Involvement of high mobility group box 1 in the auto-inflammatory process in systemic lupus erythematosus
Schaper, Fleur

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Treatment with anti-HMGB1 monoclonal antibody does not affect lupus nephritis in MRL/lpr mice

F Schaper¹, MM van Timmeren², A Petersen², G Horst¹, M Bijl³, PC Limburg⁴, J Westra¹, P Heeringa²

Departments of Rheumatology and Clinical Immunology¹, Pathology and Medical Biology², Laboratory Medicine⁴, University Medical Center Groningen, University of Groningen, the Netherlands
Department of Internal Medicine and Rheumatology³, Martini Hospital, Groningen, The Netherlands

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ABSTRACT

Objective
High Mobility Group Box 1 (HMGB1) is a nuclear DNA binding protein which acts as an alarmin when secreted. HMGB1 is increased in SLE and might represent a potential therapeutic target. We investigated whether treatment with an anti-HMGB1 antibody affects the development of lupus nephritis in MRL/lpr mice.

Materials and methods
Seven week old MRL/lpr mice were injected intraperitoneally twice weekly for 10 weeks with 50 µg monoclonal anti-HMGB1 (2G7, mouse IgG2b) (n=12) or control antibody (n=11). Control MRL/MPJ mice (n=10) were left untreated. Every two weeks blood was drawn and urine was collected at week 7, 11 and 17. Mice were sacrificed at 17 weeks for complete disease evaluation.

Results
Plasma HMGB1 and anti-HMGB1 levels were increased in MRL/lpr mice compared to control MRL/MPJ mice. There were no differences in albuminuria, urine HMGB1 and plasma levels of complement C3, anti-dsDNA and pro-inflammatory cytokines between untreated and treated mice at any time point. Lupus nephritis of mice treated with anti-HMGB1 mAb was classified as class III (n=3) and class IV (n=9), while mice treated with control mAb were classified as class II (n=4), class III (n=2) and class IV (n=5). IgG and C3 deposits in kidneys were similar in mice treated with anti-HMGB1 mAb or control mAb.

Conclusion
Treatment with monoclonal anti-HMGB-1 antibody 2G7 does not affect development of lupus nephritis, disease progression or pro-inflammatory cytokine levels in MRL/lpr mice. This indicates that blocking of HMGB1 by this neutralizing antibody does not affect lupus nephritis in MRL/lpr mice.
INTRODUCTION

Systemic Lupus Erythematosus (SLE) is a systemic autoimmune disease characterized by presence of auto-antibodies against nuclear components, leading to circulating immune complexes that are deposited in organs, causing tissue damage. SLE can affect multiple organs, including skin, joints and kidneys. Glomerulonephritis leading to persistent proteinuria and chronic renal failure is one of the most severe complications and is associated with significant mortality (1).

High mobility group box 1 (HMGB1) is a non-histone nuclear protein that has a dual function. Inside the cell it is ubiquitously expressed in the nucleus where it binds to DNA. HMGB1 can bend promoter regions and interacts directly with nucleosomes, thereby altering the accessibility of the DNA (2). HMGB1 can also be released extracellularly upon activation, damage, apoptosis or necrosis (3). It then acts as a damage-associated molecular pattern (DAMP) or alarmin and can initiate and perpetuate inflammatory responses. In several inflammatory conditions, such as sepsis and rheumatoid arthritis, serum and tissue levels of HMGB1 are increased (4-6). In animal models of autoimmune and inflammatory diseases it has been shown that blocking HMGB1 can attenuate disease. For instance, treatment with anti-HMGB1 antibodies inhibited joint destruction in arthritis models (7). In sepsis models, inhibition of HMGB1 by anti-HMGB1 antibodies suppressed pro-inflammatory cytokine release and improved survival (8,9).

In SLE patients several studies have shown that serum levels of HMGB1 are increased, and levels associate with disease activity (10-14). It has been hypothesized that impaired clearance and accumulation of apoptotic debris in SLE might lead to increased HMGB1 levels locally and systemically. Furthermore, higher levels of HMGB1 were observed in serum from patients with renal involvement compared to patients without renal involvement (10,13). Kidney biopsies from patients with lupus nephritis showed strong expression of HMGB1 at cytoplasmic and extracellular sites suggesting active release of HMGB1 (11). Collectively, these studies suggest that HMGB1 is an important factor in the pathogenesis of SLE, and that it represents a potential therapeutic target.

