Interleukin 6 (IL-6) Deficiency Delays Lupus Nephritis in MRL-*Fas*^{lpr} Mice: The IL-6 Pathway as a New Therapeutic Target in Treatment of Autoimmune Kidney Disease in Systemic Lupus Erythematosus

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ABSTRACT. ***Objective.*** To investigate the pathophysiological effect of interleukin 6 (IL-6) on lupus nephritis in MRL-*Fas*^{lpr} mice.

Methods. We generated IL-6-deficient MRL-*Fas*^{lpr} mice using a backcross/intercross breeding scheme. Renal pathology was evaluated using immunohistochemistry detection for macrophages, lymphocytes, vascular cell adhesion molecule-1 (VCAM-1), and TUNEL (terminal deoxynucleotide transferase-mediated dUTP nick end-labeling) for apoptotic cells, and renal IgG and C3 deposition by immunofluorescence staining. Expression of inflammatory markers in the spleen was analyzed by quantitative real-time reverse transcription-polymerase chain reaction. Serum cytokine concentrations were detected by FACS analysis.

Results. IL-6 deficiency was highly effective in prolonging survival and ameliorating the clinical, immunological, and histological indicators of murine systemic lupus erythematosus. During the study period of 6 months, MRL-*Fas*^{lpr} IL-6 –/– mice showed delayed onset of proteinuria and hematuria compared to IL-6-intact control mice. Survival rate was 100% in IL-6-deficient MRL-*Fas*^{lpr} mice and 25% in the control group at 6 months of age. The absence of IL-6 resulted in significant reduction of infiltrating macrophages in the kidney ($p < 0.05$), a decrease in renal IgG and C3 deposition, and a reduction of CD4+ and CD8+ lymphocytes. The parenchymal adhesion molecule VCAM-1 was found to be downregulated in kidneys of MRL-*Fas*^{lpr} IL-6 –/– compared to IL-6-intact mice. We found elevated serum levels of IL-10 and interferon-γ in IL-6-deficient mice, while splenic mRNA showed an overall downregulation of immunoregulatory genes.

Conclusion. IL-6 is a strong promoter of lupus nephritis and may be a promising new therapeutic target in the treatment of human lupus nephritis. (J Rheumatol First Release Dec 1 2009; doi:10.3899/ jrheum.090194)

Key Indexing Terms:
INTERLEUKIN 6 SYSTEMIC LUPUS ERYTHEMATOSUS
LUPUS NEPHRITIS KNOCKOUT MODEL MRL-*Fas*^{lpr}

The hallmark of systemic lupus erythematosus (SLE) includes inflammation in multiple organs and pathogenic autoantibodies. Renal disease is one of the most common and serious organ manifestations of SLE. Lupus nephritis is mediated by the infiltration of macrophages and lymphocytes and the deposition of autoantibodies in capillary walls. Advancing tubulointerstitial pathology and an increase in infiltrating leukocytes are unfavorable prognostic indicators[1]. Renal injury in MRL-*Fas*^{lpr} mice (formerly designated MRL-*lpr*) is determined by the MRL background genes. The MRL-1/1 strain develops latent, mild autoimmune renal injury. The interaction of the MRL gene

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background with single-gene mutation in *Fas* (MRL-*Fas*^{lpr}) converts this mild renal injury into a rapid and fulminant tissue-destructive process[2]. Renal pathology in MRL-*Fas*^{lpr} mice is complex; it involves interstitial, glomerular, tubular, and perivascular lesions and is mediated by antibody-dependent and cellular mechanisms[3]. Therefore, renal disease in MRL-*Fas*^{lpr} mice is a result of immune complexes and cytokine/growth factor-related events[4]. Kidney inflammation, which is evident at 3 months of age, is rapidly progressive, and by 5–8 months of age, it is fatal.

Interleukin 6 (IL-6) is an immunomodulatory pleiotropic cytokine with a wide range of biological activities. Andus, *et al* described IL-6 as the main activator of acute-phase response in liver cells[5], and Hirano, *et al* found IL-6 to be a B cell differentiation factor[6]. Further, it is a strong activator of macrophages, T cells, and B cells and also stimulates differentiation of T cells into cytotoxic T cells[7,8]. Together with IL-3, IL-6 has a synergistic influence on stimulating hemopoiesis. It also induces megakaryocyte differentiation and thus induces thrombopoiesis and activates neutrophilic granulocytes[9]. In addition, IL-6 influences osteoclast differentiation and hence can damage cartilage and bone, a phenomenon seen in rheumatoid arthritis[10]. In contrast to these proinflammatory effects, IL-6 is capable of reducing inflammatory cascades by inhibiting IL-1 and tumor necrosis factor-α (TNF-α) synthesis. Together with interferon-γ (IFN-γ), these 2 cytokines induce IL-6 synthesis, whereas it is inhibited by IL-4, IL-10, and IL-13[11]. A wide range of cells are capable of releasing IL-6, such as macrophages, B and T cells, monocytes, fibroblasts, keratinocytes, endothelial cells, and mesangial cells. In lupus nephritis, infiltrating inflammatory cells in the kidney, mainly macrophages and monocytes, are the main source of IL-6[12]. In an area of inflammation IL-6 stimulates leukocyte recruitment and monocyte/macrophage migration via the release of chemokines by endothelial cells[13]. High IL-6 concentrations are found in sera of SLE patients as well as in lupus mouse models. In addition to the high IL-6 serum levels, significant overexpression of IL-6 is found in SLE and other membranoproliferative nephritides, whereas healthy kidneys show little IL-6 expression[12]. Two studies demonstrate that IL-6 correlates with disease activity and might even be a useful biomarker of SLE[14,15]. Blocking IL-6 with anti-IL-6 antibodies was able to reduce kidney pathology in MRL-*Fas*^{lpr} and NZB/NZW mice[16,17], whereas mice with IL-6 overexpression develop mesangial proliferative glomerulonephritis[18]. A study of IL-6-deficient BALB/C mice in pristane-induced lupus, an alternative murine lupus model, revealed that autoantibody development in this lupus model is IL-6-dependent[19]. These findings led to our rationale of creating IL-6-deficient MRL-*Fas*^{lpr} mice and studying the effects on the development of lupus nephritis. No research regarding a mouse model with IL-6-deficient MRL-*Fas*^{lpr} mice has been published to date.

