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Carbon monoxide inhibits T cell activation in target organs during systemic lupus erythematosus

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Summary

Systemic lupus erythematosus is characterized by the presence of circulating anti-nuclear antibodies (ANA) and systemic damage that includes nephritis, haematological manifestations and pulmonary compromise, among others. Although major progress has been made in elucidating the molecular mechanisms responsible for autoimmunity, current therapies for lupus have not improved considerably. Because the exposure of carbon monoxide (CO) has been shown to display beneficial immunoregulatory properties in different immune-mediated diseases, we investigated whether CO therapy improves lupus-related kidney injury in lupus mice. MRL-Fas^{lpr} lupus mice were exposed to CO and disease progression was evaluated. ANA, leucocyte-infiltrating populations in spleen, kidney and lung and kidney lesions, were measured. CO therapy significantly decreased the frequency of activated B220^{+} CD4^{-} CD8^{-} T cells in kidneys and lungs, as well as serum levels of ANA. Furthermore, we observed that CO therapy reduced kidney injury by decreasing proliferative glomerular damage and immune complexes deposition, decreased proinflammatory cytokine production and finally delayed the impairment of kidney function. CO exposure ameliorates kidney and lung leucocyte infiltration and delays kidney disease in MRL-Fas^{lpr} lupus mice. Our data support the notion that CO could be explored as a potential new therapy for lupus nephritis.

Keywords: autoimmunity, carbon monoxide, systemic lupus erythematosus, T cells, therapy

Introduction

Systemic autoimmunity is characterized by an aggressive autorreactive immune response capable of injuring host tissue leading to multi-organ failure [1]. Major progress has been made in the understanding of the molecular mechanisms responsible for autoimmunity, including the identification of T helper type 17 (Th17) effector response as a key player in the pathogenesis of multiple sclerosis (MS) [2,3] and rheumatoid arthritis (RA) [4,5] and the recognition of type I interferons as critical mediators in the development of systemic lupus erythematosus (SLE) [6,7]. However, unlike the outstanding advances introduced in the treatment and prognosis of RA and MS with the development of biological agents, the field has not had a substantial breakthrough in the design of new therapies for SLE [8,9]. Furthermore, although survival of SLE patients has improved considerably in the past 50 years, they still have significantly higher mortality than healthy individuals. Thus, new therapies with enhanced efficacy and specificity that are subsequently associated with less adverse events than the current lupus treatments are needed urgently.

Haem oxygenase-1 (HO-1) is an inducible enzyme that catabolyzes haem degradation into Fe^{2+}, biliverdin and

carbon monoxide (CO) [10]. HO-1 can modulate the function of some haem proteins, including nitric oxide synthase, soluble guanylatecyclase, peroxidase and catalase through the degradation of the haem group and the release of CO, which binds to the haem group and inhibits haem protein activity [11]. HO-1 deficiency is associated with an increased susceptibility to uncontrolled inflammation with subsequent leucocytosis, splenomegaly and lymph node swelling in affected individuals [12,13]. Furthermore, we have described recently that both SLE patients, as well as lupus-prone mice, show a decreased expression of HO-1 in peripheral blood monocytes [14,15]. During immune-mediated diseases, such as MS, type 1 diabetes, collagen induced arthritis (CIA) and organ transplant rejection, several immunoregulatory functions have been attributed to CO, which is an HO-1 product [16–19]. Along these lines, we have recently reported that CO exposure prevents both monocyte population expansion and the decline of regulatory T cells (T_{regs}) in hybrid FcγRIIb knock-out (KO) mice, which develop a mild lupus-like syndrome [15].

It has been shown that HO-1 expression in T cells is induced by anti-CD3/CD28 antibodies, suggesting a possible autocrine role for CO/HO-1 in the regulation of T cell activation [20]. In addition, while forkhead box protein 3 (FoxP3)-transfected Jurkat cells increase HO-1 expression, the inhibition of HO-1 activity impairs the suppressive capacity of $CD4^+CD25^+$ T_{reg} cells. These data suggest that HO-1 can play a crucial role in the maintenance of peripheral tolerance and the modulation of immune responses [21]. Interestingly, CO exposure may limit T cell-mediated pathogenesis *in vivo* by the modulation of several immune cells, including interferon (IFN)-γ/interleukin (IL)-17-producing $CD4^+$ T cells, $CD8^+$ T cell recruitment and antigen presentation by dendritic cells (DCs) [17,22]. These observations support the notion that CO therapy can be considered as a promising treatment to decrease inflammation in systemic autoimmune diseases with dominant T cell responses, such as those seen during MS and type 1 diabetes [16,17].

Strikingly, although several groups have studied the anti-inflammatory efficacy of CO therapy in immune and autoimmune-mediated diseases, the effect of CO over T cell-mediated pathogenesis during aggressive systemic autoimmunity involving multi-organ failure has not been reported. A distinctive feature of MRL-Fas^{lpr} lupus mice is the massive lymphadenopathy and splenomegaly due mainly to unbalanced T cell expansion [23]. Furthermore, chronic kidney damage in MRL-Fas^{lpr} mice is fatal [24]. It is widely acknowledged that lupus nephritis is dependent mainly upon immune complex deposition. However, infiltrating T cells and monocytes regulated by proinflammatory cytokines also play a crucial role in disease severity [25,26]. It has been proposed that the production of IL-12 by kidney tubular cells of MRL-Fas^{lpr} mice further promotes the recruitment of multiple T cell populations such as $CD4^+$, $CD8^+$ and $B220^+CD4^-CD8^-$ T cells to this organ [27]. Interestingly, this latter population, known as double-negative (DN) T cells, is also present in human autoimmune syndromes [28,29]. This T cell subpopulation infiltrates kidneys, produces IFN-γ and contributes to cause renal damage in MRL-Fas^{lpr} lupus mice [26]. Thus, suppression of T cell infiltration into the kidneys could work as an effective therapy in lupus nephritis.

Accordingly, the aim of this study was to evaluate the therapeutic effect of CO exposure during SLE in a mouse model using MRL-Fas^{lpr} lupus mice. Importantly, we found that CO therapy decreased activated $B220^+CD4^-$ $CD8^-$ T cells in kidneys and lungs. In addition, CO decreased the levels of circulating anti-DNA and anti-Histone antibodies. Furthermore, we found that nephritogenic cytokines such as IFN-γ and IL-18 were decreased and histological alterations seen in lupus nephritis were also ameliorated in CO-treated mice. Moreover, kidney failure was delayed in lupus mice treated with CO. These data support the notion that CO exposure ameliorates kidney disease in MRL-Fas^{lpr} lupus mice and suggest that this molecule could work as a potential therapy to prevent kidney injury in autoimmune systemic diseases, such as lupus.

