

Mdm2 Promotes Systemic Lupus Erythematosus and Lupus Nephritis

Ramanjaneyulu Allam, Sufyan G. Sayyed, Onkar P. Kulkarni, Julia Lichtnekert, and Hans-Joachim Anders

Department of Nephrology, Medizinische Poliklinik, Klinikum der Universität München–Innenstadt, Munich, Germany

ABSTRACT

Systemic lupus erythematosus (SLE) is a polyclonal autoimmune syndrome directed against multiple nuclear autoantigens. Although RNA and DNA seem to have identical immunostimulatory effects on systemic and intrarenal inflammation, each seems to differ with regard to the propensity to induce mitogenic effects such as lymphoproliferation. To identify potential mechanisms by which DNA specifically contributes to the pathogenesis of lupus nephritis, we stimulated cells with immunostimulatory DNA or RNA *in vitro* and used microarray to compare the transcriptomes of RNA- and DNA-induced genes. Immunostimulatory DNA, but not RNA, induced Mdm2, which is a negative regulator of p53. *In vivo*, we observed greater expression and activation of Mdm2 in the spleen and kidneys in a mouse model of lupus (MRL-Fas^{lpr} mice) than healthy controls. Treatment of MRL-Fas^{lpr} mice with the Mdm2 inhibitor nutlin-3a prevented nephritis and lung disease and significantly prolonged survival. Inhibition of Mdm2 reduced systemic inflammation and abrogated immune complex disease by suppressing plasma cells and the production of lupus autoantibodies. In addition, nutlin-3a suppressed the abnormal expansion of all T cell subsets, including CD3⁺CD4⁻CD8⁻ T cells, which associated with attenuated systemic inflammation. However, inhibiting Mdm2 did not cause myelosuppression or affect splenic regulatory T cells, neutrophils, dendritic cells, or monocytes. Taken together, these data suggest that the induction of Mdm2 promotes the expansion of plasma cells and CD3⁺CD4⁻CD8⁻ T cells, which cause autoantibody production and immune complex disease in MRL-Fas^{lpr} mice. Antagonizing Mdm2 may have therapeutic potential in lupus nephritis.

J Am Soc Nephrol 22: 2016–2027, 2011. doi: 10.1681/ASN.2011010045

Lupus nephritis is an immune complex glomerulonephritis that develops secondary to systemic lupus erythematosus (SLE), a polyclonal autoimmune syndrome directed against multiple nuclear autoantigens.^{1,2} It is becoming increasingly obvious that SLE and lupus nephritis develop from combinations of genetic variants that impair proper apoptotic cell death and rapid clearance of apoptotic cells as a central homeostatic avenue to avoid the exposure of nuclear autoantigens to the immune system.³ The observation that antinuclear antibodies are directed against double-stranded (ds)DNA in the majority of SLE patients and in almost all lupus nephritis patients first documented dsDNA as an important lupus autoantigen. The traditional view of nuclear particles as lupus autoantigens was re-

cently broadened by the observation that nuclear particles promote lupus nephritis also by acting as autoadjuvants.^{4,5} For example, certain endogenous RNA or DNA particles activate Toll-like receptor (TLR)-7 and TLR9 in dendritic cells and B cells, which promotes lymphoproliferation and immune complex disease as well as intrarenal inflamma-

Received January 12, 2011. Accepted May 27, 2011.

Published online ahead of print. Publication date available at www.jasn.org.

Correspondence: Dr. Hans-Joachim Anders, Medizinische Poliklinik, Universität München, Pettenkoferstraße, 8a, D-80336 München, Germany. Phone: ++49-89-218075855; Fax: ++49-89-218075860; E-mail: hjanders@med.uni-muenchen.de

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tion.^{5,6} Vice versa, neutralizing TLR7 and/or TLR9 prevents and suppresses lupus nephritis.^{7–9}

Although RNA and DNA seem to have identical immunostimulatory effects on systemic and intrarenal inflammation, some observations suggest that RNA and DNA immune recognition differ in terms of their mitogenic effects. For example, RNA immune recognition drives mesangial cell apoptosis, whereas cytosolic DNA rather stimulates mesangial cell growth.¹⁰ Furthermore, administration of immunostimulatory RNA or DNA both aggravated lupus nephritis in MRL-Fas^{lpr} mice, but only DNA injections caused severe lymphoproliferation.^{11–13} We therefore speculated that, beyond its autoantigen and adjuvant effects, endogenous DNA might have also a mitogenic effect in SLE, similar to the mitogenic effect of bacterial DNA.¹⁴ Bacterial DNA was first described in 1995 as a B cell mitogen, but the underlying molecular mechanism has remained unknown. By using a comparative transcriptome analysis between RNA- and DNA-induced genes, we identified the cell cycle regulator murine double minute (Mdm)-2 to be specifically induced by DNA. Mdm2 is an E3 ubiquitin ligase that degrades several central cell cycle regulators including p53 and retinoblastoma protein.^{15,16} For example, increased levels of Mdm2 prevent the induction of genes that are required to initiate apoptosis, and Mdm2 directly activates the cell cycle, two mechanisms that are well documented to contribute to tumor progression.^{17,18} Most interestingly, Mdm2 induction by DNA viruses specifically drives B cell lymphoma,¹⁹ a mechanism that might contribute in a similar manner to lymphoproliferation in SLE, albeit initiated via self-DNA.

Therefore, we hypothesized that endogenous DNA, released from dying lymphocytes, induces Mdm2 expression during the progression of SLE, a mechanism that promotes inappropriate lymphoproliferation and immune complex disease including lupus nephritis. In fact, we found that Mdm2 expression and Mdm2 activation correlates with lymphoproliferation and lupus nephritis in MRL-Fas^{lpr} mice. Pharmacologic Mdm2 inhibition significantly reduced lymphoproliferation by specifically depleting the majority of autoreactive T cells and plasma cells without affecting hematopoiesis or granulopoiesis. Mdm2 blockade also abrogated autoantibody production, all aspects of lupus nephritis, and prolonged overall survival in MRL-Fas^{lpr} mice. These results first document mitogenic effects of self-DNA in SLE, a previously unknown disease pathomechanism, which is mediated by DNA-induced expression of the cell cycle regulator Mdm2. Our data suggest that MDM2 inhibition could be novel therapeutic approach for SLE and lupus nephritis.

RESULTS

Cytosolic DNA Triggers the Expression and Activation of Mdm2

We have recently reported that cytosolic uptake of RNA and DNA activates mesangial cells to express an almost identical transcriptome. However, DNA but not RNA induced mesan-

gial cell proliferation¹⁰; therefore, we carefully analyzed those few genes that were specifically induced by DNA. Among those were several cell cycle-regulated genes of which Mdm2 was most strongly induced only by cytosolic DNA and not by pattern recognition receptor (PRR) agonists such as poly I: poly C (pI:C) RNA (TLR3), bacterial lipoprotein (TLR2), HMGB1 (TLR2/4), and MDP (NOD1) (Figure 1, A and B). First, we compared the capacity of different immunostimulatory RNA and DNA formats to induce Mdm2 including DNA isolated from calf thymus and from late apoptotic murine T cells. All RNA and DNA formats induced IL-6 and Cxcl10 mRNA in NIH3T3 fibroblasts (Figure 1C). However, only DNA (but not RNA or lipopolysaccharide) significantly induced Mdm2 mRNA in a time- and dose-dependent manner (Figure 1C and Supplemental Figure 1). This effect was DNA-specific because DNase digestion completely prevented Mdm2 and cytokine mRNA induction by synthetic dsDNA (Figure 1D). Cytosolic DNA also induced Mdm2 protein as well as Ser-166 phosphorylation of Mdm2 (Supplemental Figure 2). Together, cytosolic DNA including necrotic cell-derived DNA triggers the expression and activation of Mdm2.