MATERIALS AND METHODS

Animals. MRL-*Fas*^{lpr} mice and IL-6 knockout BALB/C mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The mice were kept in a germ-free environment in the animal facility of the University of Mainz. We generated IL-6-deficient MRL-*Fas*^{lpr} mice (IL-6 –/– MRL-*Fas*^{lpr}) using a backcross-intercross scheme as described[20]. The progeny were screened by polymerase chain reaction (PCR) amplification of tail genomic DNA using primers for the *IL-6* wild-type gene (sense, 5'-TTC CAT CCA GTT GCC TTC TTG G -3'; antisense, 5'- TTC TCA TTT CCA CGA CGA TTT CCC -3') and *IL-6* deficiency (neomycin resistance insertion; sense, 5'-ATT GAA CAA GAT TTG GGA TTG CAC-3'; antisense, 5'-CGT CCA GAT CAT CCT GAT C-3'). Gel analysis identified the IL-6 and *neoR* gene fragments at 480 bp and 180 bp, respectively. We analyzed female B5 IL-6 –/– MRL-*Fas*^{lpr} mice, using female IL-6 +/– MRL-*Fas*^{lpr} littermate controls.

Proteinuria/hematuria, serum urea, and creatinine. The mice were tested for proteinuria and hematuria using albumin test strips (Albustix; Miles, Naperville, IL, USA) at weekly intervals starting at Week 11 (Proteinuria: 0, none; 0.5, 15 mg/dl; 1, 30 mg/dl; 1.5, 100 mg/dl; 2, 300 mg/dl; 2.5, > 2000 mg/dl. Hematuria: 0, none; 0.5, 5–10/µl; 1, 25/µl; 2, 80/µl; 3, 200/µl). Serum urea was measured using the commercial UREA/BUN kinetic UV-Test (cat. no. 1982486001V8; Roche, Mannheim, Germany). Serum creatinine was measured by the Institute of Clinical Chemistry, University Hospital of Mainz.

Histopathology. Kidneys were either frozen in OCT (Tissue Tek; Sakura, Zoeterwoude, Netherlands) for frozen sections or fixed in 10% neutral buffered formalin. Paraffin sections (4 µm) were stained with hematoxylin. We evaluated kidney pathology as described[21]. Glomerular pathology was assessed by scoring each glomerulus on a semiquantitative scale on glomerular cross-sections (gcs): 0 = normal (35–40 cells/gcs); 1 = mild; glomeruli with few lesions showing slight proliferative changes, mild hypercellularity (41–50 cells/gcs); 2 = moderate; glomeruli with moderate hypercellularity (51–60 cells/gcs), including segmental and/or diffuse proliferative changes, hyalinosis; and 3 = severe; glomeruli with segmental or global sclerosis and/or severe hypercellularity (> 60 cells/gcs), necrosis, and crescent formation. We scored 20 glomerular cross-sections per kidney.

Interstitial/tubular pathology was assessed semiquantitatively on a scale of 0–3 in 10 randomly selected high power fields. We determined the largest and average number of infiltrates and damaged tubules and adjusted the grading system accordingly: 0 = normal; 1 = mild; 2 = moderate; 3 = severe. Perivascular cell accumulation was determined semiquantitatively by scoring the number of cell layers surrounding the majority of vessel walls (0 = none; 1 = fewer than 5 cell layers; 2 = 5–10 cell layers; 3 = more than 10 cell layers). All scoring was performed on blinded slides.

Immunostaining. The phenotype of the renal infiltrating cells was analyzed by immunohistochemistry of frozen sections, as described[22]; macrophages by staining with F4/80 (MCAP497; Serotec, Martinsried, Germany), T cells by anti-CD4, anti-CD8, and B cells by anti-CD20 goat anti-mouse antibodies (SC-7735; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Vascular cell adhesion molecule-1 (VCAM-1) was detected by anti-VCAM/CD106 rat anti-mouse antibody (MCA1229; Serotec). The controls contained normal rat IgG, idiotypic controls, or rabbit serum as a substitute for the primary antibody[23]. The immunostaining was analyzed by dividing the kidney into 4 compartments (perivascular, interstitial, glomerular, periglomerular) and counting the number of F4/80, CD4-positive, CD8-positive, CD20-positive, and VCAM-1-positive cells in 10 randomly selected high power fields of each compartment.

Immunofluorescent evaluation of IgG and C3 deposits in kidney. For light microscopy of IgG deposits, renal tissue samples were fixed in 4% formaldehyde and embedded in paraffin. Sections (4 µm) were deparaffinized, rehydrated, and incubated overnight with rabbit anti-mouse IgG polyclonal antibody (end concentration 10 µg/ml; AbD Serotec, Duesseldorf, Germany). Slides were stained with FITC-conjugated goat anti-rabbit IgG secondary antibody (end concentration 10 µg/ml; AbD Serotec).

For light microscopy C3 deposits, kidneys were embedded in OCT compound and snap-frozen in liquid nitrogen. Sections (4 μm) were incubated overnight with rat anti-mouse C3 monoclonal antibody (end concentration 1 μg/ml; ABR, Golden, CA, USA) and stained with the FITC-conjugated goat anti-rat IgG secondary antibody (end concentration 15 μg/ml; Jackson Immuno Research, West Grove, PA, USA). Slides were counterstained with DAPI and were analyzed with a fluorescence microscope (Leica DMR, with a 3CCD color video camera). The extent of IgG and C3 precipitation was assessed by titrating the antibodies on serial tissue sections using dilution steps. Semiquantitative analysis was performed on at least 50 glomeruli using a 0–3 scoring system.

Identification of apoptotic cells in kidney. Apoptotic cells in kidney sections were identified by TUNEL method (terminal deoxynucleotide transferase–mediated dUTP nick end-labeling) and immunoperoxidase staining (In Situ Cell Death Detection Kit; Boehringer Mannheim, Indianapolis, IN, USA).

Cytokine and chemokine expression in spleen. Splenic RNA was isolated from two IL-6-knockout mice (test group) and two control mice (control group) using the RNeasy minikit (Qiagen); 5 μg RNA was reverse transcribed using the RT^2 First Strand Kit (SABiosciences, Frederick, MD, USA) according to the manufacturer's instructions. The real-time PCR microarray for autoimmunity and inflammation (84 relevant genes) was performed using RT^2 Profiler^{TM} PCR Array (SABiosciences; PAMM-073) and the Stratagene Mx3000P real-time cycler. Gene expression was normalized to 5 internal controls (housekeeping genes *GUSB*, *HPRT1*, *HSP90AB1*, *GAPD*, and *ACTB*) to determine changes in expression between test and control group by the $\Delta\Delta C_t$ analysis method. All data with a 3-fold increase or decrease of gene expression in comparison to the control group were considered to be significant.

Cytokine detection. Cytokine levels were measured from serum using fluorescence-activated cell sorting (FACS). To detect mouse T helper (Th_1/Th_2) cytokines we used the Cytokine FlowCytomix kit (MBS720F; Bender Medsystems, Vienna, Austria) according to the manufacturer's standards.

Determination of IgG subgroups. IgG subgroups were measured in the sera of mice by ELISA as described[24] (5300-044 + 5300-1; Southern Biotech, Birmingham, AB, USA).

Statistics. The data were analyzed using the Mann-Whitney Wilcoxon test. P values < 0.05 were considered significant.