The MRL/MpOlαHsd-Tnfrsf6\(^{lpr}\) (MRL/lpr) mouse is a frequently used mouse strain which develops a severe spontaneous autoimmune disease similar to SLE (15). The \(lpr\) mutation in the FAS gene results in loss of FAS function leading to a defect in FAS-mediated apoptosis. On the MRL genetic background this leads to extensive lymphoproliferation and generation of autoreactive T cells. The ensuing disease is characterized by lymphadenopathy, autoantibody production, complement activation, and immune complex glomerulonephritis that usually manifests between 14-18 weeks of age (16-18).
We hypothesized that HMGB1 represents a potential therapeutic target in SLE and that inhibition of HMGB1 will attenuate renal involvement in SLE. In this study we therefore investigated whether treatment with a neutralizing monoclonal anti-HMGB1 antibody could beneficially affect the development of lupus nephritis in MRL/lpr mice.

MATERIALS AND METHODS

Mice
Five week old female MRL/lpr mice were obtained from Harlan (Horst, the Netherlands) and 5 week old female MRL/MPJ were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA). Mice were group-housed in a temperature and humidity controlled environment with a 12-hour light/dark cycle. Animals were allowed ad libitum access to drinking water and standard chow. Animal experiments were approved by the local Animal Care and Experimentation Committee (DEC 6464A).

Neutralizing anti-HMGB1 antibody
Monoclonal anti-HMGB1 antibody (clone 2G7, IgG2b, gift from dr. K Tracey, the Feinstein Institute for Medical Research, NY, USA) was used for treatment in MRL/lpr mice. This antibody has been extensively characterized previously with respect to its HMGB1 neutralizing activity in in vitro and in vivo studies. The neutralizing activity of anti-HMGB1 mAb has been tested in cell culture assays, with both human and mouse macrophages, and in animal models of HMGB1-mediated damage, such as sepsis (8,19-24). Moreover, anti-HMGB1 mAb 2G7 has been shown to neutralize the cytokine isoform of HMGB1 (19).

Treatment of the mice with anti-HMGB1 antibody
Ten MRL/lpr mice were followed from week 7 till 17 without any intervention. Two groups of 12 MRL/lpr mice were injected intraperitoneally twice weekly, for 10 weeks, with 50 µg/mouse monoclonal anti-HMGB1 antibody (clone 2G7, IgG2b) or control IgG2b antibody (BioXCell, West Lebanon, USA). A control group of MRL/MPJ mice (n=10) was left untreated. Before start of treatment, at week 7, blood and urine was collected. Then, every two weeks plasma (EDTA) was collected from the saphenous vein. Urine was collected during 18 hours at 7, 11, and 17 weeks. At sacrifice, spleens and kidneys were harvested. One half of each kidney was fixed in formalin and embedded in paraffin, the remaining half was snap frozen in liquid N₂ and stored at −80°C.
Laboratory measurements
Automated total and differential white blood cell counts (WBC) were determined in whole blood obtained at sacrifice using the Sysmex XT-1800iV (Sysmex Nederland BV, Etten-Leur, Netherlands). Plasma levels of complement C3 (GenWay Biotech, San Diego, USA) and anti-dsDNA IgG (Alpha Diagnostic International, San Antonio, USA) were measured by ELISA, according to the manufacturers’ instructions. Serum blood urea nitrogen (BUN) levels were determined by the Quantichrom DIUR 500 kit (BioAssay Systems, Hayward, CA). Albuminuria was determined by ELISA (Bethyl Laboratories, Montgomery, TX), according to the manufacturer’s instructions. Plasma levels of the cytokines IL-4, IL-6, IL-17A, IFN-α, and TNF-α were measured with a Multiplex panel (ProcartaPlex Mouse Simplex; Affymetrix eBioscience, Vienna, Austria) according to the manufacturer’s instructions. Plasma levels of anti-HMGB1 were measured by an in-house developed ELISA. In short, Costar polystyrene plates were coated overnight with 1 μg/ml recombinant HMGB1 (Sigma, St. Louis, MO, USA). Mouse plasma samples were added in duplicate at 500 fold or 7500 fold (for anti-HMGB1 treated mice) dilutions. Detection of antibodies was done with rabbit anti-mouse IgG-HRP (Dako, Glostrup, Denmark). Levels of anti-HMGB1 were calculated against a standard curve of a known concentration of monoclonal anti-HMGB1 antibody (2G7, mouse IgG2b).