Disease Progression in MRL-Fas^{lpr} Mice Correlates with Increased Mdm2 Expression and Activation

MRL-Fas^{lpr} mice represent a suitable model for lupus-like systemic autoimmunity, and they also display the typical renal manifestations of diffuse proliferative lupus nephritis.²⁰ Similar to human SLE, in MRL-Fas^{lpr} mice develop splenomegaly because of splenocyte proliferation (Figure 2, A and B). Therefore, we first investigated Mdm2 expression in spleens of age-matched in MRL-Fas^{lpr} and MRL control mice. Disease progression in MRL-Fas^{lpr} mice was associated with increased Mdm2 mRNA over time compared with their age-matched MRL controls (Figure 2C). This finding was mirrored by Mdm2 protein expression in spleen as well as by an increased phosphorylation of Mdm2 at Ser-166, described to indicate Mdm2 activation in cancer cells, which was not found age-matched MRL controls (Figure 2D and Supplemental Figure 3A). However, p53 and p53-regulated genes such as ubiquitin D (Ubd) and Cdc25a were also increased in MRL-Fas^{lpr} mice (Figure 2D and Supplemental Figure 3B). Renal Mdm2 expression also increased, mainly due to glomerular Mdm2 expression, e.g., in podocytes (Figure 2E). Together, disease progression in MRL-Fas^{lpr} mice is associated with increased spleen and renal Mdm2 expression.

Mdm2 Blockade Prevents Lupus Nephritis and Prolongs Survival of in MRL-Fas^{lpr} Mice

To determine the functional role of Mdm2 in SLE and lupus nephritis we treated 12-week-old in MRL-Fas^{lpr} mice with repetitive intraperitoneal injections of either vehicle or nutlin-3a for 4 weeks. Nutlin-3a is a small molecule that disrupts Mdm2–p53 interactions and protects p53 from Mdm2 ubiquitination.²¹ Remarkably, 4 weeks of Mdm2 blockade prolonged the median survival of in MRL-Fas^{lpr} mice by 10

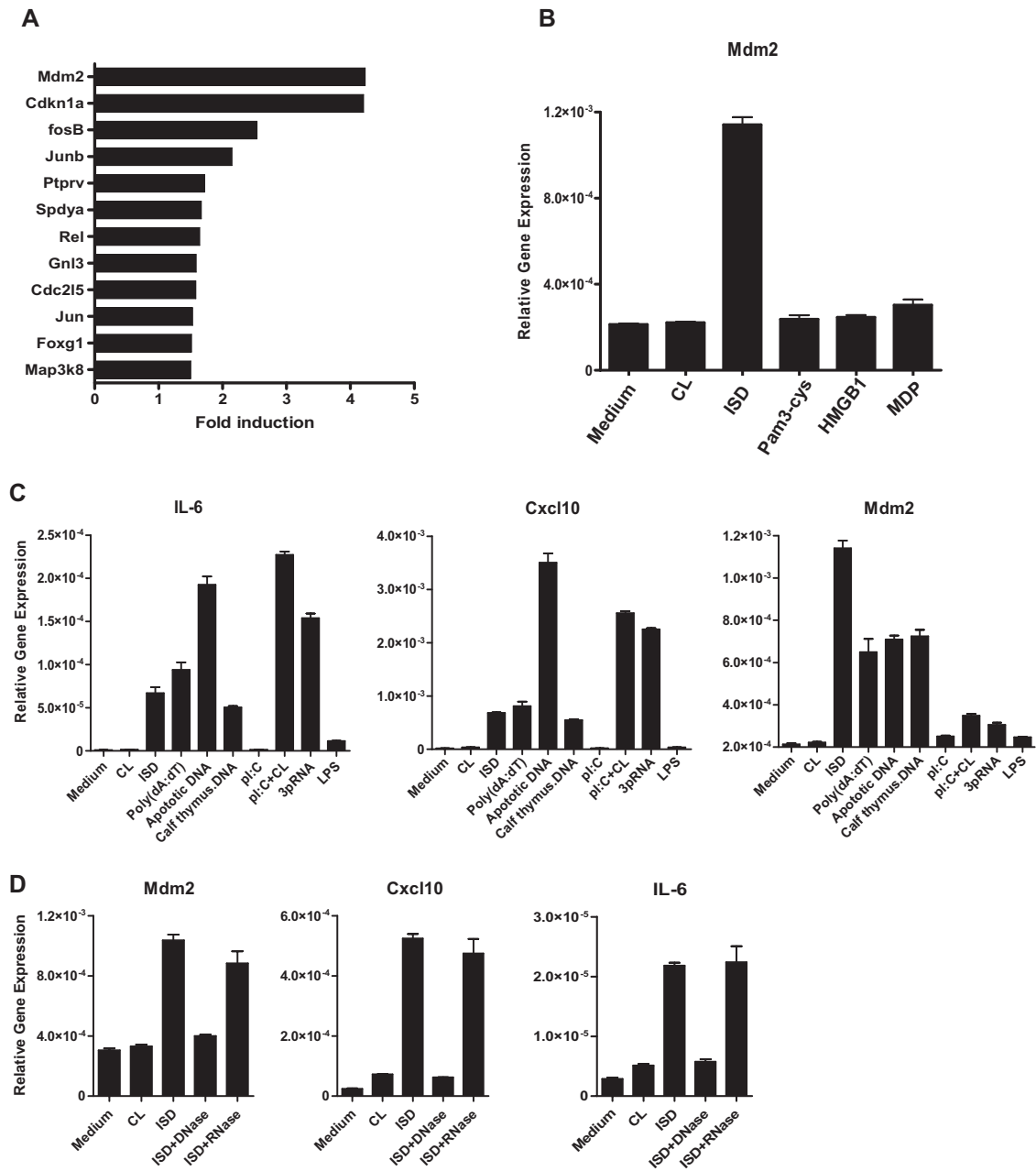


Figure 1. Cytosolic DNA induces Mdm2 expression. (A) Primary mouse mesangial cells were stimulated with 0.5 μg of 5'-triphosphate RNA (3pRNA)/cationic lipid (CL) and 30 μg of immunostimulatory DNA (ISD)/CL. Medium/CL-treated cells were used as control. After 6 hours 6 μg of total RNA from three independent preparations in each group was used for gene array analysis as described.¹⁰ The complete dataset was deposited into the GEO database (<http://www.ncbi.nlm.nih.gov/geo>; submission #GSE11898). Differentially expressed probe sets between controls and 3p-RNA/CL or DNA/CL were computed using the Microsoft Excel plug-in of SAM, version 1.21. The figure shows the genes that are being induced by cytosolic DNA but not by RNA. (B and C) NIH3T3 cells were stimulated with various formats of immunostimulatory nucleic acids complexed with or without CLs (1:1), which enhance their cytosolic uptake and other molecular patterns: double-stranded ISD (10 $\mu\text{g}/\text{ml}$); poly(dA-dT)·poly(dT-dA) [poly(dA:dT)] (6 $\mu\text{g}/\text{ml}$); apoptotic DNA (10 $\mu\text{g}/\text{ml}$); calf thymus DNA (6 $\mu\text{g}/\text{ml}$); poly I:poly C (pl:C) (5 $\mu\text{g}/\text{ml}$); double-stranded pl:C+CL RNA (5 $\mu\text{g}/\text{ml}$); 3pRNA (3 $\mu\text{g}/\text{ml}$); lipopolysaccharide (LPS) (4 $\mu\text{g}/\text{ml}$); pam3-cys (4 $\mu\text{g}/\text{ml}$); HMGB1 (0.5 $\mu\text{g}/\text{ml}$); and MDP (3 $\mu\text{g}/\text{ml}$). Total RNA was isolated after 6 hours, and mRNA expression was quantified by RT-PCR. (D) NIH3T3 cells were stimulated with 10 μg of double-stranded ISD complexed with CL that had been preincubated with either medium, DNase or RNase, as described in Concise Methods. Total RNA was isolated after 6 hours, and mRNA expression was quantified by RT-PCR. Data represent mean ratios of target mRNA to the respective 18S rRNA \pm SD.