RESULTS

IL-6-deficient MRL-*Fas*^{lpr} mice showed a milder course of lupus nephritis and considerable reduction in mortality.

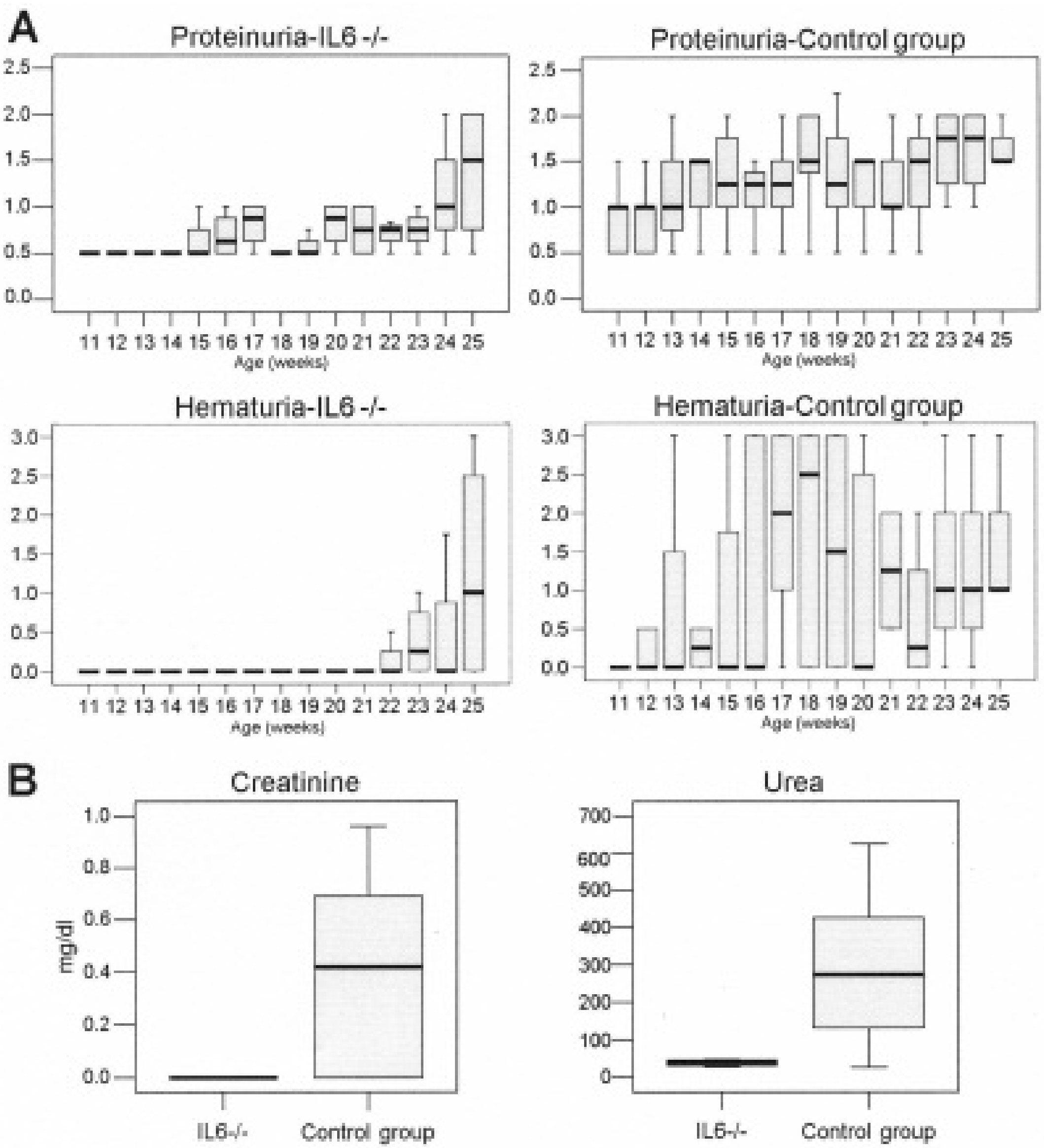
IL-6 deficiency delayed clinical manifestation of lupus nephritis. MRL-*Fas*^{lpr} IL-6 –/– mice (n = 4) showed a marked reduction of proteinuria and hematuria, with a rise only in the final 2–3 weeks of the study. The control group (n = 12) showed higher levels of proteinuria and hematuria at the beginning of the measurement, indicating an earlier decline in kidney function (Figure 1A, Table 1). In order to clinically specify renal damage, serum urea and creatinine levels were measured at the endpoint of the study. Urea levels of the female MRL-*Fas*^{lpr} IL-6 –/– mice were drastically reduced to 40 mg/dl. The control group showed median levels of 272 mg/dl (Figure 1B). In sera of IL-6-deficient MRL-*Fas*^{lpr} mice, creatinine levels were below the detection limit of 0.2 mg/dl. In contrast, the control group had median creatinine levels of 0.42 mg/dl (Figure 1B). The reduction of serum levels of urea and creatinine, together with the longterm proteinuria/hematuria data, suggests that the absence of IL-6 in the MRL-*Fas*^{lpr} lupus mouse model diminishes lupus activity, leading to a delayed onset of lupus nephritis.

Lifespans of test animals. The mortality rate of female MRL-*Fas*^{lpr} mice is known to be 50% after 6 months[24]. The IL-6-deficient MRL-*Fas*^{lpr} mice survived the observation period of 6 months, whereas only 25% of the control group survived to the end of the study (Figure 1C). About 41% died because of renal failure and 33% had to be sacrificed because of high proteinuria and rapid decline of general condition.

Reduction of kidney pathology and diminished infiltrating cells in kidneys. Renal pathology was reduced in MRL-*Fas*^{lpr} IL-6 –/– mice (n = 4) compared to the control group (n = 7). We found reduction of infiltrative cells and reduced kidney pathology (Figure 2). Upon specific immunohistochemical staining we detected a reduction in interstitial infiltration. Macrophages and CD4+ T cells were considerably reduced ($p < 0.05$; Figures 3 and 4). CD8+ T cells also showed a lower cell count ($p = 0.07$; Figure 3). CD4+ and CD8+ T cells were diminished in the periglomerular compartment (but $p > 0.05$). Detection of B cells with CD20 staining showed no difference between the 2 groups (Appendix figure 1). Apoptotic cell count was reduced in all 4 compartments (interstitial, perivascular, glomerular, and periglomerular; all $p < 0.05$; Appendix figure 2). VCAM-1 displayed perivascular and interstitial reduction ($p < 0.05$; Figure 5A, 5B). As macrophages displayed substantial reduction in all 4 compartments ($p < 0.05$), it seems that the improved kidney function was largely due to the diminished macrophage count.