Plasma and urine levels of HMGB1 were measured by Western Blotting as described previously (10), using polyclonal anti-HMGB1-biotin (Thermoscientific, Etten-Leur, the Netherlands) for detection.

Gene expression analysis by quantitative RT-PCR
Total RNA was extracted from twenty 10-µm thick kidney cryo-sections using the RNeasy Mini plus Kit (Qiagen, Westburg, Leusden, The Netherlands) according to the manufacturer’s instructions. RNA yield, cDNA synthesis and quantitative PCR amplifications were performed as described previously (25). The following Assay-On-Demand primers were used: glyceraldehyde 3-phosphatedehydrogenase (GAPDH); monocyte chemoattractant protein-1 (MCP-1), kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), IL-6 and TNF-α. The amount of target was normalized to an endogenous reference (GAPDH) and expressed as relative expression (2^(-ΔCT)).

Renal histopathology
Two-µm paraffin kidney sections were routinely stained with haematoxylin or eosin (H&E) and periodic acid-Schiff (PAS). The slides were evaluated in a blinded manner.
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by an expert renal pathologist and kidney pathology was evaluated using the lupus nephritis classification system as described by Weening et al (26). HMGB1 staining was performed as described previously (11).

Complement C3 and IgG staining was performed on 5-µm frozen kidney sections with 1 µg/ml rabbit anti-C3 antibody (Thermoscientific) followed by goat-anti-rabbit IgG-HRP (Dako). For IgG rabbit anti-mouse IgG-HRP (Dako) was used. Peroxidase activity was detected with DAB and sections were counterstained with Mayer’s hematoxylin. All sections were scored digitally after examination using a Nanozoomer Digital Pathology Scanner (NDP Scan U10074-01, Hamamatsu Photonics K.K., Japan) and quantified ((number of positive cells* 0.5) + number of strong positive cells / total cells) with software of ImageScope Viewer (V11.2.0.780 Aperio, e-Pathology Solution, CA, USA).

Statistics
Data was analyzed using SPSS (version 22, SPSS Inc, Chicago IL, USA). Data are expressed as median and range and were analyzed using Mann-Whitney U or Kruskal Wallis test. For non-continuous variables a Fisher exact test was used. Generalized estimating equations were used to analyze changes over time within mice, and the exchangeable matrix correlation structure was used. When residuals were not normally distributed, parameters were transformed (square root or logarithmic) before entered in the equation. P-values <0.05 were considered statistically significant.

RESULTS

Plasma HMGB1 and anti-HMGB1 levels increase over time in lupus-prone MRL/lpr mice

We first analyzed plasma levels of HMGB1 by Western blotting in a separate cohort of 10 MRL/lpr mice from 6 till 17 weeks of age. As shown in Figure 1, plasma HMGB1 (Figure 1A) and anti-HMGB1 (Figure 1B) were clearly detectable in 6-week-old mice. Moreover, plasma HMGB1 levels increased with age and disease progression. These results indicate that MRL/lpr mice have increasing plasma levels of HMGB1 over time and therefore represent an appropriate model to investigate whether intervention with an anti-HMGB1 antibody has beneficial effects on the development of lupus nephritis.

HMGB1 mAb treatment does not affect plasma parameters of disease activity

Plasma levels of anti-dsDNA and complement C3 were analyzed at four time points in both MRL/lpr groups and MRL/MPJ mice (Figure 2A-B) by Generalized estimating equations (GEE) analysis. At all time points, starting at week 7, MRL/lpr
mice had significantly higher levels of anti-dsDNA than MRL/MPJ mice (p<0.001, Figure 2A). In MRL/lpr mice levels of anti-dsDNA increased over time and were highest at week 17, whereas in MRL/MPJ mice the levels of anti-dsDNA increased until week 13 and remained at a similar level at week 17. Levels of anti-dsDNA were similar in MRL/lpr mice treated with anti-HMGB1 mAb or control mAb (p=0.09, Figure 2B). In MRL/lpr as well as MRL/MPJ mice, complement C3 levels decreased over time (p<0.001) and were lowest at week 17. Overall, complement C3 was significantly decreased in MRL/lpr compared to MRL/MPJ (p<0.01), but was not different between mice treated with anti-HMGB1 mAb treated mice and control mAb (p=0.55, Figure 2B).

