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Targeting transcription factor Stat4 uncovers a role for interleukin-18 in the pathogenesis of severe lupus nephritis in mice

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Abstract

Polymorphisms in the transcription factor *Stat4* gene have been implicated as risk factors for systemic lupus erythematosus. Although some polymorphisms have a strong association with autoantibodies and nephritis, their impact on pathophysiology is still unknown. To explore this further we used signal transducers and activators of transcription 4 (Stat4) knockout MRL/MpJ-*Fas*^{lpr}/*Fas*^{lpr} (MRL-*Fas*^{lpr}) mice and found that they did not differ in survival or renal function from Stat4-intact MRL-*Fas*^{lpr} mice. Circulating interleukin (IL)-18 levels, however, were elevated in Stat4-deficient compared to Stat4-intact mice, suggesting that this interleukin might contribute to the progression of lupus nephritis independent of Stat4. In a second approach, Stat4 antisense or missense oligonucleotides or vehicle were given to MRL-*Fas*^{lpr} mice with advanced nephritis. Each of these treatments temporarily ameliorated disease, although IL-18 was increased in each setting. Based on these findings, studies using gene transfer to overexpress IL-18 in MRL-*Fas*^{lpr} and IL-12p40/IL-23 knockout MRL-*Fas*^{lpr} mice reveal a critical role for IL-18 in mediating disease. Thus, the Stat4 and IL-12 (an activator of Stat4)-independent factor, IL-18, can drive autoimmune lupus nephritis in MRL-*Fas*^{lpr} mice. Temporarily blocking Stat4 during advanced nephritis ameliorates disease, suggesting a time-dependent compensatory proinflammatory mechanism.

Keywords

chronic glomerulonephritis; chronic inflammation; lupus nephritis

Systemic lupus erythematosus (SLE) is an autoimmune disease that involves increased production of autoantibodies, immune complex deposition in various organs, complement activation, and leukocyte infiltration. Until now the major cause of disease initiation remains unknown. The role of genetics in disease development is controversial. In addition to some

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DISCLOSURE

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widely known hereditary functional disorders regarding the early components of the classical complement pathway (complement 1q, complement 1r/complement 1s, complement 4, or complement 2),[1] numerous susceptibility loci for SLE have been identified in the last years. Recently, polymorphisms in the *Stat4* gene have been described as risk factors for SLE.[2] Several of the polymorphisms showed a strong association with double-stranded DNA autoantibo-dies, nephritis, early age of diagnosis,[3] and lower interferon (INF)-α activity in peripheral blood mononuclear cells in SLE patients.[4] However, the impact of these polymorphisms on the functions of the immune system and the patho-physiology of SLE remain unknown.

Stat4 was first isolated in 1994[5,6] and its expression is restricted in myeloid cells, thymus, and testis.[6] In the resting human T cells, Stat4 expression is low, however, expression can be upregulated by activation with phytohemagglutinin. Stat4 is predominantly activated by interleukin (IL)-12[7] and Stat4-deficient mice showed an impaired Th1 differentiati on with reduced INF-γ production and an impaired cell-mediated immune response (8, Ivashkiv, 2004 #18). Stat4-deficient lymphocytes showed an impaired response to IL-12, with a reduced proliferative response.[8] In numerous studies, the role of cytokines, especially that of INF-γ, IL-12, and IL-18, has been intensively investigated in mouse models for SLE disease development. The MRL/MpJ-*Fas*^{lpr}/*Fas*^{lpr} (MRL-*Fas*^{lpr}) mouse is a model of SLE characterized by many clinical (for example, glomerulonephritis, arthritis) and immunological features (for example, autoantibody formation) similar to human SLE.[9]