Reduction of immune deposits in kidneys of IL-6 –/– mice. Frozen and paraffin-embedded kidney sections were stained for IgG and C3 deposition. Glomerular immune complex deposits were detected by immunofluorescence staining in both groups. However, semiquantitative analysis showed a decrease of deposition of IgG and C3 in IL-6-knockout mice (Figure 5C; statistical data not shown).

Absence of IL-6 led to upregulation of IL-10. We measured cytokine concentrations of IL-1α, IL-2, IL-4, IL-10, IL-17, IFN-γ, TNF-α, and granulocyte-macrophage colony-stimulating factor in the sera of MRL-*Fas*^{lpr} IL-6 –/– mice and the control group. Serum levels of the antiinflammatory cytokine IL-10 were considerably elevated. MRL-*Fas*^{lpr} IL-6 –/– mice developed median IL-10 levels of 423 pg/ml compared to 186 pg/ml in control sera ($p < 0.05$; Figure 6A). IL-6-deficient MRL-*Fas*^{lpr} mice also had elevated median levels of IFN-γ compared to the control group (518 pg/ml compared to 187 pg/ml, respectively; $p < 0.05$; Figure 6A). IFN-γ is known to be a key cytokine in the development of lupus nephritis. There were no significant changes in the levels of other serum cytokines, IgG subtypes, or anti-dsDNA antibodies (Table 2).



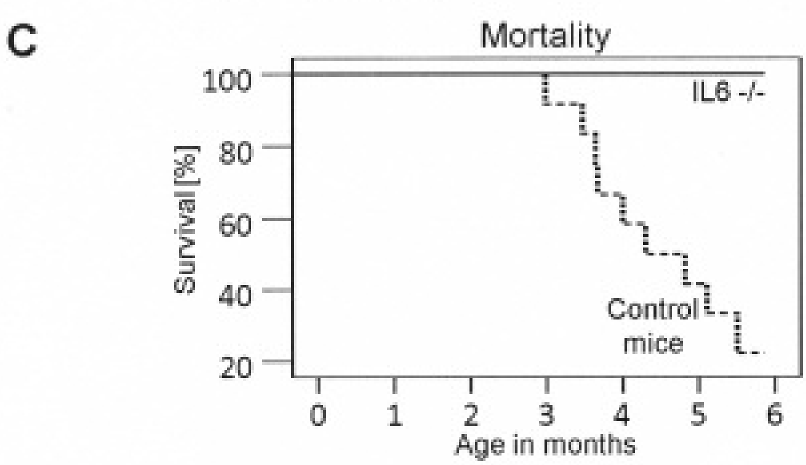


Figure 1. IL-6 deficiency in MRL-*Fas*^{lpr} mice delays lupus nephritis, shown by improved kidney function. A. IL-6 deficient MRL-*Fas*^{lpr} mice (n = 4) show delayed onset and milder course of proteinuria/hematuria. Proteinuria scale: 0, none; 0.5, 15 mg/dl; 1, 30 mg/dl; 1.5, 100 mg/dl; 2, 300 mg/dl; 2.5, > 2000 mg/dl. Hematuria: 0, none; 0.5, 5–10/μl; 1, 25/μl; 2, 80/μl; 3, 200/μl. The irregular diagram for the control group (n = 12) is due to early deaths of mice caused by severe glomerulonephritis. Proteinuria/hematuria measured semiquantitatively in weekly intervals. B. Serum urea levels were drastically reduced in MRL-*Fas*^{lpr} IL-6 –/– mice, but did not return to baseline levels (18 mg/dl) of *Fas*-intact MRL mice. Serum creatinine was below the measurement range of 0.2 mg/dl, whereas the control group shows elevated levels. C. IL-6-knockout in MRL-*Fas*^{lpr} mice improved survival. All IL-6-deficient mice (n = 4) survived the study period of 6 months compared to 25% in the control group (n = 12). MRL-*Fas*^{lpr} mice are known to have an average mortality rate of 50% after 6 months.

Table 1. Concentration of protein/erythrocyte in urine and the consecutive proteinuria/hematuria grading used in Figure 1.

	Grade of Proteinuria					
	0	0.5	1	1.5	2	2.5
Protein, mg/dl	None	15	30	100	300	> 2000

	Grade of Hematuria				
	0	0.5	1	2	3
Erythrocytes per μl	None	5–10	25	80	200

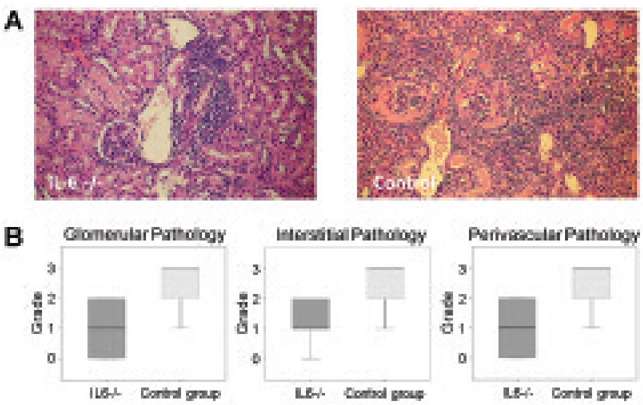


Figure 2. IL-6-deficient MRL-*Fas*^{lpr} mice showed reduced kidney pathology. A. Representative photograph of reduced cell infiltration into the kidney illustrates reduction of glomerulosclerosis and crescent formation in IL-6 deficient MRL-*Fas*^{lpr} mice (n = 4) and controls (n = 7) at the age of 6 months (H&E staining, light microscopy, original magnification ×200). B. Histological analysis of glomerular, interstitial/tubular, and perivascular pathology. Glomerular pathology: 0 = normal, 1 = mild, 2 = moderate, 3 = severe. Interstitial/tubular pathology: 0 = normal, 1 = mild, 2 = moderate, 3 = severe. Perivascular pathology: 0 = none, 1 = less than 5 cell layers, 2 = 5–10 cell layers, 3 = more than 10 cell layers.