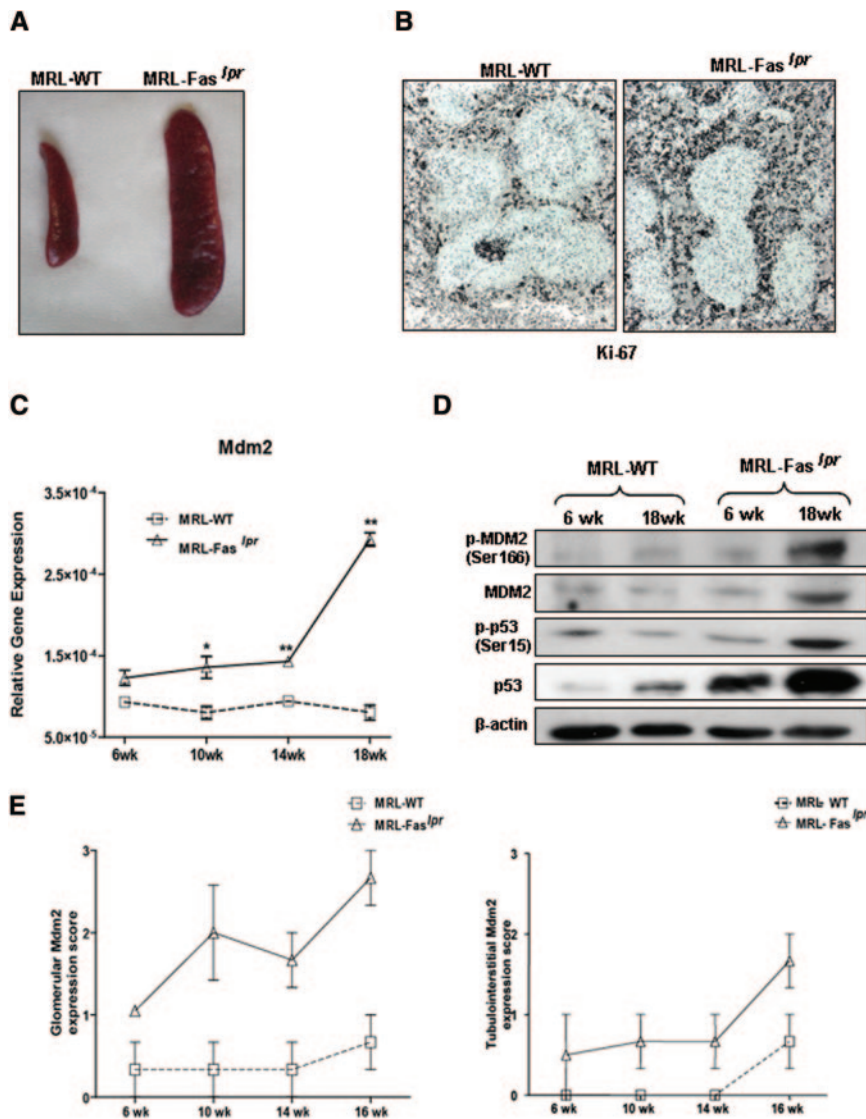


Figure 2. Mdm2 induction and activation in lupus mice. Fas deficiency in MRL mice is associated with significant splenomegaly (A) and lymphocyte proliferation, as indicated by positivity of parafollicular lymphocytes for the proliferation marker Ki-67 (B). Original magnification, $\times 100$. Images are representative for 12 mice in each group. (C) Spleen Mdm2 mRNA levels were quantified by RT-PCR in MRL wild-type (WT) and MRL-Fas^{lpr} mice at various time points as indicated ($n = 4$ for each genotype per time point). * $P < 0.05$, ** $P < 0.01$ versus age-matched WT mice. (D) Immunoblotting for spleen protein extracts of mentioned groups were performed using specific antibodies for Mdm2, Ser-166 phosphorylation of Mdm2, p53, Ser-15 phospho p53, and β -actin. (E) Semi-quantitative assessment of Mdm2 protein expression in glomerular and tubulointerstitial sections of kidney stained for Mdm2. Mdm2 staining was scored from 0 to 3 per 15 glomeruli or per 15 high-power fields.

weeks (Figure 3A). In a second cross-sectional study we obtained urine, plasma, and tissues from vehicle- and nutlin-3a-treated MRL-Fas^{lpr} mice at 16 weeks of age. A significant reduction of the urinary albumin-creatinine ratio indicated a protective effect of Mdm2 blockade on lupus nephritis (Figure 3B). In fact, Mdm2 blockade prevented the diffuse proliferative lupus nephritis of in MRL-Fas^{lpr}

mice as evidenced by a reduction of the activity and chronicity indices, normally used for scoring of human lupus nephritis (Figures 3, C and D). Mdm2 blockade also significantly reduced autoimmune peribronchitis in lungs (Figures 3, C and D). Together, Mdm2 blockade prevents lupus nephritis and prolongs survival of in MRL-Fas^{lpr} mice.

Mdm2 Blockade Suppresses the Expansion of Plasma Cells and Autoantibody Production in MRL-Fas^{lpr} Mice

Nutlin-3a treatment-related protection from lupus nephritis was associated with a significant reduction of glomerular IgG deposits, suggesting that nutlin-3a also affected systemic immune complex disease (Figure 3, C and D). SLE-related immune complex disease largely depends on the hyperactivation of plasma cells and lupus autoantibody production; hence, we tested this aspect of systemic autoimmunity in vehicle- and nutlin-3a-treated MRL-Fas^{lpr} mice. Mdm2 blockade with nutlin-3a reduced plasma IgG levels including all IgG isotypes except IgG3 (Figure 4A). Nutlin-3a also reduced plasma dsDNA autoantibodies as quantified by ELISA (Figure 4B). The latter was validated by *Crithidia luciliae* kinetoplast staining, which was almost absent in nutlin-3a-treated MRL-Fas^{lpr} mice at a plasma dilution of 1:20 (Figure 4C). In addition, plasma from vehicle-treated MRL-Fas^{lpr} mice displayed a diffuse nucleoplasmic staining pattern of interphase Hep2 cells, *i.e.*, characteristic of dsDNA autoantibodies (Figure 4C). This staining pattern was no longer observed with plasma from nutlin-3a-treated MRL-Fas^{lpr} mice so that rim-like and coarse granular nucleoplasmic staining patterns became visible. These are characteristic for antibodies directed against lamin-B along the nuclear membrane²² or against the spliceosome, *i.e.*, U1snRNP and Smith antigen, respectively

(Figure 4C). In addition, splenocyte flow cytometry revealed that Mdm2 blockade specifically reduced CD138⁺ κ light chain⁺ plasma cells (Figure 4D). To dissect this effect on long- and short-lived plasma cells, some MRL-Fas^{lpr} mice were injected with bromodeoxyuridine (BrdU) 1 week before they were killed.²³ Mdm2 blockade reduced both the BrdU negative long-lived and the BrdU positive short-lived plasma cells (Fig-