Material and methods

Antibodies and reagents

Anti-mouse CD11c-phycoerythrin (PE)/fluorescein isothiocyanate (FITC)/allophycocyanin (APC) (clone HL3), anti-mouse CD11b-PE/FITC (clone M1/70), anti-mouse lymphocyte antigen 6 complex, locus G (Ly6G) (clone RB6-8C5), anti-mouse Ly6C (clone HK1.4), anti-mouse CD4-FITC/PE/PE-cyanin 7 (Cy7) (clone GK1.5), anti-mouse CD4-peridinin chlorophyll (PerCP) (clone H129.19), anti-mouse FoxP3 Alexa Fluor 647 (clone MF3), anti-mouse CD8-APC (clone 53-6.7) and anti-mouse B220-FITC (clone RA3-6B2), anti-mouse CD19-PE (clone 1D3), anti-mouse CD69-PE/APC (clone H1.2F3) and CD45-APC (clone 30-F11) were all purchased from BD Biosciences (San Jose, CA, USA).

Mice and CO exposure

MRL/MpJ-Fas^{lpr}/J (MRL-Fas^{lpr}) mice and MRL/MpJ were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Female mice used in this study were age-matched in all the experiments. MRL/MpJ mice were used as controls, as reported previously [30]. All mice were kept under specific pathogen-free conditions at the animal facility of the Pontificia Universidad Católica de Chile. All animal work was performed according to institutional guidelines and supervised by a veterinarian. Mice were exposed to compressed CO at a concentration of 250 parts per million

(ppm) for 2 h per day from weeks 9 to 15–30 [15] (at week 15, n_{mice}/group = 5, three independent experiments; at week 30, n_{mice}/group = 5, two independent experiments). A CO analyser was used to measure and keep controlled gas concentration in chambers. We have reported previously that after CO exposure carboxyhaemoglobin levels reached 23–27% in all mice.

Urinary protein

Proteinuria was estimated by examining fresh urine with Combur Test sticks for urinalysis (Roche, Basel, Switzerland) using a scale of 0–3, where 0/trace = negative, 1 = 30 mg/dl, 2 = 100 mg/dl and 3 = 500 mg/dl. Proteinuria scores above 2 were considered to represent moderate glomerulonephritis (n_{mice}/group = 5, three independent experiments).

Histopathology

For histological studies, kidneys from mice at week 30 (n_{mice}/group = 4, two independent experiments) were harvested and fixed in 10% buffered formalin. Formalin-fixed tissue was embedded in paraffin blocks, and 3-μm sections were stained with haematoxylin and eosin and periodic acid-Schiff (PAS) reagents (Sigma-Aldrich, St Louis, MO, CA, USA). The obtained slides were evaluated by conventional light microscopy by a renal pathologist. The extension of glomerular, tubulointerstitial and vascular injury was evaluated using the current classification for patients with lupus nephritis [9]. Active lesions included glomerular hipercellularity (endocapillary and extracapillary), leucocyte infiltration, necrosis/karyorrhexis, wire-loops and/or hyaline thrombi (subendothelial and/or intraluminal immune complexes, respectively), crescents formation, interstitial inflammation, membranous-like glomerular lesions (subepithelial immune complexes), vasculitis and vascular immune complex deposition. The score for grading active lesions is a modification of the activity index used currently in renal diagnostic pathology [9,31]. The score was obtained considering the percentage of affected glomeruli in each kind of lesion, as follows: 1, 0–25%; 2, 25–50%; and 3, more than 50%. For statistical analyses, Student's *t*-test was used

Flow cytometry

Spleen, kidney and lung from MRL-Fas^{lpr} and MRL/MpJ mice from different groups at weeks 15 or 30 were harvested, weighed and minced in phosphate-buffered saline (PBS) supplemented with 10% fetal calf serum (FCS), until a homogeneous cell suspension was reached (spleen, n_{mice}/group = 5, two independent experiments; kidney and lung, n_{mice}/group = 5, three independent experiments). No changes in kidney, lung, spleen or inguinal lymphatic node weight were observed between untreated and CO-treated groups (Supporting information, Fig. S3a–d). Total cell count was measured by counting cells with Türk solution in Neubauer chamber. Absolute counts are presented as cells $\times 10^6$ per spleen. Tissue homogenates were filtered through a 70-mm nylon mesh filter (BD Biosciences). Cells were washed with PBS–1% bovine serum albumin (BSA), resuspended at 2×10^6 cells/ml (50 μl/tube) and incubated with FITC-, PE-, PerCP-, PE-Cy7- and APC-conjugated antibodies for 30 min at 4°C. For FoxP3 intracellular staining, fixed cells were incubated with an Alexa 647-conjugated anti-FoxP3 antibody in permeabilization buffer (PBS/BSA 3%–saponin 0.5%) for 4 h. Cells were washed with permeabilization buffer and acquired using a fluorescence activated cell sorter (FACS)Canto II flow cytometer (BD Biosciences). To discriminate between CD45$^+$ single cells and doublets or cell debris, events were gated sequentially on side-scatter (SSC)-A and forward-scatter (FSC)-A, FSC-W and FSC-H, SSC-W and SSC-H and SSC-A and CD45-APC plots. Representative flow cytometry data to illustrate the gating strategy are shown in Supporting information, Fig. S1.

Enzyme-linked immunosorbent assay (ELISA)

Serum samples from different groups of mice were obtained by cardiac puncture at the time of euthanasia (n_{mice}/group = 5, three independent experiments). Autoantibodies against DNA (Invitrogen, Boston, MA, USA) and histones (Calbiochem, La Jolla, CA, USA) were quantified by ELISA. Briefly, ELISA plates were coated overnight at 4°C with 2 μg/ml DNA or 2 μg/m histones in PBS buffer, washed and then blocked with PBS/BSA 10% for 2 h at room temperature. After washing three times, serum samples were diluted in PBS/BSA1% starting at 1 : 100 to 1 : 10000 and incubated for 2 h at room temperature. Immunoglobulin (Ig)G1 and IgG2a (Biolegend, San Diego, CA, USA) were detected with specific goat anti-mouse IgG1 or IgG2a antibodies conjugated with biotin. Next, plates were incubated with streptavidin–horseradish peroxidase (HRP) (Invitrogen). After washing three times, HRP substrate was added (3,30,5,50-tetramethylbenzidine; Sigma, St Louis, MO, USA) and optical density (OD) at 450 nm was measured on a microplate reader.

Real-time PCR

RNA was extracted from kidneys using Trizol reagent (Invitrogen) according to the manufacturer's instructions from mice at week 30 (n_{mice}/group = 4, two independent experiments). Reverse transcription–polymerase chain reaction RT–PCR) and cDNA synthesis were performed using oligo dTprimers (ImProm-II; Promega, Madison, WI, USA). Real-time PCR reactions were carried out using a StepOne plus thermal cycler (Applied Biosystems, Foster City, CA, USA). To corroborate amplification specificity, PCR products were subjected to a melting curve programme. Abundance of proinflammatory genes including IFN-γ, IL-1β,

IL-6, IL-12, IL-18 and FoxP3 mRNA was determined by relative expression to β-actin and glyceraldehyde 3-phosphate dehydrogenase (GADPH) by the $2^{-\Delta\Delta Ct}$ method. IL-1β: sense 5′-GCA ACT GTT CCT GAA CTC AAC T-3′, anti-sense 5′-ATC TTT TGG GGT CCG TCA ACT-3′; IL-6: sense 5′- TAG TCC TTC CTA CCC CAA TTT CC-3′, anti-sense 5′-TTG GTC CTT AGC CAC TCC TTC-3′; IL-12: sense 5′-ACA GGT GAG GTT CAC TGT TCC T-3′, anti-sense 5′-TGG TTT GCC ATG GTT TTG CTG-3′; GAPDH: sense 5′-AGG TCG GTG TGA ACG GAT TTG-3′, anti-sense 5′-TGT AGA CCA TGT AGT TGA GGT CA-3′; β-actin sense 5′-ACC TTC TAC AAT GAG CTG GG-3′, anti-sense 5′-CTG GAT GGC TAC GTA CAT GG-3′; IFN-γ: sense 5′-ATG AAC GCT ACA CAC TGC ATC-3′, anti-sense 5′-TCT AGG CTT TCA ATG ACT GTG C-3′; IL-18: sense 5′-CAG GCC TGA CAT CTT CTG CAA-3′, anti-sense 5′-TCT GAC ATG GCA GCC ATT GT-3′; and FoxP3: sense 5′-CAG CTG CCT ACA GTG CCC CTA G-3′, anti-sense 5′-CAT TTG CCA GCA GTG GGT AG-3′.