Expression of markers for autoimmunity and inflammation in splenocytes. Detectable PCR products were obtained for 71/84 genes (defined as requiring < 35 cycles for both groups). Of these 71 genes, 5 were not altered, 23 were upregulated, and 43 were downregulated (Figure 6B). The ΔΔCt values of the 5 housekeeping genes (*GUSB*, *HPRT1*, *HSP90AB1*, *GAPD*, and *ACTB*) were similar between IL-6 –/– mice and the control group. While IL-6 was not detectable in IL-6-deficient mice, the expression of IFN-γ was elevated (1.7-fold); as well, 5 genes were expressed at significantly higher levels (> 3-fold upregulation) in IL-6 –/– mice compared with controls, including the proinflammatory cytokines *IL2* (3.1-fold) and *TNFα* (5.0-fold) and the interferon-stimulated exonuclease *ISG20* (8.2-fold), and the chemokines *CCL2* (MCP-1, 10.6-fold) and *CXCL5* (ENA-78, 3.9-fold). More than 3-fold downregulation was found for *CCL22* (–4.5-fold), *CXCL2* (–14.9-fold), *FOXP3* (–3.2-fold), *IL17f* (–6.6-fold), *IL6ra* (–4.2-fold), *IL7r* (–5.7-fold), *STAT4* (–4.3), and *TRAF6* (–3.3-fold) (Figure 6D), suggesting a broad immunomodulatory effect of IL-6 signaling. Of the T cell line-specific transcription factors, only Tbx21 (T-bet, TH1) was upregulated (2.4-fold) in IL-6-knockout mice (Figure 6C). All other factors tested were downregulated [RORγt (–1.9-fold, TH17), Gata-3 (–1.7-fold, TH2), Foxp3 (–3.2-fold, Tregs), as well as the factors C/EBP beta (–2.45-fold) and NFATc2 (–1.6-fold)] or were not altered (nuclear factor-κB, –1.1-fold).

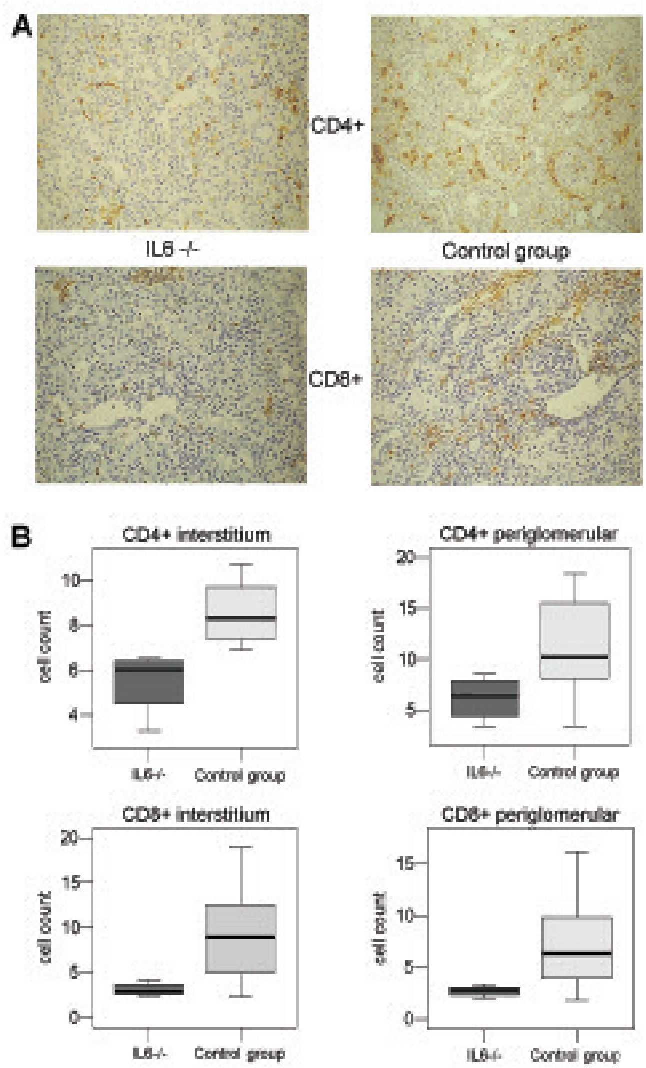


Figure 3. MRL-*Fas*^{lpr} IL-6 –/– mice showed reduction of infiltrating CD4 and CD8 cells in the kidney. A. Representative photographs of CD4+ cells (dark brown cells) from kidney of IL-6-deficient mice and controls (CD4+ and CD8+ staining, light microscopy, original magnification ×200). B. Histological analysis of interstitial and periglomerular reduction of infiltrating CD4+ and CD8+ cells in the kidney ($p < 0.05$, Mann-Whitney test).

Extrarenal lupus manifestation. Thirty percent of the control group developed skin lesions, whereas the IL-6-deficient mice did not. The weights of kidney and spleen showed no differences between the groups (Table 3).

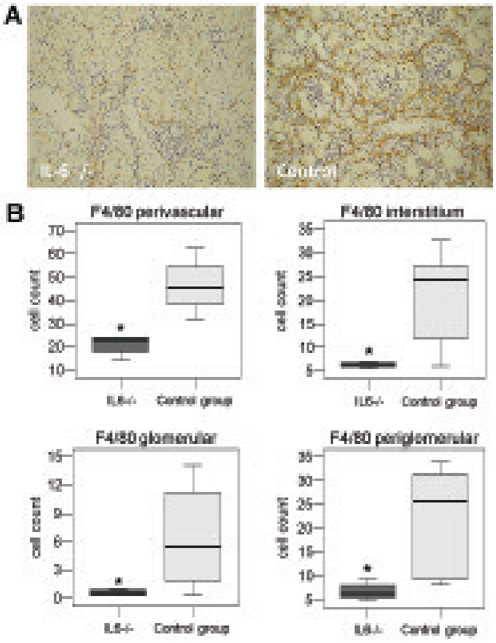


Figure 4. IL-6 deficiency drastically reduced renal macrophage count in kidneys of MRL-*Fas*^{lpr} mice. A. Representative photo of macrophages in the kidney of IL-6-deficient mice and controls (F4/80 staining, dark brown cells; original magnification ×200). Macrophages showed the greatest reduction of infiltrating cells. B. IL-6 –/– MRL-*Fas*^{lpr} mice had drastic reduction of infiltrating macrophages in all 4 kidney compartments (*p < 0.05, Mann-Whitney test).

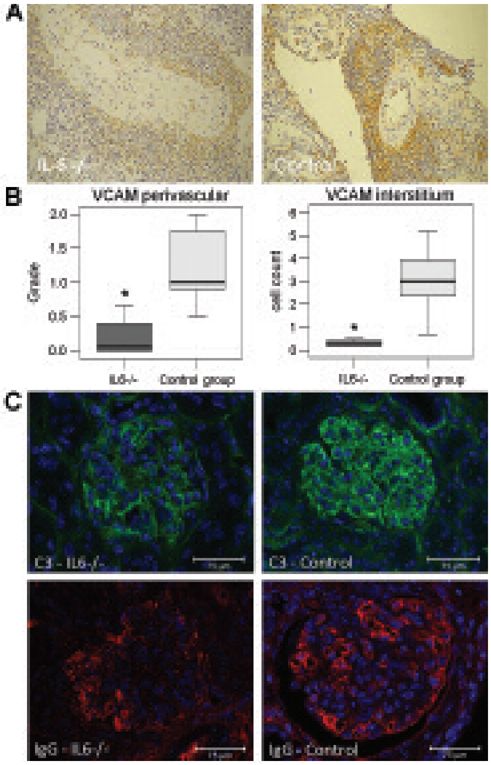


Figure 5. Reduction of VCAM-1 and IgG/C3 deposition in kidneys of MRL-*Fas*^{lpr} IL-6 –/– mice. A. Representative photograph of perivascular VCAM-1 (VCAM-1 staining with DAB substrate, dark brown staining); original magnification ×200). B. Histological analysis of perivascular and interstitial VCAM expression (*p < 0.02, Mann-Whitney test). IL-6-deficient MRL-*Fas*^{lpr} mice had markedly diminished perivascular and interstitial VCAM-1. C. Representative immunofluorescence images of C3 (green fluorescence) and IgG (red) immune deposits in the glomeruli of IL-6-knockout mice (left panels) and controls (right panels). Slides were counterstained with DAPI. IL-6 deficiency resulted in reduction of autoimmune complex deposition for both C3 and IgG.