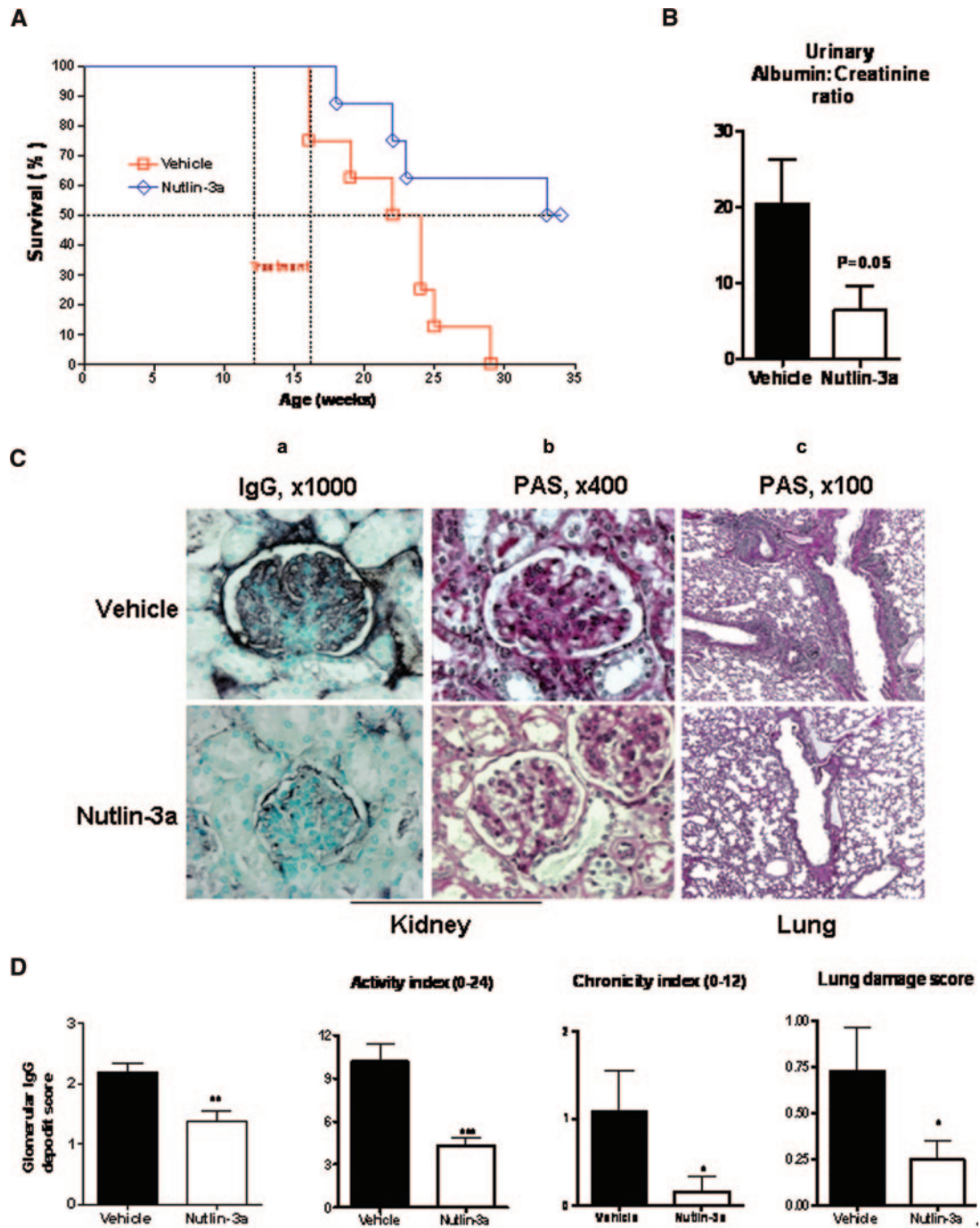


Figure 3. Mdm2 blockade reduces kidney as well as lung injury and mortality in experimental lupus. (A) Survival was shown as Kaplan-Meier curve, and the treatment period is indicated by dotted lines. Note that 4 weeks of Mdm2 blockade extended median survival by 10 weeks. $n = 8$ mice in each group. (B) Albuminuria, a functional parameter of lupus nephritis, was quantified in urine samples from at least 12 mice in each group. The ratio of urinary albumin per respective creatinine excretion was calculated to correct for the variability of urine concentration by different fluid intake as done in human urine analysis. (C) Renal (a and b) and lung (c) sections were stained with PAS or IgG as indicated. Images represent at least 12 mice in each group. Note that nutlin-3a reduced glomerular IgG staining (a), diffuse proliferative lupus nephritis (b), and peribronchial inflammation (c) in MRL-Fas^{lpr} mice. (D) Lupus nephritis activity and chronicity scores were assessed on Periodic acid-Schiff (PAS)-stained renal and lung sections as described in Concise Methods (score ranged from 0 to 24 and 0 to 12, respectively). Data represent means \pm SD from at least 12 mice in each group. * $P < 0.05$ versus vehicle-treated MRL-Fas^{lpr} mice; ** $P < 0.01$; *** $P < 0.001$.

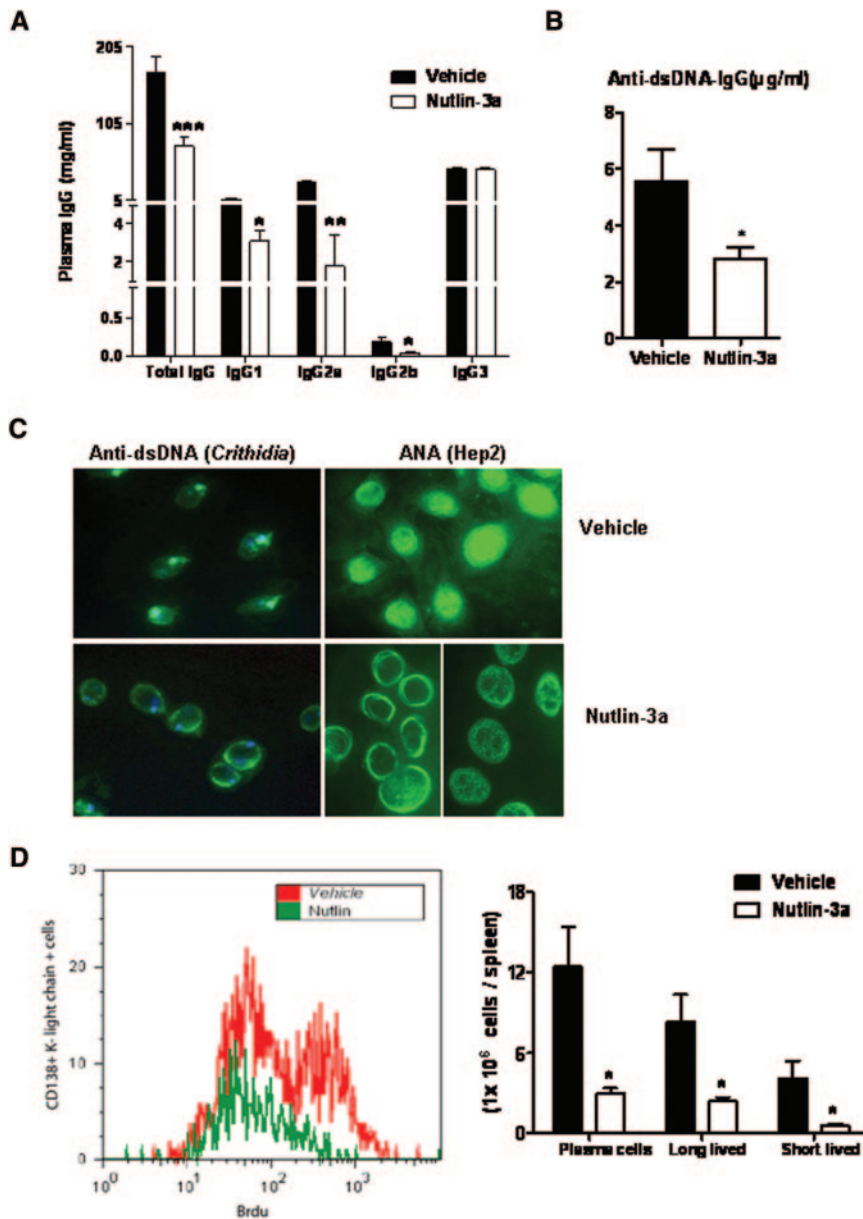


Figure 4. Mdm2 blockade affects autoantibodies in experimental lupus. (A) Plasma levels of total IgG or respective IgG isotype antibodies were quantified by ELISA. Data are means of at least 12 mice in each group \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus vehicle-treated MRL-Fas^{lpr} mice. (B) Plasma anti-double-stranded DNA (dsDNA) IgG was quantified by ELISA from at least 12 mice in each group. (C) The specificity of anti-dsDNA IgG was confirmed by positive kinetoplast staining (in green) of *Crithidia luciliae*, evident by costaining with nuclear DAPI signals (in blue). Note that nutlin-3a reduced the green IgG (but not the blue DAPI) signal (left panel). Hep2 cell staining (right panel) identified antinuclear antibodies with different patterns in both of the groups (see text for details). Images are representative for at least 12 mice in each group. (D) Splenocyte plasma cell subsets in vehicle- or nutlin-3a-treated MRL-Fas^{lpr} mice were quantified by flow cytometry. BrdU uptake was used to discriminate long- and short-lived forms of CD138⁺ κ light chain⁺ plasma cells in spleen. Histogram profiles of bromodeoxyuridine (BrdU) intensity of gated CD138⁺ κ light chain⁺ plasma cells (left) and the quantitative BrdU plasma cell data (right). Data are means \pm SD from at least six mice in each group. * $P < 0.05$, ** $P < 0.01$ versus vehicle-treated MRL-Fas^{lpr} mice. $n = 6$.