Statistics

Data and statistical analyses were performed using Prism 5 software (Graph Pad Software, Inc., San Diego, CA, USA). For statistical analyses, analysis of variance (ANOVA) (Bonferroni's post-test) and Student's *t*-test were used. *P*-values below 0.05 were considered statistically significant. Flow cytometry data were analysed using FACS Diva (BD Biosciences) and FlowJo software (TreeStar Inc., Ashland, OR, USA).

Results

CO decreases activated T cell accumulation in kidneys and lungs of lupus mice

One of the most important features of MRL-Fas^{lpr} lupus mice is the systemic expansion of different subpopulations of T cells [23]. In this lupus animal model the most affected tissues are kidneys, lungs and skin, which show proliferative glomerulonephritis, interstitial pneumonitis and dermatitis, respectively [1,32]. Kidneys and lungs from MRL-Fas^{lpr} lupus mice display an intense inflammatory infiltrate [32,33]. Infiltrating T cells play an important role in organ damage by secreting cytokines such as IFN-γ, which may be deleterious for stromal cell function, including glomerulus-associated cells [34]. To evaluate whether CO treatment could ameliorate leucocyte infiltration in kidneys and lungs, MRL-Fas^{lpr} lupus mice were exposed to 250 ppm of CO gas from weeks 9 to 15 of age. We found that CO treatment mildly decreased $CD45^+$ cell infiltration in kidneys (without significant effect in the lungs) of MRL-Fas^{lpr} lupus mice when compared to untreated lupus mice (Fig. 1a,d; Supporting information, Fig. S2a,b). We next analysed leucocyte subpopulations by flow cytometry, and found that CO treatment decreased the frequency of activated T cells infiltrating the kidneys (Fig. 1b,c,g) and lungs (Fig. 1e,f,j). Interestingly, the effect of CO treatment seemed to be specific for T cells, because other leucocyte subpopulations, such as B cells ($CD19^+$ cells), monocytes ($CD11^+Ly6G^-$ cells), neutrophils ($CD11^+Ly6G^+$ cells) and DCs ($CD11c^+$ cells), were not altered in the kidneys (Fig. 1h,i,m–o,s,t) and lungs (Fig. 1k,l,p–r,v,w) of SLE mice (Supporting information, Fig. S2c,e). Similarly, when FoxP3 mRNA levels were evaluated in kidneys by real time PCR as a parameter of T_{reg} frequency, we found that CO treatment did not modulate this cell type (Fig. 1u). It is noteworthy that CO treatment mildly decreased the frequency of monocytes in the lungs of lupus mice compared to untreated control mice (Fig. 1q).

When these T cells were studied, we found that CO treatment decreased both the amount of $B220^+CD4^-CD3^+$ T cells number as well as activated $CD69^+B220^+CD4^-CD3^+$ T cells in kidneys from MRL-Fas^{lpr} lupus mice compared to untreated mice (Fig. 2a,b,e,f). In addition, exposure to CO also reduced the frequency of activated $B220^+CD4^-CD3^+$ T cells in the lungs of these animals (Fig. 2c,d,g,h; Supporting information, Fig. S2d,f).

Conversely, CO treatment did not reduce hyperplasia of lymphatic tissues in MRL-Fas^{lpr} (Supporting information, Fig. S3c,d). CO exposure did not prevent leucocyte spleen expansion including $CD3^+$ T cells, $CD4^+CD25^+$ T_{regs}, $CD19^+$ B cells, $CD11b^+Ly6G^-$ monocytes, $CD11c^+$ dendritic cells and $CD11b^+Ly6G^+$ neutrophils compared to untreated control mice (Fig. 3a,b,i–r and Supporting information, Fig. S4a). Similarly, frequency and absolute number of $B220^+CD3^+CD4^-$ T cells subpopulations in spleen of MRL-Fas^{lpr} lupus-prone mice were not affected by CO therapy (Fig. 3e–h). Although it has been reported that CO could modulate T cell function [35], CO did not affect the frequency of activated T cells in spleen (Fig. 3c,d and Supporting information, Fig. S4b).

CO exposure decreases autoantibody production in lupus mice

One of the most important features of SLE pathogenesis is the production of ANAs, including anti-dsDNA and anti-histone IgGs, which conform circulating immunocomplexes that usually deposit in glomeruli leading to proliferative glomerulonephritis and subsequently to kidney failure [32]. MRL-Fas^{lpr} lupus mice were exposed to CO from weeks 9 to 30 of age. Strikingly, we found that CO treatment decreased both IgG2a and IgG1 isotypes of anti anti-histone autoantibodies compared to untreated control mice (Fig. 4a,b). In addition, CO therapy also decreased IgG2a anti-dsDNA titres, compared to untreated mice (Fig. 4c,d). Both wild-type mice groups did not develop ANA (Fig. 4a–d). These findings suggest that CO treatment could eventually reduce organ failure by decreasing autoantibody-mediated tissue injury.