![Figure 1](https://via.placeholder.com/150)

**Figure 1:** Plasma HMGB1 and anti-HMGB1 antibody levels increase with age in MRL/lpr mice. 
**A:** Plasma HMGB1 levels were measured by Western Blot, at week 6, 7, 9, 11, 13, 15 and 17 in MRL/lpr mice (n=11). HMGB1 levels are presented as values of fluorescence intensity and were normalized against a standard of jurkat cell lysate. 
**B:** Plasma anti-HMGB levels (ng/ml) were measured by ELISA at week 6, 7, 9, 11, 13, 15 and 17 in MRL/lpr mice (n=11). Anti-HMGB1 levels are presented as values of fluorescence intensity and were normalized against a standard of monoclonal anti-HMGB1 antibody.
Anti-HMGB1 treatment does not affect lupus nephritis in lupus mice.

Figure 2: Anti-HMGB1 mAb treatment of MRL/lpr mice does not affect plasma levels of HMGB1, anti-dsDNA antibodies and complement C3, while increasing HMGB1 antibody levels.

A: Plasma anti-dsDNA (units/ml) was measured by ELISA in MRL/MPJ (n=10) and MRL/lpr mice (n=10-12) at week 7, 9, 13 and 17.

B: Plasma Complement C3 (mg/ml) was measured by ELISA in MRL/MPJ (n=10) and MRL/lpr mice (n=10-12) at week 7, 9, 13 and 17.

C: Plasma HMGB1 was measured by Western Blotting in MRL/MPJ (n=10) and MRL/lpr mice (n=10-12) at week 7, 9, 11, 13, 15 and 17. HMGB1 levels are presented as values of fluorescence intensity and were normalized against a standard of jurkat cell lysate.

D: Plasma anti-HMGB1 (ng/ml) was measured by ELISA in MRL/MPJ (n=10) and MRL/lpr mice (n=10-12) at week 7, 9, 11, 13, 15 and 17. Anti-HMGB1 levels are presented as values of fluorescence intensity and were normalized against a standard of monoclonal anti-HMGB1 antibody. Depicted are the median values with range at each time point.

Plasma levels of HMGB1 were analyzed at 6 time points (Figure 2C). GEE analysis demonstrated that HMGB1 significantly increased over time in all three groups (p<0.001). As expected, at all time points MRL/lpr mice had higher levels of HMGB1 than MRL/MPJ mice (p<0.05), however no significant differences were observed between MRL/lpr mice treated with anti-HMGB1 mAb or control mAb (p=0.32).

Plasma levels of anti-HMGB1 were measured at six time points by ELISA (Figure 2D). Anti-HMGB1 significantly increased over time in MRL/lpr mice (p<0.001) but not in MRL/MPJ mice. At week 7, before start of treatment in MRL/lpr mice, plasma levels of anti-HMGB1 were similar in all MRL/lpr and MRL/MPJ mice. At all of the time points anti-HMGB1 levels were substantially higher in MRL/lpr mice treated with anti-HMGB1 mAb than with control mAb (p<0.001, Figure 2D). In MRL/MPJ mice levels of anti-HMGB1 did not change over time, whereas in MRL/lpr mice...
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Plasma levels of several proinflammatory cytokines were analyzed with a multiplex assay as shown in Figure 3. At week 7, the levels of IFN-α, IL-17A and IL-6 were similar in MRL/lpr and MRL/MPJ mice, whereas TNF-α was higher in MRL/lpr mice (p<0.001). IL-6 levels (Figure 3A) increased over time in MRL/MPJ as well as in MRL/lpr mice, however no difference were observed between MRL/lpr mice treated with anti-HMGB1 mAb or control mAb. IL-17A levels (Figure 3B) decreased over time in MRL/MPJ as well as MRL/lpr mice, however no differences were observed between treated groups. In MRL/lpr mice, but not in MRL/MPJ mice, IFN-α (Figure 3C) and TNF-α (Figure 3D) levels increased significantly (p<0.01) over time, however, no differences were observed between MRL/lpr mice treated with anti-HMGB1 mAb or mice treated with control mAb. To summarize, although MRL/lpr mice treated with anti-HMGB-1 mAb displayed massively increased circulating anti-HMGB-1 antibody levels, no effect was observed on plasma levels of HMGB1, anti-dsDNA, complement C3 and pro-inflammatory cytokines.

Figure 3: Anti-HMGB1 mAb treatment of MRL/lpr mice does not affect plasma levels of pro-inflammatory cytokines.