ure 4D), indicating that Mdm2 is a nonredundant factor for plasma cell proliferation in SLE. Together, Mdm2 blockade protects from lupus nephritis because it suppresses the hyperproliferation of plasma cells, lupus autoantibody production, and lupus-like immune complex disease of the kidney.

Mdm2 Blockade Also Depletes CD4/CD8 Double-Negative T Cells without Causing Myelosuppression

The profound effect of Mdm2 blockade on plasma cell proliferation raises the question whether these antiproliferative effects are specific or rather unspecific, the latter involving the risk for B and T cell ablation and diffuse myelosuppression. We used flow cytometry to determine which splenocyte populations were affected by Mdm2 blockade. Nutlin-3a had no effect on B220⁺ IgD⁺ IgM⁺ mature B cells, B220⁺ CD21^{low} CD23^{high} follicular B cells, or B220⁺ CD21^{high} CD23^{low} marginal zone B cells, but it reduced CD3⁺ CD4⁺, CD3⁺ CD8⁺, and CD3⁺/CD4⁻ CD8⁻ double-negative T cells (Table 1). This nutlin-3a effect was sufficient to substantially reduce the spleen weight size and showed a trend toward lower weights of mesenteric lymph nodes, documenting that Mdm2 drives lymphoproliferation in SLE (Supplemental Figure 4, A and B). However, it is of note that Mdm2 blockade did not affect the numbers of CD4⁺/CD25⁺/Foxp3⁺ “regulatory” T cells, CD11c⁺ dendritic cells, monocyte/macrophages, and neutrophils (Table 1). Furthermore, nutlin-3a treatment did not affect the total number of cells in femur bone marrow or the numbers of bone marrow monocyte/macrophages, neutrophils, and plasma cells as well as white and red cell counts in peripheral blood (Table 1). Thus, Mdm2 blockade prevents from lupus nephritis by suppressing the expansion of CD3⁺/CD4⁻ CD8⁻ double-negative T cells and plasma cells in lymphoid organs, but it does not affect myelo- or hematopoiesis.

Necrotic Cells Induce Mdm2 Expression

So far our data show that Mdm2 induction drives lymphoproliferation and autoimmunity in SLE from which lupus ne-

Table 1. Different cell population in vehicle- and nutlin-3a-treated MRL-Fas^{lpr} mice

	Vehicle	Nutlin-3a
Spleen (cells/million)		
Total number of cells	460.61 ± 57.15	291.21 ± 43.55 ^a
Marginal zone B cells	10.34 ± 4.27	10.57 ± 2.31
Follicular B cells	20.32 ± 7.79	19.45 ± 1.66
Mature B cells	37.34 ± 12.40	38.42 ± 7.86
Monocytes	0.97 ± 0.31	0.83 ± 0.12
Neutrophils	0.94 ± 0.28	0.51 ± 0.14
CD3 ⁺ CD4 ⁺ cells	10.59 ± 3.10	4.60 ± 0.70 ^a
CD3 ⁺ CD8 ⁺ cells	2.16 ± 0.45	0.90 ± 0.11 ^a
CD3 ⁺ CD4 ⁻ CD8 ⁻ cells	19.19 ± 3.29	6.27 ± 2.43 ^a
Regulatory T Cells	11.55 ± 2.87	9.13 ± 0.85
Dendritic cells	19.15 ± 4.03	12.54 ± 3.56
Macrophages	18.27 ± 3.43	16.10 ± 1.94
Bone marrow (cells/million) in femur		
Total number of cells	61.84 ± 9.77	83.25 ± 8.29
Monocytes	5.25 ± 1.20	9.95 ± 1.11
Neutrophils	35.02 ± 5.46	49.76 ± 3.59
Plasma cells	0.26 ± 0.10	0.28 ± 0.03
Short-lived plasma cells	0.11 ± 0.03	0.09 ± 0.02
Long-lived plasma cells	0.14 ± 0.06	0.19 ± 0.01
Blood		
Neutrophils (cells/ μ l)	2096.27 ± 221.38	2123.6 ± 389.12
Lymphocytes (cells/ μ l)	4029.72 ± 872.10	4176.8 ± 1103.5
Monocytes (cells/ μ l)	427.9 ± 110.1	462.08 ± 164.3
Eosinophils (cells/ μ l)	54.27 ± 14.7	28.25 ± 9.8
Leukocytes (10 ⁹ cells/L)	6.37 ± 1.1	6.79 ± 1.4
Erythrocytes (10 ¹² cells/L)	9.18 ± 0.15	8.96 ± 0.23
Thrombocytes (10 ⁹ cells/L)	954 ± 63.8	1187 ± 82.1

n = 6 per group; data are means ± SEM; ^a*P* < 0.05 versus vehicle.

phritis develops. However, what is the source of immunostimulatory DNA in MRL-Fas^{lpr} mice? Dead cell clearance defects and increased levels of extracellular nuclear autoantigen are a hallmark of systemic lupus^{24,25} and drive disease activity, *e.g.*, via the immunostimulatory effects of endogenous nucleic acids.^{4,5} In MRL-Fas^{lpr} mice necrotic (propidium iodide positive) cells accumulate in spleens compared with their wild-type counter parts (Figure 5A). This phenomenon associates with increased mRNA levels of IFN- β and IFN- γ in the same organ (Figure 5B). Therefore, we speculated that necrotic cell-derived DNA can induce the expression of Mdm2. To test this, we exposed necrotic supernatants to cultured NIH3T3 fibroblast cells and measured Mdm2 expression. Interestingly necrotic cell supernatants induced Mdm2 expression along with inflammatory molecules such as Cxcl10 and IL-6, all of which were significantly reduced by DNase digestion of the necrotic cell supernatants (Figure 5C). Immunostimulatory DNA (but not RNA) increased cell proliferation, which was inhibited by nutlin-3a (Figure 5D). In support of these *in vitro* results, Mdm2 blockade significantly reduced the amount of propidium iodide-positive “secondary necrotic” cells in spleens of MRL-Fas^{lpr} mice and increased the number of annexin

V-positive “apoptotic” cells (Figure 6A), suggesting that the cell cycle inhibitory and proapoptotic effect of nutlin-3a not only inhibits splenocyte proliferation but also secondary splenocyte necrosis as a stimulus for DNA-mediated Mdm2 induction. Less splenocyte necrosis with nutlin-3a treatment was also associated lower spleen mRNA expression levels of TNF, IFN- β and IFN- γ , which mirrors the reduction of the respective serum levels of these factors in the plasma of nutlin-3a-treated MRL-Fas^{lpr} mice (Figure 6, B and C). Together, MRL-Fas^{lpr} mice secondary-necrotic splenocytes releases DNA, which induced Mdm2. Mdm2 drives splenocyte proliferation as well as additional splenocyte necrosis. Nutlin-3a blocks both mechanisms, which reduced systemic inflammation as well as lymphoproliferation, autoimmunity, and lupus nephritis.