DISCUSSION

MRL-*Fas*^{lpr} mice develop autoantibodies, immune complex renal injury, and disease features that closely resemble those in human SLE[25]. The pathogenesis of this disease is characterized by the interplay of humoral, T helper (Th_2)-mediated[26], and cellular (Th_1) autoimmune responses[27]. Yet the complex pathophysiological details of SLE remain uncertain.

IL-6 is believed to be an essential modulator of various inflammatory diseases, with a broad range of bioactivities on its target cells. Elevated concentrations of this cytokine have been found in patients with different inflammatory and autoimmune diseases[28]. Studies showed that IL-6 correlates with disease activity[29] and might even function as a biomarker for SLE activity[14]. In a study by Liang, *et al*, NZB/NZW mice treated with anti-IL-6 monoclonal antibody showed beneficial effects on autoimmunity in murine SLE[17].

We tested the hypothesis that IL-6 plays an important role in the development of murine lupus nephritis. We generated IL-6-deficient MRL-*Fas*^{lpr} mice and monitored them for a period of 6 months. IL-6 deficiency in MRL-*Fas*^{lpr} mice diminishes lupus activity, leading to delayed onset of lupus nephritis. Further, it resulted in a reduction of infiltrating cells and immune complex deposition. The reduction of infiltrating cells can be explained by 2 mechanisms. First, the absence of IL-6, which is a strong activator of T cells, B cells, and macrophages, also stimulates differentiation of immature T cells to cytotoxic T cells and seems to inhibit the development of regulatory T cells[7-9,30]. Romano, *et al* were able to show that in an area of inflammation IL-6 excites leukocyte recruitment and monocyte migration via the release of chemokines by endothelial cells[13]. Thus, the reduction of these IL-6-dependent mechanisms can explain the considerable reduction of infiltrated renal macrophages, which showed the greatest reduction of infiltrating cells in all 4 renal compartments as well as the decrease of CD4+ and CD8+ T cells. IL-6-deficient mice showed decreased glomerular deposition of IgG and C3, which could also account for the prolonged survival. This finding emphasizes the improved immune status of IL-6 –/– mice in comparison to controls.

The absence of IL-6 led to upregulation of IL-10 in sera of MRL-*Fas*^{lpr} IL-6 –/– mice, a cytokine known to have antiinflammatory effects. Thus the second mechanism: high serum levels of IL-10 might also account for our results. Data from a study with rats that overexpressed IL-10[31] correlate with our findings that renal interstitial infiltration and renal expression of monocytes/macrophages, CD8+, and