Plasma levels of the cytokines IL-17 (A), IL-6 (B), IFN-α (C) and TNF-α (D) were measured by multiplex in MRL/MPJ and MRL/lpr mice at week 7, 9, 13 and 17. Box and Whiskers plot, median and interquartile range are shown for n= 6-8 mice per group. Separate dots indicate outliers.
**HMGB1 mAb treatment does not affect body and spleen weight and distribution of white blood cells**

To investigate whether treatment with anti-HMGB1 had an effect on body weight, mice were weighed every week. As shown in Supplementary Figure 1A, all three groups of mice had increasing body weight from week 7 until sacrifice at week 17 and no differences between the groups were observed. As expected, average spleen weight at sacrifice was increased in MRL/lpr mice compared to MRL/MPJ mice (p<0.0001), however average spleen weight was similar between MRL/lpr mice treated with anti-HMGB1 mAb or control mAb (Supplementary Figure 1B). Total white blood cell count and differential white blood cell counts were also similar between anti-HMGB1 and control mAb treated MRL/lpr mice (Supplementary Table 1). These results demonstrate that treatment with monoclonal anti-HMGB1 antibody does not influence body and spleen weight or white blood cell composition.

**HMGB1 mAb treatment does not affect kidney pathology and proteinuria**

Kidney pathology was evaluated using the lupus nephritis classification system as described by Weening et al (26). MRL/MPJ mice, as expected, did not show any lupus nephritis, while both groups of MRL/lpr did (Figure 4A). Lupus nephritis of MRL/lpr mice treated with anti-HMGB1 mAb was classified as class III (n=3) and class IV (n=9), while mice treated with control mAb were classified as class II (n=4), class III (n=2) and class IV (n=5). In all mice mostly proliferative lesions were found and all displayed mononuclear cell infiltration. In both groups 2 mice showed vasculitis. In addition, chronicity was low, two mice in both groups showed sclerosing lupus nephritis. None of the mice developed tubulointerstitial disease, or glomerular and/or fibrous crescents.

Renal deposits of IgG and complement C3 were determined by immunohistochemistry (Figure 4A-B). As expected, extensive glomerular IgG and C3 deposits were detected in MRL/lpr mice when compared to the control MRL/MPJ mice. However, the extent of glomerular IgG and C3 deposits was similar in MRL/lpr mice treated with anti-HMGB1 mAb or control mAb. As expected, HMGB1 staining showed nuclear staining in kidneys from MRL/MPJ mice (Supplementary Figure 2). In kidneys from MRL/lpr mice extracellular HMGB1 was observed. Moreover, there were also HMGB1 negative nuclei (blue staining) present, which suggests active release of HMGB1 from these cells, but there was no difference between mice treated with anti-HMGB1 mAb or control mAb.

Renal function was determined by measuring blood urea nitrogen (BUN) levels at week 7, 11 and 17 (figure 5A). At week 7 BUN levels were similar between in MRL/lpr and MRL/MPJ mice, while at week 11 and 17 BUN levels were increased...
Figure 4: Anti HMGB1 mAb treatment of MRL/lpr mice does not affect renal pathology and renal immune complex deposition. 
A: Representative pictures of PAS, HE, C3 and IgG staining in kidney sections of 17 weeks old MRL/MPJ and MRL/lpr mice (10x). Inserts show a glomerulus in detail (40x). B: Quantitative analysis of C3 and IgG deposition in kidney sections of 17 weeks old MRL/MPJ and MRL/lpr mice. Box and Whiskers plot, median and interquartile range are shown for n= 8-11 mice per group. A Kruskal Wallis test was used to test for overall differences between the three groups. To investigate which group was different further testing was performed to compare groups separately using Mann-Whitney. *P<0.05
Anti-HMGB1 treatment does not affect lupus nephritis in lupus mice.

Renal injury was also evaluated by 18-hour albumin excretion into the urine (Figure 5B). At week 7 and week 11, urinary albumin excretion was similar in MRL/lpr and MRL/MPJ mice. At week 17, urinary albumin excretion was highly increased in the MRL/lpr mice but not in MRL/MPJ mice (p<0.0001). However, albuminuria was similar in MRL/lpr mice treated with anti-HMGB1 mAb or control mAb. Urine HMGB1 levels were analyzed by Western blotting (Figure 5C). At week 7 and week 11, urine HMGB1 was undetectable in all mice. At week 17, urine

**Figure 5:** HMGB1mAb treatment of MRL/lpr mice does not affect albuminuria and HMGB1 urine levels.

A: Renal function was determined by measuring blood urea nitrogen (BUN) levels at week 7, 11 and 17. B: Albumin was measured by ELISA in 18-hours urine of MRL/MPJ (n=10) and MRL/lpr mice (n=10-12) at week 7, 11 and 17. C: HMGB1 was measured by Western Blotting in 18-hours urine of MRL/MPJ (n=10) and MRL/lpr mice (n=10-12) at week 7, 11 and 17. HMGB1 levels are presented as values of fluorescence intensity and were normalized against a standard of Jurkat cell lysate.