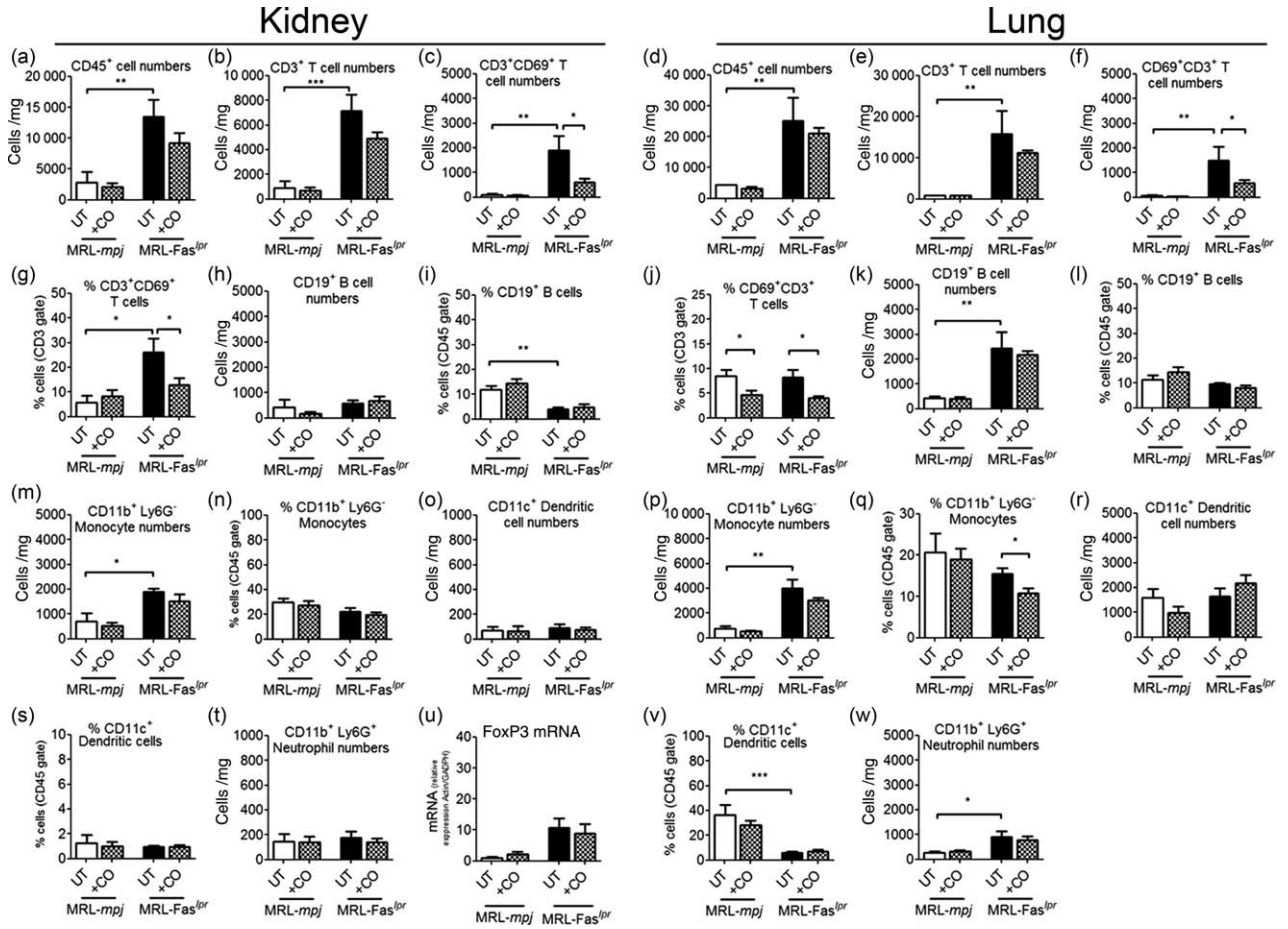

Fig. 1. Carbon monoxide (CO) treatment decreases kidney infiltrating activated T cells from MRL-Fas^{lpr} mice. MRL-Fas^{lpr} and MRL-MpJ mice were treated with CO 250 parts per million (ppm) 5 days/week from weeks 9 to 15. Graph represents percentage and absolute counts of infiltrating CD45$^+$ leucocytes in kidneys (a) and lungs (d). Percentage, absolute counts of different leucocyte populations (CD45 gate) in kidney: CD3$^+$ (b), CD3$^+$ CD69$^+$ (c,g) (CD3 gate), CD19$^+$ B cells (h,i), CD11b$^+$ Ly6G$^-$ monocytes (m,n), CD11c$^+$ dendritic cells (o,s) and neutrophils (t). Regulatory T cells [forkhead box protein 3 (FoxP3) mRNA levels by real-time polymerase chain reaction (PCR)] in kidney (u). In lung: CD3$^+$ (e), CD3$^+$ CD69$^+$ (f,j) (CD3 gate), CD19$^+$ B cells (k,l), CD11b$^+$ Ly6G$^-$ monocytes (p,q), CD11c$^+$ dendritic cells (r,v) and neutrophils (w). Absolute counts are presented as cells $\times 10^6$ per mg of tissue of three independent experiments; n_{mice}/group = 5, three independent experiments. $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$ by one-way analysis of variance (ANOVA) test. Data are shown as mean ± standard error of the mean.

CO exposure ameliorates histological alterations in lupus nephritis

Because CO therapy decreased anti-dsDNA and anti-histone autoantibodies, we next evaluated the therapeutic effect of CO in the histological changes observed during lupus nephritis. Renal disease in human lupus, as well as in animal models such as MRL-Fas^{lpr} lupus mice, is characterized by proliferative glomerulonephritis caused by infiltrating inflammatory cells that disrupt glomerular structure and function (Fig. 5 and Table 1) [9]. As described above, wild-type mice and MRL-Fas^{lpr} lupus mice were exposed to CO from weeks 9 to 30 of age. Kidney damage was assessed by histological analysis of the active lesions in the tissue as described in Material and methods. Although not statistically significant (Student's *t*-test, MRL-Fas^{lpr} untreated *versus* +CO), MRL-Fas^{lpr} lupus-prone mice exposed to CO showed a decrease of several pathological features of the glomeruli such as endocapillary proliferation, extension of wire-loop and hyaline thrombi, crescent formations, vascular immune complex deposition and membranous changes along the peripheral capillary walls (Fig. 5a–d and Table 1). In contrast, vasculitis and interstitial inflammation was not modulated by CO treatment (Fig. 5e and Table 1). In addition, glomerular indemnity was evaluated periodically by determining the presence of proteinuria. CO exposed MRL-Fas^{lpr} lupus-prone mice showed a sligth delay in proteinuria onset compared with untreated control mice (Fig. 5f). These observations may explain the reason why CO-treated mice also developed kidney injury. In contrast, CO