DISCUSSION

Our experiments identify a previously unknown pathogenic effect of self-DNA in lupus nephritis, *i.e.*, a mitogenic effect that is specifically mediated via the induction of Mdm2. So far the pathogenic role of self DNA for lupus nephritis was referred to its functional properties as an autoantigen as well as an adjuvant.^{2,5} Recently, it was concluded that lupus develops because genetic variants of dead cell clearance allow the exposure of potential nuclear autoantigens to the immune system where they are—by mistake—taken as viral particles, elicit adjuvant effects, and thereby trigger complex pseudoantiviral immune responses.⁴ The mitogenic effect of self-DNA, which we describe here, adds another DNA-related pathomechanism and identifies Mdm2 as a novel target gene in lupus nephritis.

The current pathogenic concept of lymphoproliferation in SLE is based on the antigenic properties of nucleosomes and ribonucleoproteins released from dying cells, which drive the expansion of epitope-specific autoreactive lymphocytes.²⁵ However, epitope spreading and polyclonal autoimmunity suggest additional mechanisms of lymphoproliferation in SLE.^{1,25} We found splenocyte necrosis to be associated with splenocyte proliferation, both correlated with the induction and activation of Mdm2. These findings differ from a previous report describing that DNA breaks induced by radiation inhibit Mdm2 and promote p53-dependent cell death.²⁶ The causal role of Mdm2 in the relationship of splenocyte necrosis and proliferation was documented by Mdm2 inhibition, which reduced splenocyte necrosis and increased “immunologically silent” apoptotic cell death. We assume that Mdm2 inhibition disrupted the vicious cycle for additional release of immunostimulatory molecules.²⁷ The antiproliferative effect of nutlin-3a affected CD4/CD8 double-negative T cells and plasma cells but not B cells in spleen or myelo- and hematopoiesis in the bone marrow. The decline in B220+ cells in spleen might also result from a reduction of natural killer (NK) cells,

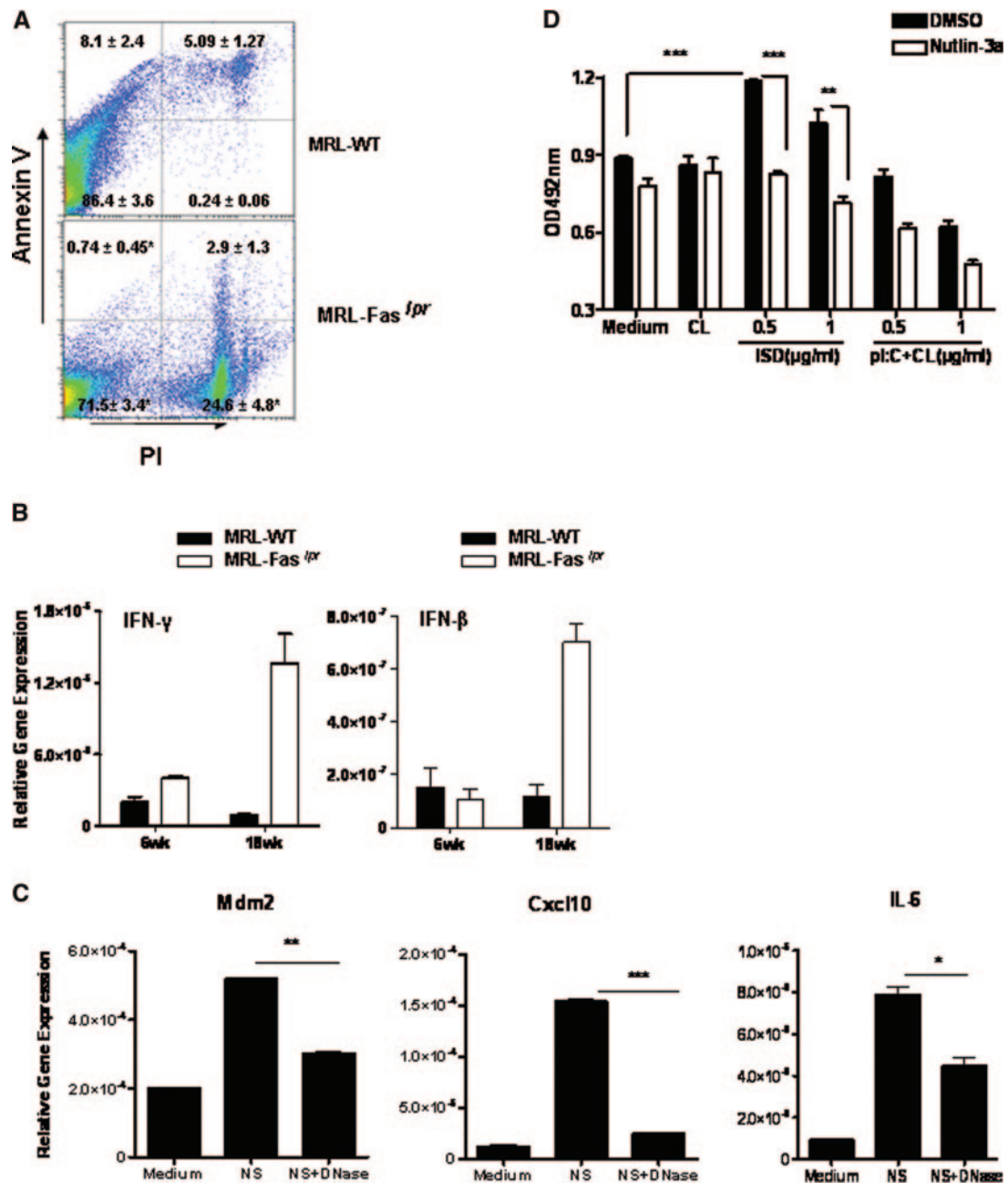


Figure 5. DNA content of necrotic cells induced Mdm2 expression. (A) The graph shows annexin V- and/or propidium iodide (PI)-positive splenocytes from 18-week-old MRL-wild-type (WT) and MRL-Fas^{Ipr} mice that were quantified by flow cytometry and are shown in representative dot blots ($n = 4$ mice). (B) IFN- γ and IFN- β expression in spleens of MRL-Fas^{Ipr} mice and MRL-wild-type (WT) mice. Data represent in mean ratios of target mRNA to the respective 18S rRNA \pm SD. (C) NIH3T3 cells were stimulated with necrotic cell supernatants (NS) complexed with cationic lipids (CLs) and DNase-treated necrotic supernatants (NS+DNase). Total RNA was isolated after 6 hours, and mRNA expression was quantified by RT-PCR. (D) Nutlin-3a-treated or untreated NIH3T3 cells were stimulated with double-stranded immunostimulatory DNA (ISD) complexed with CL or double-stranded poly I:poly C (pI:C) RNA complexed with CL (Lipofectamine 2000 1:1) at the indicated concentrations for 48 hours, and cell proliferation was quantified with 96 Aqueous One Solution Cell Proliferation Assay. The data represent the mean \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, t test, *** $P < 0.001$.

which we did not specifically quantify in our analysis. Bone marrow long-lived plasma cells that assure the humoral component of immune memory were also not affected. The latter could be explained by low numbers of necrotic cells in

bone marrow and the fact that the nutlin-3a concentrations reached in bone marrow are much lower compared with other organs.²⁸ The specific effect of Mdm2 inhibition on lymphocyte expansion and the reduction of autoantibodies