A Kruskal Wallis test was used to test for overall differences between the three groups at each time point. To investigate which group was different further testing was performed to compare groups separately using Mann-Whitney U.

Box and Whiskers plot, median and interquartile range are shown for n= 8-12 mice per group. Separate dots indicate outliers. *** P<0.0001
HMGB1 levels were increased in MRL/lpr mice, however no difference between treated groups were seen. To summarize, MRL/lpr mice displayed renal injury characteristic of lupus nephritis which was unaffected by treatment with anti-HMGB1 mAb.

**HMGB1 mAb treatment does not affect pro-inflammatory cytokine and injury-related gene expression in the kidney**

To further investigate renal inflammation, mRNA levels of the pro-inflammatory cytokines IL-6, TNF-α and MCP-1 and of renal injury-related biomarkers NGAL and KIM-1 were assessed in kidneys (Figure 6). As expected, levels of IL-6, TNF-α, NGAL, and MCP-1 were higher in MRL/lpr mice compared to MRL/MPJ mice (p<0.05). However, KIM-1 levels (Figure 6E) were comparable between MRL/MPJ and MRL/lpr mice, confirming renal histology in which no tubulointerstitial disease was observed. No differences were found in expression levels of all investigated genes between MRL/lpr mice treated with anti-HMGB1 mAb or control mAb (Figure 6), confirming the results of renal histology that treatment with anti-HMGB1 mAb does not influence kidney damage.

**Figure 6:** Anti HMGB1mAb treatment of MRL/lpr mice does not affect renal mRNA levels of IL-6, TNF, MCP-1, NGAL or KIM-1.

Expression levels of TNF-α (A), IL-6 (B), MCP-1 (C), NGAL (D) and KIM-1 (E) mRNA were analysed in kidney tissues of MRL/MPJ mice (n=8) and MRL/lpr mice treated with control mice (n=11) and mice treated with monoclonal anti-HMGB1 (n=9). Data are shown as relative expression compared to GAPDH. Box and Whiskers plot, median and interquartile range are shown. Separate dots indicate outliers. A Kruskal Wallis test was used to test for overall differences between the three groups. To investigate which group was different further testing was performed to compare groups separately using Mann-Whitney U. *P<0.05 ** P<0.01
DISCUSSION

Lupus nephritis is one of the most severe complications of SLE and is associated with significant mortality. In SLE, HMGB1 serum levels are associated with disease activity and renal involvement, and therefore it has been proposed that HMGB1 might represent a potential therapeutic target in SLE (11,12,27). Using the lupus-prone MRL/lpr mouse model, we show that administration of neutralizing monoclonal anti-HMGB1 antibodies does not affect development of lupus nephritis, disease progression or pro-inflammatory cytokine expression.

In accordance with observations in patients with SLE (10-14), plasma HMGB1 levels were increased in lupus-prone MRL/lpr mice compared to the control MRL/MPJ mice that do not develop disease. Moreover, HMGB1 levels increased with disease progression, which again is in agreement with observations in SLE patients where serum HMGB1 levels correlate with the SLE disease activity index (SLEDAI) (10,14). Upon development of lupus nephritis at week 17, HMGB1 was also detectable in urine as was also shown in patients (11). In line with renal HMGB1 immunohistochemistry in patients, we observed HMGB1 expression at extracellular sites in kidneys from MRL/lpr mice, which suggests active release of HMGB1. Collectively, these data indicate that HMGB1 release occurs in the MRL/lpr mouse model and that, also with respect to HMGB1, this mouse model reflects human disease.