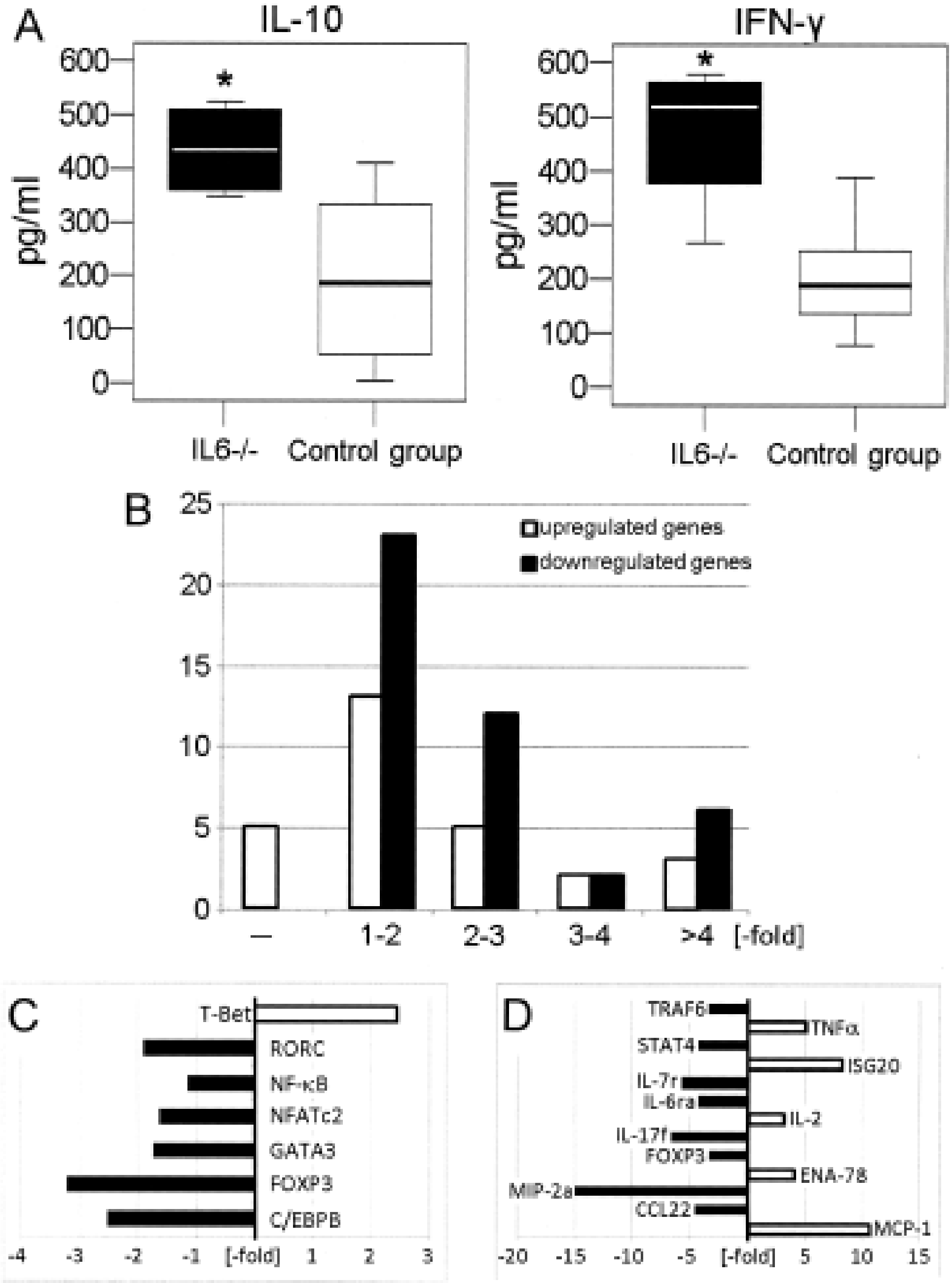


Figure 6. Elevation of IL-10 and IFN-γ in MRL-*Fas*^{lpr} IL-6 –/– mice. Real-time PCR analysis of splenic mRNA of IL-6 –/– mice shows reduction of genes involved in inflammation and autoimmunity. A. Absence of IL-6 in MRL-*Fas*^{lpr} mice led to high serum levels of IL-10 (*$p < 0.05$, Mann-Whitney test). IL-10 is capable of inhibiting mesangial proliferative glomerulonephritis through inhibition of macrophage-induced glomerular injury. The control group had lower IL-10 levels. IL-6-deficient MRL-*Fas*^{lpr} mice showed upregulation of IFN-γ ($p < 0.05$, Mann-Whitney test). Despite high IFN-γ levels, MRL-*Fas*^{lpr} IL-6 –/– mice had improvement of kidney function and reduction of renal pathology. B. Expression of splenic genes involved in immune regulation in IL-6-knockout and control mice. Compared to controls, 43 genes were downregulated, 23 upregulated, and 5 were unchanged (71 genes tested). C. Splenic expression of T cell subset-specific transcription factors: whereas most were downregulated, upregulation of the Th_1-specific factor Tbx21 (T-bet) might indicate a reactive stimulation of the Th_1 pathway. D. Splenic gene expression of IL-6-knockout and control mice: genes that show more than 3-fold altered expression in comparison to controls.

CD4+ T cells were reduced. Further studies showed that IL-10 is capable of inhibiting mesangial proliferative glomerulonephritis through inhibition of macrophage-induced glomerular injury[32,33]. MRL-*Fas*^{lpr} mice with IL-10 deficiency developed exacerbated disease with heavy glomerulonephritis[34]. Despite these antiinflammatory effects of IL-10, it is presumed to be an important modulator of disease activity in human and murine SLE[35]; several

Table 2. IL-6-knockout in MRL-Fas^{lpr} mice had no effect on serum IgG subtype or dsDNA antibody levels. Serum IgG levels measured by ELISA. Levels of dsDNA antibodies were tested at the Laboratory of Clinical Chemistry, University Hospital Mainz. All data p > 0.05, Mann-Whitney test.

	Antibodies				
	IgG1, mg/ml	IgG2a, mg/ml	IgG2b, mg/ml	IgG3, mg/ml	dsDNA, IU/ml
IL-6 –/–	1.8 ± 0.2	1.8 ± 0.6	1.2 ± 0.3	0.5 ± 0.2	12.9 ± 8.7
Controls	1.4 ± 0.5	2.1 ± 0.5	0.8 ± 0.3	0.7 ± 0.3	11.2 ± 4.4

investigations found a correlation of IL-10 to disease activity. Whereas some authors were not able to detect a correlation of IL-10 with SLE activity, a study by Llorente, *et al* with 7 lupus patients showed elevated serum levels of IL-10[36]. On the other hand, a study by Ripley, *et al* with 171 lupus patients showed no correlation of IL-10 with SLE disease activity[29]. Attempting to explain these discrepant results, Yin, *et al* proposed that IL-10 is needed in the early stages of the disease in order to prevent inflammation, but in later phases overproduction of IL-10 might lead to extensive autoantibody production and thus promote disease activity[34]. In contradiction of this thesis, Tyrrell-Price, *et al* showed that peripheral blood mononuclear cells (PBMC) from lupus patients that were stimulated with IL-10 reacted differently depending on the patient's disease activity. PBMC from patients with inactive SLE had an increase of anti-single-strand DNA and anti-double-strand DNA antibody production, whereas PBMC from patients with active disease reacted to IL-10 stimulation with reduction of autoantibodies[37]. This finding favors an antiinflammatory role of IL-10 in active SLE. Median anti-ds-DNA antibody levels in the MRL-*Fas*^{lpr} IL-6 –/– mice showed slight reduction. Our results suggest that IL-10 is able to reduce kidney pathology through these mechanisms.

Analysis of mRNA in spleen revealed significant reduction of inflammatory gene expression in MRL-*Fas*^{lpr} IL-6 –/– mice. Of the transcription factors specific for the different subsets of T cells, only Tbx21 (T-bet) was upregulated. Tbx21 is specific for Th_1 cells and thus might indicate a reactive upregulation of the Th_1 pathway. This finding is consistent with the upregulation of Th1 cytokines IL-2, IFN-γ, TNF-α, and ISG20 in splenic mRNA analysis as well as upregulation of IFN-γ in sera of IL-6-deficient mice. One can speculate that the high levels of Th_1 cytokines and the chemokine MCP-1 can be seen as an alternative immunoreactive pathway due to IL-6 deficiency in MRL-*Fas*^{lpr} mice. These results suggest that the majority of the genes involved in maintenance of the proinflammatory status of MRL-*Fas*^{lpr} mice are downregulated in IL-6-deficient mice, leading to a longer lifespan of these animals. Cytokine measurement revealed elevated serum levels of IFN-γ in the MRL-*Fas*^{lpr} IL-6 –/– mice. Pathogenic mechanisms regulated by IFN-γ are central to autoimmune kidney disease in MRL-*Fas*^{lpr} mice[38]. Surprisingly, the high IFN-γ levels in IL-6 –/– mice were not associated with exacerbated renal disease. In addition, VCAM-1 that is normally upregulated by IFN-γ showed reduced renal expression. This phenomenon noted in our study suggests that IFN-γ's mediation of lupus nephritis is at least partially dependent on IL-6 signaling.

IFN-γ also displays antiinflammatory effects. A study showed that IFN-γ limits macrophage expansion in MRL-*Fas*^{lpr} interstitial nephritis through a negative regulatory pathway[20]. In experimental rheumatoid arthritis, IFN-γ was found to be protective against the development of destructive joint disease[39]. Our data indicate that the proinflammatory effects of IFN-γ are outweighed by the antiinflammatory mechanisms of IL-10 and the loss of IL-6 activity. Given that IFN-γ triggers IL-6, high levels of IFN-γ could also be caused by the disruption of a negative feedback loop in MRL-*Fas*^{lpr} mice with IL-6 deficiency. Our finding of fewer apoptotic cells in kidneys of MRL-*Fas*^{lpr} IL-6 –/– mice correlates with reduction of the total number of infiltrating cells in the organ. Cytotoxic T cells are able to induce apoptosis directly, and since IL-6 stimulates the differentiation of T cells to cytotoxic T cells, this can also explain reduced apoptosis in kidneys of MRL-*Fas*^{lpr} IL-6 –/– mice.