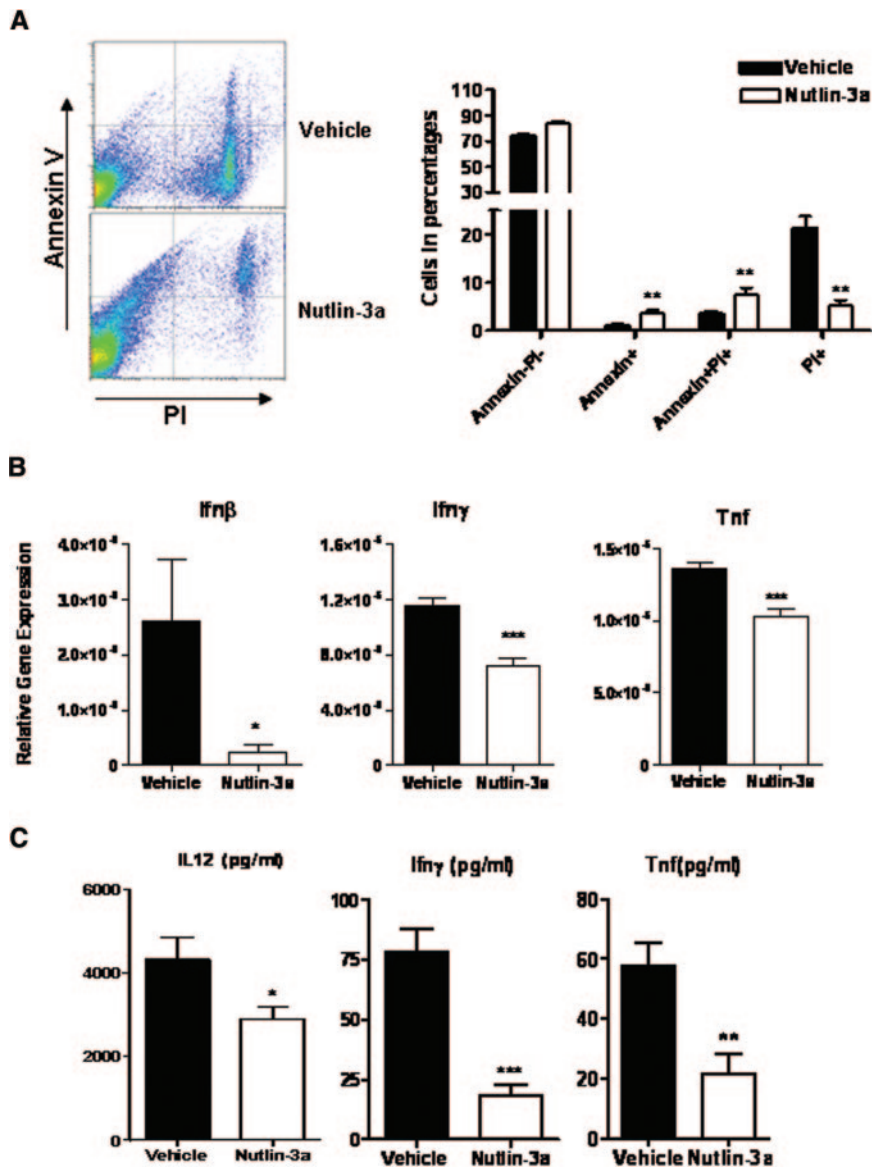


Figure 6. Nutlin-3a reduced necrotic cells in MRL-Fas^{lpr} mice. (A) Annexin V and/or propidium iodide (PI)-positive splenocytes from 16-week-old vehicle- or nutlin-3a-treated MRL-Fas^{lpr} mice were quantified by flow cytometry and are shown in representative dot blots (left) and as a histogram (right). (B) Twelve-week-old MRL-Fas^{lpr} mice were treated either with vehicle or nutlin-3a for 4 weeks, and spleen mRNA levels of IFN-β, IFN-γ, and TNF were quantified by RT-PCR. Data represent mean ratios of cytokine mRNA/18s rRNA ± SD from at least 12 mice in each group. (C) Plasma levels of IL-12, IFN-γ, and TNF were measured by ELISA. Data are means of at least 12 mice in each group ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 versus vehicle-treated MRL-Fas^{lpr} mice.

indicate the therapeutic potential of Mdm2 inhibitors in SLE without systemic immunosuppression. Consistent with many other interventional lupus nephritis studies the reduction of autoreactive lymphocytes correlated with reduced production of immune complex disease and lupus nephritis. Finally, the functional role of Mdm2 induction in lupus was documented by a significant prolongation of overall life span of MRL-Fas^{lpr} mice, although few mice in

the nutlin-treated group died and the underlying cause could not be determined. However, the survival benefit of nutlin-3a-treated over untreated MRL-Fas^{lpr} mice exceeded the treatment period three times; hence, we conclude that Mdm2 blockade has a profound effect on the phenotype of MRL-Fas^{lpr} mice.

The finding that necrotic cell-derived DNA induces cell proliferation via Mdm2 induction could represent a basic physiologic mechanism to trigger tissue regeneration after necrotic cell injury.²⁹ This mechanism would be in contrast to the homeostatic process of apoptotic cell death, which avoids DNA release and which is not associated with cell proliferation.³⁰ However, Mdm2-induced cell proliferation is already well known to turn into a maladaptive pathomechanism, *i.e.*, in cancer malignancy. A broad literature describes the pathogenic role of Mdm2-mediated p53 stabilization for an uncontrolled proliferation of malignant cells.^{17,18} It is of note that several DNA (not RNA) viruses have the potential for an oncogenic transformation of infected cells, such as Epstein-Barr virus and Kaposi's sarcoma herpesvirus for B cell lymphoma, a process that can also be prevented by Mdm2 inhibition.^{19,31,32} In fact, the central role of Mdm2 in cancer served as the rationale for the development of Mdm2 inhibitors, which were proven to be effective in inducing cancer cell apoptosis.^{21,33–35} Hence, nutlin-3a is currently in clinical trials for the treatment of various cancers.^{34,35}

Our data document an inappropriate induction of Mdm2 in murine SLE, which contributes to the autoimmune polyclonal lymphoproliferation, similar to virus-induced lymphoma. SLE may therefore represent a second example how DNA-induced Mdm2 and subsequent cell proliferation can turn into a maladaptive pathomechanism. This process would be similar to the molecular mimicry of self and viral nucleic acids at the level of the Toll-like receptors by which nuclear particles induce those autoadjuvant effects that drive the presentation of lupus autoantigens.^{5,6,36} In addition, it might be possible that persistent DNA virus replication, *e.g.*, Epstein-Barr virus, serves as a second source of mitogenic DNA driving lymphoproliferation in lupus as it has been proven for Epstein-Barr virus-induced

lymphomas.^{19,36} Furthermore, it is intriguing to speculate that Mdm2 induction is also responsible for the recently described phenomenon of TLR7/9-mediated expansion and steroid resistance of plasmacytoid dendritic cells in lupus.³⁷

Together, the progression of SLE is associated with Mdm2 induction, most likely triggered by necrotic cell-released DNA. Mdm2 induction promotes the abnormal polyclonal expansion of autoreactive lymphocytes, which causes autoantibody production and immune complex disease in murine lupus. We conclude that the mitogenic effect of self-DNA via Mdm2 induction is a previously unknown pathomechanism in SLE and lupus nephritis. Hence, Mdm2 inhibition might be suitable to suppress lupus nephritis without myelosuppressive side effects.

CONCISE METHODS

Animal Experiments

Six-week-old female MRL-Fas^{lpr} mice from Harlan Winkelmann (Borchen, Germany). At 12 weeks of age the mice received intraperitoneal injections every other day with either the Mdm2 antagonist nutlin-3a (Alexis Biochemicals, San Diego, CA) at a dose of 20 mg/kg body wt in DMSO or vehicle only for 4 weeks. All experimental procedures were performed according to the German animal care and ethics legislation and had been approved by the local government authorities. All mice were killed by cervical dislocation at the end of the 16th week of age, and tissues, urine, and plasma were harvested for a comparative analysis. In a second identical experiment survival was assessed beyond the aforementioned treatment interval.