Renal injury in MRL-*Fas*^{lpr} mice is complex and consists of glomerular, interstitial, and perivascular disease mediated by immune complex deposition and infiltration of monocytes and lymphocytes. INF-γ is crucial for disease development and drives the autoimmune kidney destruction in MRL-*Fas*^{lpr}(ref. 10). IL-12 is important for the regulation of INF-g production, and IL-12 secreted by renal tubular epithelial cells (TECs) has been shown to promote the autoimmune kidney destruction by expanding IFN-γ-secreting T cells.[11,12] IL-18, another potent IFN-γ-inducing cytokine expressed by TECs and mononuclear cells, is related to the IL-1 family in terms of its structure, receptor family, and function (see below).[13] IL-18 is involved in Th1 clone development and IFN-γ production in T cells and therefore involved in inflammatory and autoimmune disorders.[13,14] The importance of IL-18 in the lupus-like syndrome of MRL-*Fas*^{lpr} mice was demonstrated in a study showing that the overexpression of an IL-18 receptor accessory chain (IL-18Rβ) on lymphocytes from MRL-*Fas*^{lpr} mice could account for the hyperresponsiveness of these cells to IL-18, resulting in enhanced IFN-γ secretion.[15] Furthermore, a correlation of tubular IL-18 expression and disease activity could be shown.[16] Thus, IL-18 has many functional properties that are similar to that of IL-12. In mice deficient for IL-18, IFN-γ production was suppressed despite the presence of IL-12.[17] These data suggest that there is an important interplay between IL-12 and IL-18 for optimal IFN-γ production.

Although functionally similar, differences in the downstream signaling pathway of IL-12 and IL-18 receptors have been reported.[18] In contrast to IL-18, IL-12 is an important activator of Stat4 and signals via the receptor-associated Janus kinases, Janus kinases 2 and TYK2.[19] Thus, although IL-12 and IL-18 seem to act synergistically, they use independent pathways of intracellular signaling.

In the present study, we report that targeting Stat4 in severe lupus nephritis reveals compensatory proinflammatory mechanisms proven by two approaches: the knockout approach of *Stat4* gene and the knockdown approach of the Stat4 mRNA. Knocking out the *Stat4* gene does not affect clinical features compared with wild-type (WT) mice, whereas treatment with antisense (AS) oligonucleotides for a period of 3 weeks ameliorates the advanced lupus nephritis in MRL-*Fas*^{lpr} mice. Interestingly, we show that IL-18 functions

independently of IL-12 to incite kidney injury in MRL-*Fas*^{lpr} mice. We conclude that the compensatory upregulation of IL-18 in the Stat4 knockout approach mediates kidney disease in MRL-*Fas*^{lpr} mice. However, the AS oligonucleotide treatment to knock down Stat4 temporarily during advanced renal injury resulted in an amelioration of kidney diseases.

RESULTS

Nephritis, systemic disease, and survival are not altered in Stat4$^{-/-}$MRL-*Fas*^{lpr} mice

To evaluate the relevance of Stat4 in initiation and acceleration of kidney and systemic disease in MRL-*Fas*^{lpr} mice, we generated Stat4$^{-/-}$MRL-*Fas*^{lpr} mice.

We did not detect a difference in the survival of Stat4$^{-/-}$ MRL-*Fas*^{lpr} mice compared with WT mice (Figure 1a). Furthermore, we did not determine a difference in lymphadenopathy and splenomegaly in Stat4$^{-/-}$ compared with Stat4$^{+/+}$MRL-*Fas*^{lpr} mice (data not shown) and renal damage (renal function; Figure 1b). The severity of glomerular, interstitial, and perivascular pathologies was similar in Stat4$^{-/-}$ and Stat4$^{+/+}$MRL-*Fas*^{lpr} mice (data not shown). Similarly, there was no difference in the number of infiltrating leukocytes (CD4^{+}, CD8^{+}, CD45/B220^{+}, and F4/80^{+} cells; Figure 1c). However, we detected significantly higher serum levels of IL-18 and IL-12, but no difference in INF-γ serum levels in Stat4$^{-/-}$ compared with Stat4$^{+/+}$ MRL-*Fas*^{lpr} mice (Figure 1d). The analysis of immunoglobulin (Ig) G subclasses revealed significantly higher serum titres of IgG1 in Stat4$^{-/-}$ mice, whereas serum levels of IgG2a, IgG2b, and IgG3 showed comparable values in Stat4$^{-/-}$ and Stat4$^{+/+}$MRL-*Fas*^{lpr} mice (Figure 1e). Taken together, deletion of Stat4 did not result in an improvement of renal disease in Stat4$^{-/-}$MRL-*Fas*^{lpr} mice. However, we detected a compensatory upregulation of IL-18 and IL-12 in Stat4$^{-/-}$ MRL-*Fas*^{lpr} mice, suggesting that IL-18 independent of IL-12-Stat4 signaling mediates kidney injury.

