abs cross-react with other renal Abs such as collagen or α -actinin (36, 37). Whether binding of anti-dsDNA Abs to these Abs might have been changed in our experiments remains an open question.

As a consequence of the postulated lesser binding of anti-dsDNA Abs, we assumed the prevention of apoptosis in the kidneys of lupus-prone mice. According to this hypothesis, we found TUNEL staining as a marker for apoptosis-induced dsDNA breaks in saline-treated mice, but not in irinotecan-treated mice. Corresponding to TUNEL, caspase-3 activity was lower in irinotecan-treated mice, suggesting that impaired apoptosis was critically involved in irinotecan-mediated effects in NZB/W F1 mice. However, the role of apoptosis in systemic lupus erythematosus is not fully understood yet. Whereas apoptosis is widely accepted to be an important factor in the pathogenesis of lupus erythematosus (38–40), the influence of renal cell apoptosis on the course of the disease has been much less investigated. In this context, it has been shown that pan-inhibition of caspases ameliorated lupus-associated nephritis in mice (41). Other data described glomerular cell apoptosis in patients with lupus erythematosus, which was correlated with disease activity (42). Moreover, a study published during the preparation of this article demonstrated elevated active caspase-3 in the kidneys of NZB/W F1 mice older than 26 wk (43). In our experimental setup, inhibition of apoptotic cell death most likely retained the integrity of glomerular basement membranes, explaining the demonstrated prevention of sub-endothelial IgG deposits.

Table III. Comparison of different studies demonstrating survival and remission rates after treatment of established lupus nephritis in NZB/W F1 mice

	Median Survival in Weeks (Median Time to Death after Onset of Proteinuria in Weeks)			Remission of Proteinuria Grade 3+ (4+) (%)	Median Time to Relapse Grade 3+ (4+) in Weeks
	Grade 3+	Grade 4+	Grade 3+ and 4+		
CTX (11)	—	—	—	10 ^a	—
Azathioprine (12)	—	—	—	18 ^a	—
CTX + azathioprine + methylprednisolone (14)	—	—	44	0	—
C-reactive protein (16)	—	46	—	100	—
CTX + CTLA4Ig and CTX + CTLA4Ig + α -CD154 (15)	—	—	—	60–80 ^{b,c}	—
Irinotecan	70 (35)	76 (39)	73 (35)	75 (55) ^{b,c}	9 (12)

^aGrade of proteinuria before treatment not indicated, for CTX alone at least 5 animals with grade 2+.

^bDefined as 30 mg/dl of proteinuria or less maintained for at least 3 wk.

^cRate of remission owing to the first treatment cycle.

In conclusion, our data clearly show that the topoisomerase I inhibitor irinotecan reverses lupus nephritis and prolongs survival of NZB/W F1 mice. This effect was accompanied by the induction of ssDNA breaks, by inhibition of renal cell apoptosis, and by the prevention of subendothelial IgG deposits; it is also different from what is known about irinotecan-mediated effects in anticancer therapy. Thus, we provide evidence for irinotecan as a new option for the treatment of systemic lupus erythematosus.

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Disclosures

S.F. has filed a patent with the European patent office for the treatment of autoimmune diseases by topoisomerase I inhibitors.

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