In Vitro Studies

Primary mesangial cells were cultured as described previously.¹⁰ NIH3T3 cell line obtained from ATCC (American Type Culture Collection). Cells were maintained in Dulbecco's modified Eagle's (DME) medium (Biochrom KG, Berlin, Germany) supplemented with 10% fetal bovine serum, penicillin 100 U/ml, and streptomycin 100 µg/ml at 37°C in a humidified incubator containing 5% CO₂. Necrotic cell supernatants were prepared from EL4 cells either by repeated freezing and thawing or staurosporine treatment for 24 hours.³⁸ In some experiments necrotic soup was treated with DNase (Qiagen) for 1 hour at 37°C. Apoptotic DNA was prepared from staurosporine-treated EL4 cells for 12 hours, and DNA was isolated with apoptotic DNA ladder kit (Roche, Mannheim, Germany). We purchased calf thymus DNA, pA:dT [poly(dA-dT)·poly(dT-dA)] sodium salt (Sigma-Aldrich) pI:C RNA, Pam3Cys, MDP (Invivogen), and HMGB1 protein (Novus Biologicals). Immunostimulatory DNA and 5'-triphosphate RNA were prepared as described previously.¹¹ Cell proliferation was determined using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI). Briefly, NIH3TC cells were grown in 96-well plates for 24 hours before stimulation with immunostimulatory DNA and pI:C + cationic lipid (CL). Cells were treated with nutlin-3a (1 µM) before 1 hour of stimulation. After 72 hours

the solution was added, and cells were incubated at 37°C in 5% CO₂ for 2 hours before absorbance was determined at a wavelength of 492 nm.

Microarray Studies

RNA was prepared from murine primary mesangial cells that had been stimulated with 0.5 µg of 3P-RNA/CL and 30 µg of non-CpG DNA/CL for 6 hours as described.¹⁰ Biotin-labeled cRNA samples were hybridized with MOE 430Av2 arrays and analyzed using the Affymetrix GeneChip Operating software (GCOS1.0). Assay quality and normalization was performed, and probe set signals were logarithmized to base 2 as described.¹⁰ To exclude probe sets in the lower end of the signal range, which have large signal variation, a background filter cutoff value was defined as the maximal signal value obtained from nonhuman Affymetrix-control probe sets multiplied by a factor of 1.2. Probe sets with a signal below cutoff in every array of the corresponding comparison as well as Affymetrix control probe sets were excluded from the analysis. The dataset was deposited into the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>; submission #GSE11898). Differentially expressed probe sets between controls and 3P-RNA/CL or non-CpG DNA/CL were computed using the Microsoft Excel plug-in of SAM, version 1.21.

Immunoblotting

Immunoblotting was performed for Mdm2 (Abcam, Cambridge, U.K.), Ser-166 Phospho Mdm2, p53, Ser-15 phospho p53, and β-actin (Cell Signaling Technology, Beverly, MA) as described previously.¹⁰

Flow Cytometric Analysis

We prepared single-cell suspensions from femoral bone marrow and spleen. We performed flow cytometric analyses of splenocytes and bone marrow cells using fluorochrome-conjugated antibodies to mouse CD3, CD4, CD8, CD25, CD21, CD23, B220, CD138, CD11c, CD40, IgD, IgM, 7/4 (neutrophils), and Ly6G (all purchased from BD Biosciences, Heidelberg, Germany) and F4/80 (Ebioscience, Frankfurt, Germany). To detect plasma cells and regulatory T cells, we performed, respectively, intracellular κ light chain (BD Biosciences, Heidelberg, Germany) and Foxp3 (BioLegend, San Diego, CA) staining with Fix&Perm Cell Permeabilization Kit (BD Biosciences). Respective isotype antibodies were used to demonstrate specific staining of cell subpopulations as described.³⁹ Cells were counted using Caltag counting beads (Invitrogen, Karlsruhe, Germany) on an FACS Calibur flow cytometer (BD Biosciences). For discrimination between short- and long-lived plasma cells, we injected the MRL-Fas^{lpr} mice with 1 mg BrdU intraperitoneally per mouse daily for 7 days before killing.⁴⁰ After surface antibody staining of splenocytes, we performed intracellular staining for κ light chain and incorporated BrdU with the BrdU-Flow-Kit (BD Biosciences) according to the manufacturer's instructions.

Enzyme-Linked Immunosorbent Assay

Murine IL-12p70, IFN-γ (both OptEiA, BD) and TNF (BioLegend, San Diego, CA) were measured by ELISA. Plasma antibodies IgG, IgG1, IgG2a, IgG2b, IgG3, and dsDNA IgG antibodies (Bethyl Labs,

Montgomery, TX) were also measured by ELISA as described previously.¹¹

RNA Analysis

RNA extraction and reverse transcription were performed as described previously.¹⁰ Quantitative RT-PCR analysis was performed with Light cycler 480 (Roche) and SYBR Green system. 18S rRNA was used as an internal control. The following SYBR Green forward (f) and reverse (r) oligonucleotide sequences were used in the study: 18SrRNA: 5'GCAATTATCCCCATGAACG3'(f), 5'AGGGCCTCACTAAACCATCC3'(r); Mdm2: 5'TGTGAAGGAGCACAGGAAAA3'(f), 5'TCCTTCAGATCACTCCCACC3'(r); IL-6: 5'TGATGCACTTGACAGAAAACA3'(f), 5'ACCAGAGGAAATTTCAATAGGC3'(r); Cxcl10: 5'CACCATGAA-TCAAAGTGCAG3'(f), 5'GCTGATGCAGGTACAGCGT3'(r); IFN β : 5'TCCCTATGGAGATGACGGAG3'(f), 5'ACCCAGTGCTGGAGAAATTG3'(r); TNF: 5'CCACCACGCTCTTCTGTCTAC3'(f), 5'AGG-GTCTGGGCCATAGAAGT3'(r); IFN γ : 5'ACAGCAAGGCGAAAAAGGAT3'(f), 5'TGAGCTCATTGAATGCTTGG3'(r); Ubd: 5'AAGCAGACACAAGGATCTTTTCTT3'(f), 5'AAGGTCATTA-ACCGCCATTG(r); and Cdc25a: 5'TTCAGAAGACCCAATG-GAGTG 3'(f), 5'CTGAATGGCGTTCATGTAC (r).

Autoantibody Assays

Diluted plasma, 1:20, was used to detect DNA antibodies with *C. luciliae* slides. The method was described previously.¹¹ Anti-nuclear antibody staining pattern was assessed by incubating plasma samples (1:20 dilutions) with Hep-2 slides as described previously.¹¹

Analysis of Tissue Injury

Spleen, lung, and kidney organs from all mice were fixed in 10% buffered formalin and embedded in paraffin. Sections at 2- μ m for periodic acid-Schiff (PAS) stains were prepared following routine protocols. The severity of the renal lesions was graded using the indices for activity and chronicity as described.¹¹ IgG immunostaining was performed on paraffin-embedded sections as described¹¹ using anti-mouse IgG (1:100, M32015, Caltag Laboratories, Burlingame, CA) and anti-Mdm2 (1:400, Abcam). Negative controls included incubation with a respective isotype antibody. Semiquantitative scoring of glomerular IgG or Mdm2 deposits from 0 to 3+ was performed on 15 cortical glomeruli. Tubulointerstitial Mdm2 staining was scored on 15 high-power fields, accordingly. Ki-67 staining was performed on these paraffin-embedded spleen sections. Measurement of urinary albumin and creatinine were done as described previously.¹¹

Evaluation of Peripheral Blood Cells

Peripheral blood was collected from the mice on the day they were killed. The numbers of monocytes, lymphocytes, eosinophils, leukocytes, erythrocytes, and thrombocytes were determined by an automatic cell analyzer (Synlab, Augsburg, Germany).

Statistical Analysis

Data were expressed as mean \pm SD. Comparison between two groups was performed by two-tailed *t* test. A value of *P* < 0.05 was considered to be statistically significant. Survival curves were compared by Kaplan-Meier analysis using log-rank two-tailed test. All statistical analyses were calculated using GraphPad Prism, 4.03 version.

ACKNOWLEDGMENTS

This work was funded by grants from the Deutsche Forschungsgemeinschaft (GRK1202 and AN372/12-1). We thank Bruno Luckow, University of Munich, Germany, for sharing NIH3T3 cells. We thank Ewa Radomska and Dan Draganovic for their expert technical assistance. The authors declare no competing financial interests.

DISCLOSURES

None.

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