AS oligonucleotides are potent inhibitors of IL-12-induced INF-γ production

To confirm the efficacy of oligonucleotide treatment, splenocytes from Balb/c Stat4$^{+/+}$ mice were stimulated with IL-12, resulting in a significant increase in INF-γ production (Figure 2). After co-incubation with IL-12 and AS oligonucleotides, the level of INF-γ was markedly reduced than stimulation with IL-12 alone. Co-stimulation with IL-12 and anti-IL-18 showed decreased INF-γ levels than stimulation with IL-12. Thus, AS oligonucleotides are capable of sufficient inhibition of IL-12-induced INF-γ production.

Stat4 blockade temporarily ameliorates advanced kidney and systemic disease in MRL-*Fas*^{lpr} mice

To determine whether Stat4 modulates disease activity and progression, we treated MRL-*Fas*^{lpr} mice at 5 months of age with severe kidney and systemic disease using Stat4 AS oligonucleotides compared with mice treated with missense (MS) oligonucleotides and phosphate-buffered saline for control. After 3 weeks of treatment, renal function was improved in AS oligonucleotide-treated compared with MS oligonucleotide-treated MRL-*Fas*^{lpr} mice, as determined by proteinuria, blood urea nitrogen (BUN), and creatinine (Figure 3a).

In correlation to our clinical findings, we detected less glomerular damage (Figure 3b) and a decreased number of infiltrating leukocytes in kidneys of AS oligonucleotide-treated vs MS oligonucleotide-treated MRL-*Fas*^{lpr} mice (Figure 3c). Immunostaining revealed a tendency to a lower number of CD4^{+} infiltrating cells glomerularly and interstitially. Furthermore, a significantly lower number of glomerular and interstitial F4/80^{+}, CD8^{+}, and CD45/B220^{+} cells could be found in mice treated with AS oligonucleotides compared with MS oligonucleotide-treated mice or controls (Figure 3c). As expected from our *in vitro*

experiments, we detected decreased INF-γ levels following AS oligonucleotide treatment compared with MS oligonucleo-tide-treated and control MRL-*Fas*^{lpr} mice (AS: 20.3±16.3; MS: 36.3±10.5; controls: 26.3±$_{26.3}$ pg/ml). Surprisingly, we found significantly higher levels of IL-18 in mice treated with AS oligonucleotides compared with mice MS oligonucleotide- or phosphate-buffered saline-treated mice (Figure 3d). Treatment with AS or MS oligonucleotides had no significant effect on serum levels of IgG and its subclasses. The role of IgGs in mediating kidney and systemic disease is discussed controversially; our findings would support the concept that the extent of circulating IgGs and auto-antibodies does not exclusively correlate with kidney damage.[20,21] In conclusion, Stat4 knockout did not affect the disease initiation and acceleration, whereas temporary treatment with AS oligonucleotides ameliorates the disease activity and progression in MRL-*Fas*^{lpr} mice.

Co-incubation with AS oligonucleotides and anti-IL-18 greatly suppresses spontaneous INF-γ production in splenocytes, but Stat4$^{-/-}$ mice achieve the IFN-γ production through IL-18 expression

To clarify the underlying mechanisms of our *in vivo* approach, we investigated the INF-γ production in splenocytes isolated from MRL-*Fas*^{lpr} mice. Incubation with IL-12 led to very high levels of INF-γ production (Figure 4a). By comparison, IL-12-induced INF-γ production in splenocytes of MRL-*Fas*^{lpr} mice treated with AS oligonucleotides was greatly reduced, and not affected by MS treatment. Interestingly, splenocytes of MRL-*Fas*^{lpr} mice showed a higher baseline production of INF-γ compared with BALB/c mice. Stimulation with AS oligonucleotides and anti-IL-18 was able to reduce INF-γ production below baseline levels compared with stimulation with AS oligonucleotides alone. In conclusion, Stat4-signaling and IL-18 appear to be the most potent mediators of INF-γ production.

Using splenocytes of MRL-*Fas*^{lpr} Stat4$^{-/-}$ and Stat4$^{+/+}$ mice, we determined that the INF-γ production in response to IL-18 was comparable in MRL-*Fas*^{lpr} Stat4$^{-/-}$ and Stat4$^{+/+}$ mice. Moreover, following stimulation with IL-12, IL-18 production in MRL-*Fas*^{lpr} was independent of Stat4. In addition, the IFN-γ and IL-18 expression could be blocked by anti-IL-18 treatment. Thus, the *in vitro* data support the importance of IL-18 in initiation and progression of lupus nephritis in MRL-*Fas*^{lpr}.