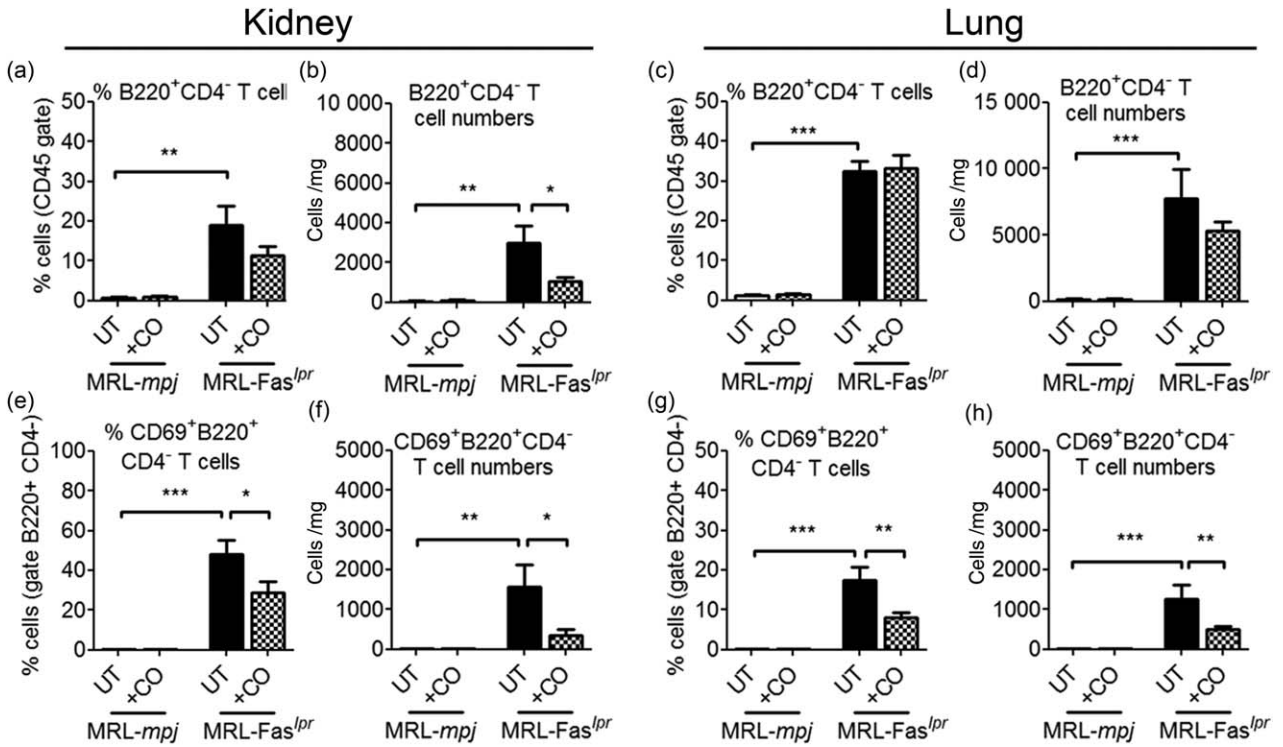

Fig. 2. Reduced $CD3^+$ $B220^+$ $CD4^-$ $CD69^+$ T cell subpopulation in kidneys and lungs from carbon monoxide (CO)-treated lupus mice. Murphy Roths large (MRL)-Fas^{lpr} and MRL-MpJ mice were treated with CO 250 parts per million (ppm) 5 days/week from weeks 9 to 15. Graphs represent the percentage and absolute counts of: $CD3^+$ $B220^+$ $CD4^-$ T cells from kidney (a,b) and lung (c,d); $CD3^+B220^+CD4^-CD69^+$ T cells from kidney (e,f) and lung (g,h). Absolute counts are presented as cells $\times 10^6$ per mg of tissue of three independent experiments; n_{mice}/group = 5, three independent experiments. $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$ by one-way analysis of variance (ANOVA) test. Data shown are the mean ± standard error of the mean.

treatment could not improve survival of MRL-Fas^{lpr} lupus-prone mice (data not shown). To evaluate further the contribution of CO therapy in lupus glomerulonephritis we measured mRNA levels of several proinflammatory genes in kidney tissue. We found that IFN-γ, IL-1β, IL-6, IL-12 and IL-18 mRNA levels in kidneys were increased in MRL-Fas^{lpr} lupus mice compared to MRL-Mpj control mice. Interestingly, although not statistically significant, we found that CO-treated MRL-Fas^{lpr} lupus mice showed a marked decrease in mRNA levels of several proinflammatory cytokines, such as IFN-γ, IL-6, IL-12 and IL-18 that was not seen in untreated mice (Fig. 5g–j). Strikingly, CO treatment reduced IFN-γ mRNA levels in 1-log. In contrast, no major changes were observed in IL-1β mRNA levels from CO-treated lupus mice (Fig. 5k). Therapeutic effects of CO treatment may be related to a decrease in T cell activation that, in turn, may produce lower levels of proinflammatory cytokines such as IFN-γ.

Discussion

In this study, we have provided evidence showing that CO exposure decreased autoantibody levels and the number of activated T cells infiltrating target tissues, such as kidneys and lungs in lupus mice. However no significant effects of CO were observed in other leucocyte subpopulations. Furthermore, treatment with CO may prevent glomerulus deterioration and contribute to maintaining organ structure. To our knowledge, this study is the first to report that CO exposure reduces T cell activation in tissues that are targeted during lupus-related inflammation.

Although it has been reported that CO may exert anti-inflammatory effects in different autoimmune diseases, there are no reports evaluating the role of this treatment in T cells during aggressive autoimmune disorders, such as that observed in MRL-Fas^{lpr} lupus mice [15]. Furthermore, this is the first report showing that CO can modulate $B220^+CD3^+CD4^-$ T cells infiltrating kidneys and lungs in autoimmunity. The importance of these findings is underscored by the observation that this T cell subset is also found in human autoimmune disorders (DN T cells) due mainly to a dysfunction of the Fas apoptotic pathway [28,36]. It has been reported previously that CO can modulate T cell proliferation directly by means of blocking extracellular-regulated kinase (ERK) signalling and decreasing IL-2 production triggered by CD3/CD28 stimulation