However, as we describe here, intraperitoneal administration of the monoclonal anti-HMGB1 antibody 2G7 to lupus-prone MRL/lpr mice (50 µg/mouse twice weekly from week 7 till 17) did not ameliorate development of lupus nephritis. Previously, the monoclonal anti-HMGB1 antibody 2G7 has been successfully used in various models of inflammation in which a pro-inflammatory effect of HMGB1 was suspected. For example, intraperitoneal administration of mAb 2G7 in a mouse model of Rheumatoid Arthritis (RA) ameliorated development of arthritis. In this specific study either 100 µg/mouse was injected every second day for 5 weeks or 70 µg/mouse daily for 1 week (7,28). Moreover, intraperitoneal administration of a single dose of mAb 2G7 (10 µg/mouse) 24 hours after cecal ligation and puncture, significantly improved survival in this sepsis model (29). In addition, mAb 2G7 has been shown to prevent TLR4-mediated early graft failure after pancreatic islet graft transplantation, although in this specific study the antibody (50 µg/mouse) was injected into the portal vein of the liver together with the transplanted islets (28). Collectively, these studies demonstrate that the anti-HMGB1 mAb 2G7, in comparable dosages and employing similar administration routes, is able to confer protective effects in various inflammatory disease models.

In addition, a number of studies, albeit using different anti-HMGB1 antibodies which also recognize a different epitope, have shown beneficial effects of treatment
with anti-HMGB1 antibodies in mouse models of sepsis and renal ischemia/reperfusion (8,30). In these models, it has been demonstrated that HMGB1 is an early mediator of damage and that neutralization of HMGB1 inhibits downstream effects, such as release of pro-inflammatory cytokines. However, these models represent reversible acute disease models, whereas our lupus nephritis model represents a chronic irreversible disease. In acute diseases, HMGB1 may act as an early alarmin that can initiate downstream pro-inflammatory signaling. In SLE, disease development occurs gradually and is multifactorial, and hence HMGB1 represents only one of the many pro-inflammatory factors which can activate the immune system and cause damage. This contention is supported by our finding that neutralization of HMGB1 does not halt or improve lupus nephritis development.

Inhibition of HMGB1 activity can be achieved in different ways. In our study we used anti-HMGB1 antibodies to neutralize HMGB1. An alternative approach is to use the competitive receptor antagonist Box A in order to prevent binding of HMGB1 to its receptor RAGE. In mouse models of arthritis (31,32) and sepsis (8) administration of Box A exerted beneficial effects on disease progression. It should be noted, however, that RAGE is only one of the receptors for HMGB1, and hence it is likely that not all effects of HMGB1 will be inhibited when using Box A.

In addition, Li et al. recently reported that blocking serum HMGB1 with glycyrrhizin, a traditional herbal medicine which blocks HMGB1, alleviated lupus nephritis in ALD-DNA induced lupus mice (33). In this model, lupus nephritis is induced by active immunization of mice with DNA from lymphocytes that have undergone activation-induced cell death (ALD-DNA) in CFA. After induction, serum HMGB1 levels gradually increased. Glycyrrhizin treatment attenuated HMGB1 as well as circulating proinflammatory cytokine levels and alleviated renal pathology. However, for glycyrrhizin a range of biological effects have been reported, including anti-inflammatory, antioxidant, free radical-scavenging, antiulcer, antihepatotoxic, antimicrobial, cytoprotective and cytotoxic activities (34,35). Therefore, it is unclear whether glycyrrhizin exerts its beneficial effects solely via blocking of HMGB1 in this ALD-DNA induced lupus mouse model.

The results presented here appear to contradict a recent study by Zhang et al. showing that treatment with anti-HMGB1 antibodies reduced lupus-like disease, including proteinuria and glomerulonephritis, in lupus-prone BXSB mice (36). Lupus in BXSB mice is characterized by lymph node and spleen enlargement, hypergammaglobulinemia, anti-nuclear antibodies, and immune complex glomerulonephritis, which usually becomes evident at around 5 months of age. A duplicated copy of the TLR7 gene is primarily responsible for the autoimmune phenotype. In MRL/lpr we demonstrated that plasma HMGB1 levels are elevated upon disease development, but it is unclear whether the same holds true for the
BXSB lupus mouse model. Moreover, the source of the anti-HMGB1 antibody used in the study by Zhang et al is different from ours and a different treatment regimen was employed consisting of 10 µg/mouse injected intraperitoneally three times a week from week 16 till week 26. Therefore, the differences between our study and the data from Zhang et al might be explained by differences in mouse model, anti-HMGB1 antibody and treatment regimen. These inconclusive or contradictory results warrant further studies in different mouse models of SLE such as mice that lack MFGE8.