Gene transfer of IL-18 into MRL-*Fas*^{lpr} kidneys induced nephritis

On the basis of the results in MRL-*Fas*^{lpr} Stat4$^{-/-}$ mice and AS oligonucleotide treatment, we evaluated the relevance of IL-18 during kidney disease in MRL-*Fas*^{lpr} mice by overexpressing IL-18 locally in the kidney of MRL-*Fas*^{lpr} mice using ‘IL-18-carrier cells’. ‘IL-18-carrier cells’ constitutively secrete high levels of IL-18 in culture as compared with ‘carrier cells’ (Figure 5a). Furthermore, we confirmed successful gene transfer of IL-18 into MRL-*Fas*^{lpr} TECs by the presence of EGFP (Figure 5a). Of note, in the IL-18 overexpression study, we analyzed mice at day 28 after ‘carrier cell’ implantation, unless otherwise stated. Intrarenal IL-18 transcripts increased following ‘IL-18-carrier cell’ implantation as compared with ‘carrier cell’ implantation and untreated kidneys at 3 months of age (Figure 5a, right panel). Following intrarenal IL-18 overexpression, we detected massive accumulation of leukocytes within the implant site that extended into the adjacent intrarenal area (Figure 5b). Furthermore, we detected a notable increase in glomerular and tubular damage following ‘IL-18-carrier cell’ compared with ‘carrier cell’ implantation (Figure 5b). The increase in kidney pathology is accompanied by accumulation of multiple leukocyte populations in the kidney following ‘IL-18-carrier cell’ compared with ‘carrier cell’ implantation (Figure 5c). To determine whether the increase in renal pathology and intrarenal leukocytes following overexpression of IL-18 was accompanied by impaired renal function, we compared proteinuria and serum BUN levels. In fact, proteinuria and BUN levels rose in mice following ‘IL-18-carrier cell’ compared with ‘carrier cell’ implantation

to levels comparable to that of nephritic MRL-*Fas*^{lpr} mice at 5 months of age. Thus, intrarenal overexpression of IL-18 initiates kidney disease in MRL-*Fas*^{lpr} mice.

Increasing intrarenal IL-18 initiates systemic disease in MRL-*Fas*^{lpr} mice

To determine whether intrarenal IL-18 expression could be responsible for systemic disease in MRL-*Fas*^{lpr} mice, we assessed several cytokines in the circulation, as well as the impact of IL-18 overexpression on contralateral kidney and lung pathology. We detected a rise in circulating IL-18, IFN-γ, and tumor necrosis factor-α following intrarenal 'IL-18-carrier cell' compared with 'carrier cell' implantation (Figure 6a) that are comparable to levels detected in MRL-*Fas*^{lpr} mice with advanced kidney disease (data not shown). Interestingly, other pro-inflammatory cytokines, such as IL-12 and IL-1β, were not affected by the intrarenal IL-18 overexpression. To establish whether these enhanced cytokine levels were a reflection of accelerated systemic disease, we evaluated intrarenal leukocytes in the contralateral kidney. Interestingly, we detected an increase of multiple leukocyte populations in the contralateral kidney following 'IL-18-carrier cell' compared with 'carrier cell' implantation (Figure 6b). Moreover, we could detect an accumulation of perivascular and interstitial leukocytes in the lung following intrarenal 'IL-18-carrier cell' compared with 'carrier cell' implantation (intra-pulmonary 2.25±0.89 vs 1.17±0.41, **P*<0.05; perivascular 2.75±0.46 vs 1.83±0.75, **P*<0.05; peribronchial 2.63±0.52 vs 2.00±0.63). Thus, the rise in the level of circulating cytokines and increased leukocyte infiltration in the contra-lateral kidney and lung suggests that local intrarenal IL-18 expression initiates systemic disease in MRL-*Fas*^{lpr} mice.

IL-18 elicits renal injury independent of IL-12 and/or IL-23 in MRL-*Fas*^{lpr} mice

To clarify whether the presence of IL-12 and/or IL-23 is required for the IL-18 induced nephritis, we implanted 'IL-18-carrier cells' into IL-12p40/IL-23$^{-/-}$ and intact MRL-*Fas*^{lpr} mice. We detected a comparable influx of leukocytes in IL-12p40/IL-23$^{-/-}$ and MRL-*Fas*^{lpr} mice assessed at the implantation site, periglomerular, perivascular, and interstitial (Figure 7a). Furthermore, circulating IL-18 and IFN-γ levels were increased whereas level of IL-12 was not detectable (data not shown). The increase in intrarenal leukocytes in IL-12p40/IL-23$^{-/-}$ and MRL-*Fas*^{lpr} was accompanied by impaired renal function. Proteinuria and BUN levels rose to levels comparable to nephritic MRL-*Fas*^{lpr} mice at 5 months of age (Figure 7b). Thus, IL-18 initiates and accelerates kidney disease in MRL-*Fas*^{lpr} mice independent of IL-12p40 and/or IL-23.