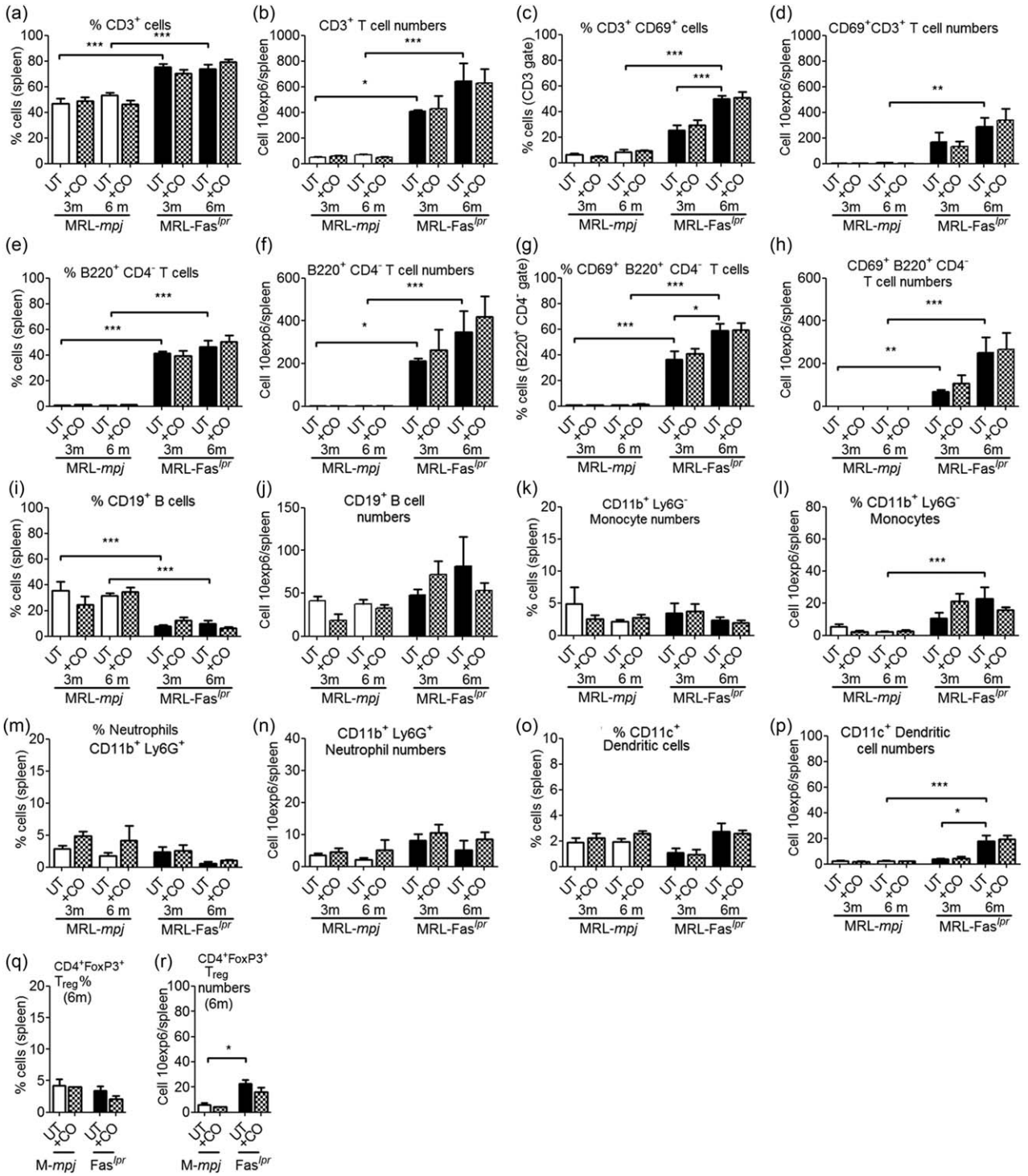

Fig. 3. Carbon monoxide (CO) treatment does not reduce T cell populations in spleens from Murphy Roths large (MRL)-Fas^{lpr} lupus mice. MRL-Fas^{lpr} and MRL-MpJ mice were treated with CO 250 parts per million (ppm) 5 days/week from weeks 9 to 15 or 30. Graphs represent the percentage and absolute counts of different leucocyte populations: $CD3^+$ (a,b), $CD3^+CD69^+$ (c,d), $CD3^+B220^+CD4^-$ (e,f), $CD3^+B220^+CD4^-CD69^+$ (g,h), $CD19^+$ B cells (i,j), $CD11b^+Ly6G^-$ monocytes (k,l), $CD11b^+Ly6G^+$ neutrophils (m,n) and $CD11c^+$ dendritic cells (o,p); regulatory T cells (T_{regs}) $CD3^+$ $CD4^+$forkhead box protein 3 $(FoxP3)^+$ (q,r). Absolute counts are presented as cells $\times 10^6$ per spleen of two independent experiments; n_{mice}/group = 5, two independent experiments. $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$ by one-way analysis of variance (ANOVA) test. The data shown are the mean ± standard error of the mean.

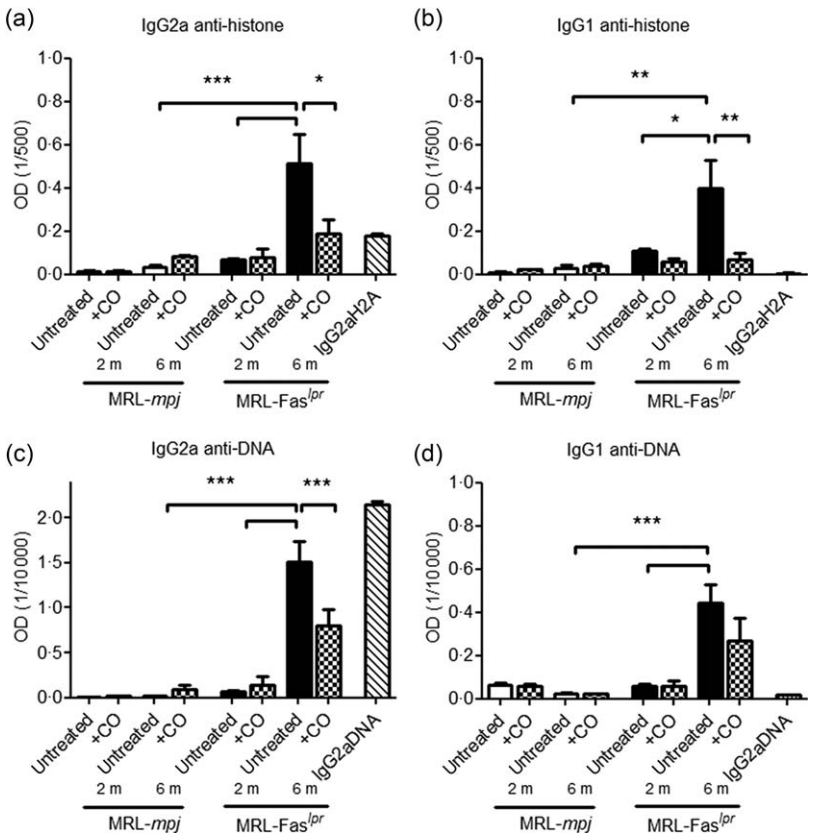

Fig. 4. Decreased autoantibody production in carbon monoxide (CO)-treated Murphy Roths large (MRL)-Fas^{lpr} lupus mice. MRL-Fas^{lpr}and MRL-MpJ mice were treated with CO 250 parts per million (ppm) 5 days/week from weeks 9 to 30. Serums were collected and specific anti-histone and anti-DNA immunoglobulin (Ig)G1 and IgG2a isotype levels were evaluated by enztme-linked immunosorbent assay (ELISA). (a,b) Serum IgG2a and IgG1 anti-histone levels at 30 weeks of age diluted 1/500. (c,d) Serum IgG2a and IgG1 levels of anti-DNA at 30 weeks of age diluted 1/10000; n_{mice}/group = 5 (three independent experiments). $^{***}P < 0{\cdot}001$, $^{**}P < 0{\cdot}01$ and $^{*}P < 0{\cdot}05$ by one-way analysis of variance (ANOVA) test. Data are shown as mean ± standard error of the mean.

[35]. We suggest that CO exposure could directly impair cellular signalling in T cells, thus limiting proinflammatory cytokines production in target tissue. It is known that IFN-γ, probably produced by CD4$^+$, CD8$^+$ and DN T cells, is crucial for the onset of lupus nephritis [26]. Therefore, we propose that CO confines B220$^+$CD3$^+$CD4$^-$ T cell activation, causing the decrease of IFN-γ mRNA levels which, in turn, would shape the proinflammatory local environment, similarly to what has been observed in animal models of type 1 diabetes [17]. Despite the encouraging finding that CO produced a marked decrease in IFN-γ mRNA levels in kidney, this reduction did not achieve statistical significance ($P = 0.0577$). In addition, CO treatment would also modulate the activation and maturation of DCs and macrophages leading to reduced levels of proinflammatory cytokines such as IL-6, IL-12 and IL-18, as observed in CO-treated mice [37]. However, the precise contribution of activated B220$^+$CD3$^+$CD4$^-$ T cells to kidney and lung injury remains to be elucidated, as well as whether CO could modulate activated T cell recruitment directly in glomerulus. It has been reported that in an anoxia–reoxygenation model using rat pulmonary endothelial cells, CO produced by HO-1 enzymatic activity limits the activity of caspase-3 and inhibits cell death [38]. Our data cannot rule out an anti-inflammatory effect of CO over stromal cells such as podocytes, endothelial or tubular kidney cells which, in turn, could modulate either T cell activation or recruitment through chemokine production [27].