Plasma HMGB1 levels, measured by Western Blotting, were unaffected in MRL/lpr mice treated with neutralizing monoclonal anti-HMGB1 antibodies. We used Western Blotting to detect HMGB1 since we and others have previously shown that serum proteins and antibodies interfere in the HMGB1 ELISA (10,37). However, due to denaturation and high temperatures in Western blotting, immune complexes are dissociated and the HMGB1 measured probably consists of free HMGB1 and previously complexed HMGB1. Our findings seem to implicate that complexes of HMGB1-anti-HMGB1 that are formed during treatment are not cleared from the circulation, which can be explained by the fact that the antibody used is a neutralizing but not a depleting antibody.

MRL/lpr mice develop lymphadenopathy leading to the presence of autoreactive lymphocytes. Activated lymphocytes are a source of extracellular HMGB1 (38), which can contribute to high levels of HMGB1 in the spleen or lymph nodes. Therefore, due to high levels of HMGB1 locally, the dose of mAb 2G7 used here may have been insufficient to neutralize HMGB1 completely. Although we cannot exclude this possibility, previous studies in BXSB lupus mice and a sepsis model have clearly shown a positive effect of anti-HMGB1 antibody treatment despite the development of splenomegaly (21,36).

In untreated MRL/lpr mice, plasma levels of HMGB1 and anti-HMGB1 increase over time with disease progression, which is in line with data from SLE patients (10,39). In the disease models described by Li et al and Zhang et al, which do show a positive effect of blocking HMGB1, it is unclear whether anti-HMGB1 antibodies are also present (33,36). At present the role of endogenously formed anti-HMGB1 antibodies in disease pathogenesis is unclear and warrants further investigation. It is also possible that rheumatoid factors, which can be present in serum from MRL/lpr mice, can interfere with the activity of the anti-HMGB1 mAb. However, earlier studies using this antibody in animal models of RA did not observe a reduced effect of anti-HMGB1 mAb (7).

Since HMGB1 is known to induce pro-inflammatory cytokine release from immune cells we also measured plasma pro-inflammatory cytokine levels. In vitro, HMGB1 has been shown to induce TNF-α release from mouse macrophages (40,41).
and IFN-α production from dendritic cells (42). In MRL/lpr mice we observed increasing plasma levels of TNF-α and IFN-α over time. However, it is unclear whether these pro-inflammatory cytokines are induced solely by HMGB1 and/or are derived from autoreactive lymphocytes that are continuously present in MRL/lpr mice. Regardless of the source, we observed no effect of anti-HMGB1 antibody treatment on plasma pro-inflammatory cytokine levels.

Taken together, our data show that, although HMGB1 levels are increased in MRL/lpr mice, treatment with a monoclonal anti-HMGB1 antibody does not inhibit development or affect progression of lupus nephritis. Further studies are needed to validate these results in other mouse models of SLE.

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mobility group box 1 (HMGB1) and anti-HMGB1 antibodies and their relation to disease characteristics in systemic lupus erythematosus. 13: R71.


SUPPLEMENTARY MATERIAL

**Supplemental figure 1:** HMGB1mAb treatment of MRL/lpr mice does not affect bodyweight or spleen weight.
A: Body weight (gram) of MRL/MPJ (n=10) and MRL/lpr (n=12) mice, lines represent median with interquartile range. Mice were weighed every week from week 7 till 17. B: Spleen weight (gram) of MRL/MPJ (n=10) and MRL/lpr (n=12) mice at week 17, Box and Whiskers plot, median and interquartile range are shown. A Kruskal Wallis test was used to test for overall differences between the three groups. To investigate which group was different further testing was performed to compare groups separately using Mann-Whitney U. *** P<0.0001

**Supplemental figure 2:** HMGB1mAb treatment of MRL/lpr mice does not affect renal HMGB1 protein expression.
Representative pictures of HMGB1 staining in kidney sections of 17 weeks old MRL/MPJ and MRL/lpr mice (10x). Arrows indicate extracellular release of HMGB1, which is present in MRL/lpr mice but not in MRL/MPJ mice.
Supplementary table 1: HMGB1mAb treatment of MRL/lpr mice does not affect the composition and number of circulating white blood cells.

<table>
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<th>MRL/lpr + control mAb</th>
<th>MRL/lpr + HMGB1 mAb</th>
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<td>Eosinophils and basophils (%)</td>
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<td>0.9 (0.1-2.3)</td>
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