DISCUSSION

In the present study, we dissected the role of Stat4 on lupus nephritis by two approaches. *Stat4* gene deletion does not affect disease initiation and progression in MRL-*Fas*^{lpr} compared with WT mice, whereas temporary treatment with AS oligonucleo-tides ameliorates advanced lupus nephritis in MRL-*Fas*^{lpr} mice. On the basis of the finding that in both approaches a compensatory IL-18 upregulation was detectable, studies using gene transfer to overexpress IL-18 in MRL-*Fas*^{lpr} and IL-12p40/IL-23$^{-/-}$MRL-*Fas*^{lpr} mice reveal a critical role for IL-18 in mediating kidney and systemic disease in MRL-*Fas*^{lpr} mice.

In MRL-*Fas*^{lpr} mice, INF-γ deficiency led to the absence of lupus disease,[10] as well as in NZB/NZW mice, another mouse model for lupus.[22] In experiments using a mouse model for SLE based on NZB/NZW mice, Stat4$^{-/-}$ New Zealand mixed mice developed accelerated kidney disease with an increase in proteinuria and mortality despite decreased IFN-γ serum levels.[23,24] In contrast, administration of exogenous IFN-γ accelerates renal disease in (NZB × NZW)F1 mice. Surprisingly, we did not detect any amelioration or acceleration of lupus disease in MRL-*Fas*^{lpr}Stat4$^{-/-}$ mice, moreover, Stat4 deficiency had no impact on INF-γ

production. These differences between MRL-*Fas*^{lpr} and NZB/NZW mice could be explained by the different role of type I IFNs in these models: in NZB/NZW models, type-I IFN receptor deficiency reduced lupus-like disease,[25] whereas the type I IFN, IFN-β, turned out to be therapeutic in MRL-*Fas*^{lpr} mice.[26] In human SLE patients, Stat4 polymorphisms were recently described in patients who showed decreased IFN-α activity in peripheral blood mononuclear cells but increased expression of IFN-α-induced genes.[4] Stat4 showed a dominant influence on the sensitivity of peripheral blood mononuclear cells to serum IFN-α and seems to be an important mediator in type-I IFN pathway.

In fact, Stat4 deficiency does not mediate initiation of lupus nephritis in MRL-*Fas*^{lpr} mice. Thus, we speculated that different cytokines might be responsible for this observation and we focused on IL-18. We choose to focus on IL-18 as lymphocytes from MRL-*Fas*^{lpr} mice have been shown to be hyperresponsive to IL-18, resulting in enhanced IFN-γ secretion.

The importance of IL-18 in the lupus-like syndrome of MRL-*Fas*^{lpr} mice was demonstrated in a study showing that lymphocytes of MRL-*Fas*^{lpr} mice are hyperresponsive to IL-18, which results in enhanced IFN-γ secretion.[15] Further support for the concept of IL-18 as a sufficient inducer of a TH1 response comes from recent studies in our lab on overexpression and blocking of IL-18. Local intrarenal overexpression of IL-18 incites lupus nephritis and systemic disease, a blockade of IL-18 activation ameliorates kidney and systemic disease (manuscript submitted). IL-18 receptor-a-deficient MRL-*Fas*^{lpr} mice have markedly reduced autoimmune disease,[27] and IL-18 complementary DNA vaccination protects MRL-*Fas*^{lpr} mice from spontaneous lupus-like autoimmune disease.[28] Furthermore, IL-18-deficient mice show an ameliorated kidney disease in a model of induced autoimmune nephritis (nephrotoxic serum nephritis).[29] In addition, it has been shown that tubular IL-18 expression correlates with disease activity in MRL-*Fas*^{lpr} mice.[16] We found elevated levels of IL-18 in MRL-*Fas*^{lpr}Stat4$^{-/-}$ mice and, therefore, we speculate that IL-18 bypasses the *Stat4* gene defect in MRL-*Fas*^{lpr}Stat4$^{-/-}$ mice and leads to similar levels of INF-γ compared with WT. The results of our *in vitro* approach support that both Stat4 and IL-18 contribute to spontaneous INF-γ production and that IL-18 works independently of IL-12 to promote a sufficient IFN-γ production to mediate disease.