In contrast, unlike what we reported in a mild model for lupus (FcγRIIb KO mice in an hybrid background), no significant changes in splenic leucocyte populations were observed as a result of CO effect in this study [15]. This apparent discrepancy might be due to genetic background differences between the lupus mice models used in each study conferring distinctive characteristics to each model [39]. Although CO could modulate CD11b$^+$ cell expansion in spleen from lupus FcγRIIb KO mice, CO treatment in MRL-Fas^{lpr} lupus mice does not modulate this cell type population. As it has been reported that Fas deficiency plays an active role in different processes, such as keeping circulating monocyte numbers, regulating macrophage activation and migration, it is likely that the lack of Fas provides a more severe impairment in cell regulation than the deficiency of FcγRIIb that cannot be modulated by CO treatment [40]. The fact that CO treatment could not improve MRL-Fas^{lpr} lupus mice survival could be associated with the damage in other tissues, such as in heart and blood vessels, driven by the massive lymphoproliferation [32].

It has been shown that HO-1 induction by haem, which increases endogenous production of CO, modulates B cells by reducing the production of antigen-specific IgM *in vivo* [41]. This notion is consistent with the decrease in anti-dsDNA and anti-histone levels observed during CO

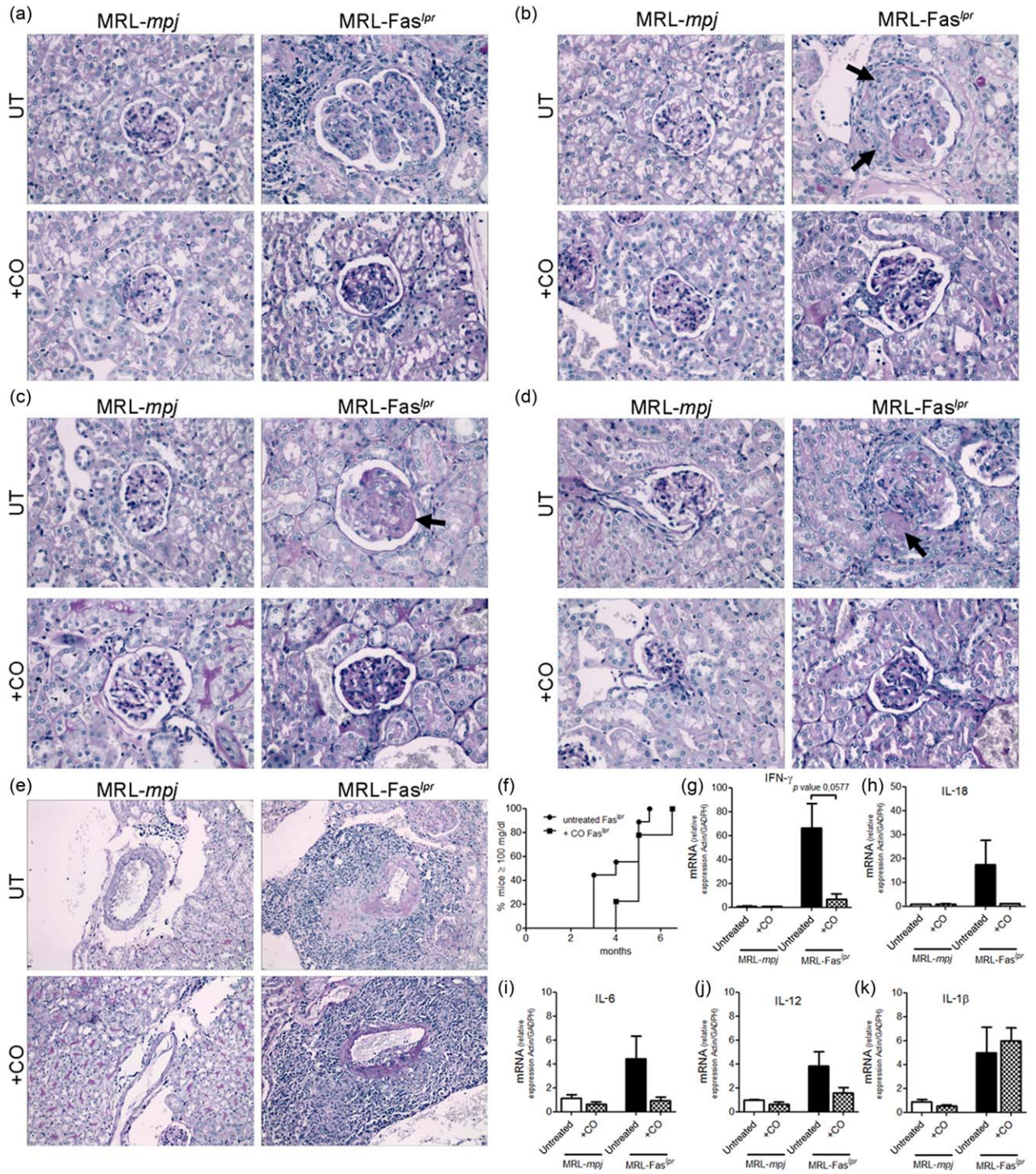

Fig. 5. Carbon monoxide (CO) treatment improves histological alterations in lupus nephritis. Murphy Roths large (MRL)-Fas^{lpr} and MRL-MpJ mice were treated with CO 250 parts per million (ppm) 5 days/week from weeks 9 to 30. The score was obtained from the percentage of affected glomeruli by each kind of lesion, as follows: 1 = 0–25%; 2 = 25–50%; and 3 = more than 50%. Representative photomicrographs (×400) of periodic acid-Schiff (PAS)-stained kidney sections from untreated and CO-treated MRL-Fas^{lpr} and MRL-MpJ mice show: (a) glomerular hipercellularity; (b) crescents formation; (c) wire-loops lesions; (d) vascular immune complexes deposition; and (e) vasculitis lesions (small size arteries). Arrows indicate active lesions. (f) Presence of proteinuria in MRL-Fas^{lpr} mice treated with ●CO, or ■ untreated over time. RNA from kidneys was extracted. Then real-time polymerase chain reaction (PCR) and cDNA synthesis were performed to evaluate mRNA abundance of interferon (IFN)-γ (g), interleukin (IL)-18 (h), IL-6 (i), IL-12 (j) and IL-1β (k) (n_{mice}/group = 4; two independent experiments). Data shown are the mean ± standard error of the mean. Data of proteinuria are shown by a Kaplan–Meier survival fractions profile.

Table 1. Therapeutic effect of carbon monoxide (CO) in glomerulonephritis lesions from lupus mice

Mice group		Hypercellularity	Wire-loop lesions	Crescent formations	Interstitial inflammation	Vasculitis (%)	Membranous changes (%)	Immunecomplex deposition in vascular walls (%)	Hyalin arteriolosclerosis (%)
MRL-Mpj	Untreated	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0	0	0	50
MRL-Mpj	+CO	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0·0 ± 0·0	0	0	0	50
MRL-Fas^{lpr}	Untreated	2·5 ± 0·5[a]	1·25 ± 0·5[a]	1·5 ± 1·0[a]	1· ± 0·8[a]	50	25	50	100
MRL-Fas^{lpr}	+CO	2·0 ± 0·8[a]	1·0 ± 0·0[a]	1·0 ± 1·0[a]	1·1 ± 0·7[a]	67	17	33	33

Score from affected glomeruli: 1 = 0–25%; 2 = 25–50%; and 3 = more than 50%; % of total mice. Mean ± standard deviation. Student's *t*-test was used.