IL-6-deficient MRL-*Fas*^{lpr} mice showed a perivascular and interstitial reduction of this adhesion molecule. VCAM-1 mediates the adhesion of lymphocytes, neutrophils, and monocytes to endothelial cells and thus plays a crucial role in the development of inflammation. It is known that VCAM-1 is associated with the activity and severity of glomerulonephritis in SLE[40]. Whereas VCAM-1 is upregulated by IL-1 and TNF-α[41], IL-10 is capable of downregulating VCAM-1 expression[42]. Studies with IL-6 deficiency

Table 3. Weights of body, spleen, and kidney in IL-6-deficient and control mice did not differ significantly and there were no differences in grade of lymphadenopathy.

	Body Weight, g	Spleen Weight, g	Kidney Weight, g	Lymph Node abd/ing	Lymph Node med/ax/cerv
IL-6 –/–	39 ± 4.3	0.5 ± 0.4	0.26 ± 0.05	1 ± 2	5.9 ± 5.1
Controls	37 ± 5.6	0.3 ± 0.3	0.25 ± 0.03	0.8 ± 1.5	6.3 ± 4.1

All data p < 0.05, Mann-Whitney test. abd: abdominal; ing: inguinal; med: mediastinal; ax: axillary; cerv: cervical.

models and treatment of Crohn's disease with anti-IL-6 antibodies resulted in downregulation of VCAM-1[43,44].

IL-6 promotes cellular responses through a receptor complex consisting of at least one subunit of the signal-transducing glycoprotein gp130[45]. IL-6 activates gp130 through a membrane-bound cognate IL-6 receptor (IL-6R) that is mainly expressed on hepatocytes and leukocytes[46]. So how can the many biological activities assigned to IL-6 be explained? A soluble IL-6 receptor (sIL-6R) provides IL-6 with an alternative mechanism of gp130 activation. The IL-6/sIL-6R complex binds directly to cellular gp130, and thus enables IL-6 to stimulate cells that would otherwise remain unresponsive to IL-6 itself[47]. This alternative IL-6 signaling is termed "IL-6 transsignaling." IL-6 transsignaling is known to play a role in a number of inflammatory events[48,49]. Suzuki, *et al* showed that serum sIL-6R levels correlated with serum IL-6 levels in MRL^{lpr} mice[50]. Thus IL-6 transsignaling also contributes to the development of lupus nephritis. For example, mononuclear cell recruitment and VCAM-1 expression are known to be modulated by IL-6 transsignaling[48].

IL-6 deficiency was able to reduce kidney pathology and was capable of diminishing lupus activity in MRL-*Fas*^{lpr} mice. More research will be needed to understand the complex immune regulation of cytokines by IL-6 and IL-6 transsignaling in systemic lupus erythematosus. With the advent of the new drug tocilizumab, a humanized anti-human IL-6 receptor antibody, IL-6 now becomes a realistic new option for SLE treatment. Tocilizumab reduced disease activity in a group of 419 patients with rheumatoid arthritis[51] and is currently in preliminary trials in patients with SLE. Another option for treatment might be found in selective blockade of IL-6 transsignaling. Hence, IL-6 will continue to be in the focus of SLE research and therapy.

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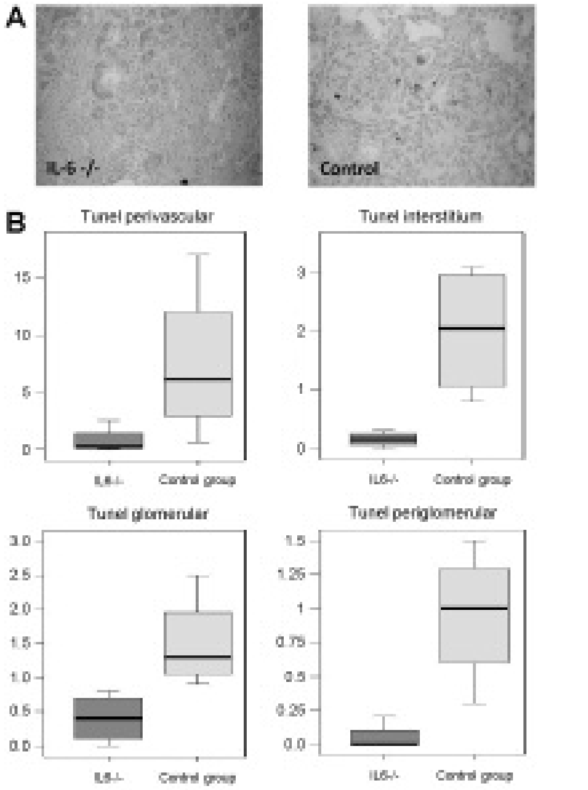
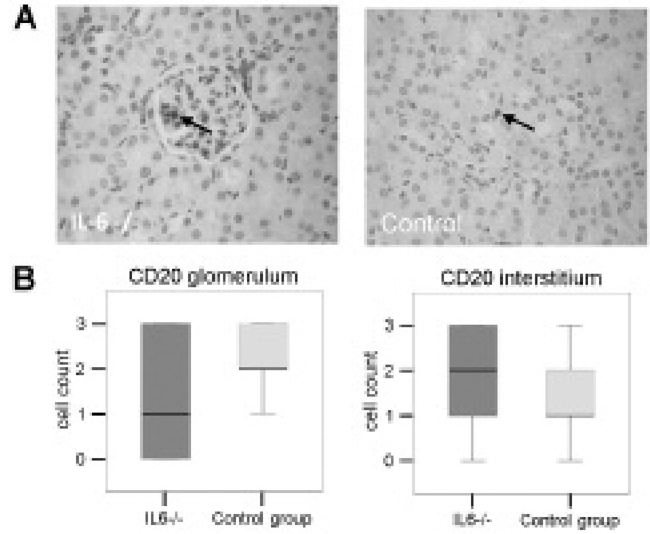


Appendix figure 1. TUNEL staining. A. Representative photograph of reduction of apoptotic cells in kidney of IL-6-deficient MRL-*Fas*^{lpr} mice (n = 4) and control group (n = 7) at age 6 months. B. IL-6-deficient MRL-*Fas*^{lpr} mice exhibited a drastic reduction of apoptotic cells in all 4 compartments of the kidney.



Appendix figure 2. Immunohistochemical staining for CD20+ cells showed no group-specific differences. A. Representative photograph of CD20+ cells (dark cells) in kidney of IL-6-deficient mice and control group (CD20+ staining, original magnification ×400). B. Histological analysis of glomerular and interstitial reduction of infiltrating CD20+ cells in the kidney.

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