*$P < 0·05$, MRL-Fas^{lpr} untreated *versus* MRL-MpJ untreated.

†Non-statistical significance, MRL-Fas^{lpr} untreated *versus* +CO.

treatment in this lupus mouse model. These results are consistent with previous studies from our group showing that CO treatment decreases anti-histone IgGs levels in serum [15]. Furthermore, CO may reduce the availability of apoptotic antigens during lupus because CO/HO-1 induction prevents apoptosis by modulating different signalling molecules, such as caspase 3, Bcl-2, Bad, Bcl-xL and Bag-1 [42–46]. Lower anti-DNA/histone antibody levels induced by CO treatment may lead to less immune complex deposition in glomeruli and vascular walls, which subsequently reduces organ failure [1]. In addition, the reduce kidney damage observed during CO treatment may be due to a decreased production of IFN-γ by $B220^+CD3^+CD4^-$ T cells or to a decrease local infiltration of leucocytes in the glomerulus. However, diverse mechanisms could contribute to the therapeutic effect of CO in kidney, including one driven by stromal cells [47]. We cannot rule out an effect of CO over the production of other proinflammatory cytokines released by infiltrating myeloid cells or glomerular cells such as podocytes that could also impact kidney function and membranous changes [48,49]. Along these lines, the beneficial consequences of CO treatment could be explained by a blocking effect of CO on different factors that may participate in nephritis pathogenesis in SLE patients and murine models, such as the presence of stromal cell apoptosis [26,50,51]. Accordingly, CO could promote cell survival of endothelial cells, as has been observed with ischaemia and cytostatic drugs [47,52]. Similarly, as reported previously by our group, there is a trend showing that CO treatment delays kidney damage during systemic autoimmunity [15]. Unfortunately, the mechanism responsible for the effect mediated by CO remains to be determined. Further research would be required to evaluate whether CO can decrease apoptosis in stromal kidney cells during lupus pathogenesis.

Although CO treatment decreased the amount of activated $B220^+CD3^+CD4^-$ T cells in lungs and kidneys, the precise role of this subpopulation in lupus pathogenesis remains to be determined. However, it is remarkable that CO exposure decreased circulating anti-dsDNA and anti-histone antibodies and improved glomerular status which could, in turn, ameliorate lupus nephritis. In terms of clinical projection of our data, it is important to emphasize that the arrival of CO-releasing molecules has increased significantly actual knowledge about anti-inflammatory properties of CO, avoiding side effects associated with secondary hypoxia due to inhaled CO [16]. Clinical trials conducted in healthy volunteers suggest that doses of CO similar to those used in our study have no adverse effects (ClinicalTrials.gov identifier: NCT00094406). Moreover, clinical trials based on CO therapy are being performed to prevent lung inflammation (ClinicalTrials.gov identifier: NCT00094406; ClinicalTrials.gov Identifier: NCT00122694) and transplant rejection (ClinicalTrials.gov Identifier: NCT00531856). Although not evaluated in this study, we suggest that the decrease in

activated $B220^+CD3^+CD4^-$ T cells observed in CO-treated mice may promote a better performance of lungs during systemic autoimmunity [1]. A more detailed analysis is needed to evaluate whether there is a pivotal role for $B220^+CD3^+CD4^-$ T cell subpopulation during lupus pathogenesis.

To our knowledge, this is the first report characterizing the therapeutic properties of CO exposure in MRL-Fas^{lpr} lupus mice. Our data suggest that the anti-inflammatory effects of CO in kidneys could be the result of a decrease in circulating autoantibodies together with the inhibition of T cell activation that would, in turn, reduce local proinflammatory cytokines such as IFN-γ, IL-6, IL-12 and IL-18. None the less, our data indicate that a CO-based therapy may be a potential tool for rheumatic diseases through different humoral and cellular mechanisms. Moreover, these results pose CO treatment as a potential effective therapy in T cell-mediated diseases as well as in non-oncological lymphoproliferative disorders through the inhibition of T cell activation. The understanding of molecular pathways underlying the beneficial effects of CO may provide new insights into treating or preventing autoimmunity and inflammation-based disease.

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Disclosure

A patent application for the use of CO to treat SLE has been submitted.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Gating strategy and fluorescence activated cell sorter (FACS) analysis. Spleen, kidney and lung from Murphy Roths large (MRL)-Fas^{lpr} and MRL/MpJ mice

from different groups were harvested and minced in phosphate-buffered saline (PBS) supplemented with 10% fetal calf serum (FCS) until a homogeneous cell suspension was reached. Tissue homogenates were filtered through a 70-mm nylon mesh filter (BD Biosciences, San Jose, CA, USA). Cells were washed with PBS-1% bovine serum albumin (BSA), resuspended at 2×10^6 cells/ml (50 μl/tube) and incubated with fluorophero-conjugated antibodies for 30 min at 4°C. Then cells were washed and acquired using a FACS Canto II flow cytometer (BD Biosciences). To discriminate between $CD45^+$ single cells and doublets or cell debris, events were gated sequentially on side-scatter (SSC)-A and forward-scatter (FSC)-A, FSC-W and FSC-H, SSC-W and SSC-H, and SSC-A and CD45-allophycocyanin (APC) plots. Representative flow cytometry data to illustrate the gating strategy are shown.

Fig. S2. Representative fluorescence activated cell sorter (FACS) analysis of leucocytes in lung and kidney. Graphs represents histograms and density plots of $CD45^+$ leucocytes in kidneys (a) and lungs (b). (c,e) Representative density plots of $CD3^+CD69^+$ cells (CD3 gate) from kidney and lung. (d,f) Representative density plots of $CD69^+$ cells from $CD3^+$ $B220^+$ $CD4^-$ gate.

Fig. S3. Carbon monoxide (CO) treatment did not modulate kidney, lung and lymphatic tissue weights in Murphy Roths large (MRL)-Fas^{lpr} and MRL-MpJ mice. MRL-Fas^{lpr} and MRL-MpJ mice were treated with CO 250 parts per million (ppm) 5 days/week from weeks 9 to 15 or 30. Spleen, inguinal lymphatic nodes, kidney and lung from CO or untreated MRL-Fas^{lpr} and MRL-MpJ mice were harvested and the weights were registered. Graph represents weight of kidney (a), lung (b), spleen (c) and inguinal lymphatic node (d). $***P < 0.001$ by one-way analysis of variance (ANOVA) test. The data shown are the mean ± standard error of the mean.

Fig. S4. Representative fluorescence activated cell sorter (FACS) analysis of leucocytes in spleen. Representative density plots of $CD69^+$ cells from $CD3^+$ $B220^+$ $CD4^-$ gate (a) and neutrophils $CD11b^+$ $Ly6G^+$ and monocytes $CD11b^+$ $Ly6G^-$.