Bearing in mind the limitations of performing studies in gene-targeted mice,[30] we used AS oligonucleotide treatment as a posttranscriptional modification of gene expression to knock down Stat4 *in vivo*. We found that AS oligo-nucleotide therapy resulted in an amelioration of advanced kidney disease. This technique offers a realistic option as a therapeutic treatment in human SLE patients. AS oligonucleotides have mainly been tested in the field of oncology in phase II and III studies. A combination with oblimersen sodium, a Bcl-2 AS oligonucleotide, and rituximab was shown to be effective in patients with recurrent B-cell non-Hodgkin's lymphoma.[31] In contrast, aprinocarsen, a protein kinase C-alpha AS oligonucleotide, did not enhance survival and other efficacy measures in patients with advanced non-small cell lung cancer treated with gemcitabine and cisplatin.[32] Future studies will likely prove the efficacy of AS oligonucleotide treatment and give a rationale for the application in patients with autoimmune disease.

In the present study, treating MRL-*Fas*^{lpr} mice for 3 weeks resulted in the amelioration of lupus nephritis. However, we also observed a concomitant increase in serum levels of IL-18. One possibility for the differing effect of the inhibition of Stat4 signaling is the time of blockade. The increased levels of IL-18 in the AS oligonucleotide-treated group suggest that extended treatment periods will have no sufficient effect because of the compensatory disease-mediating IL-18 upregulation. Therefore, future studies should evaluate a combination therapy using Stat4 and IL-18 blockade.

In conclusion, Stat4-independent factors can drive auto-immune lupus nephritis in Stat4$^{-/-}$MRL-*Fas*^{lpr} mice, while blocking Stat4 temporarily in advanced nephritis can ameliorate disease suggesting time-dependent compensatory proinflammatory mechanisms. The AS oligonucleotide treatment to block Stat4 in mice with lupus-like disease suggests a clinically relevant therapy that should be further investigated for potential and immediate benefits to human SLE patients.

MATERIALS AND METHODS

Generation of Stat4$^{-/-}$MRL-*Fas*^{lpr} mice

To investigate the influence of Stat4 in early stages of murine SLE, we generated Stat4-deficient ($^{-/-}$) MRL-*Fas*^{lpr} mice. MRL-*Fas*^{lpr} mice and Stat4$^{-/-}$ BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, Maine), and Stat4$^{-/-}$ MRL-*Fas*^{lpr} mice were derived by a series of genetic backcrosses using the backcross–intercross scheme. MRL-*Fas*^{lpr} mice were mated with Stat4$^{-/-}$ Balb/c mice to yield heterozygous F1 offspring. We intercrossed F1 mice and screened the progeny by PCR amplification of tail genomic DNA for the *Fas*^{lpr} mutation (primer: WT sense 5′-CCAACTCAGACAGCAACTGG-3′, AS 5′-GCTCTTTGAGCAGGGATG-3′) and Stat4 (using the linked neomycin resistance (*neo*) gene, primer: *neo* sense: 5′-ATTGAACAAGATTTGCAC-3′, *neo* AS: 5′-CGTCCAGATCATCCTGATC-3′). Double homozygous (*Fas*^{lpr}/*Fas*^{lpr} Stat4$^{-/-}$) N1F1 progeny were backcrossed with MRL-*Fas*^{lpr}/*Fas*^{lpr} mice. B1 progeny, homozygous for the *Fas*^{lpr} mutation and heterozygous for the Stat4, was intercrossed, and mice homozygous for the Stat4$^{-/-}$ mutation were selected by PCR typing for continued backcrossing. After five generations of backcross matings, this breeding scheme generated a colony of MRL-*Fas*^{lpr} mice (>95–97.8% MRL background) homozygous and heterozygous for the mutated Stat4. We analyzed the F6 generation as it was previously established that there are sufficient MRL-*Fas*^{lpr} background genes even by the F3 generation to result in phenotypic changes characteristic of the MRL-*Fas*^{lpr} strain.[33] Moreover, we performed a genome scan to ensure 495% MRL background in used mice (Supplementary Figure 1). MRL-*Fas*^{lpr} Stat4$^{+/+}$ (n = 22) and MRL-*Fas*^{lpr} Stat4$^{-/-}$ (n = 13) were under observation for 6 months to determine the effect of Stat4 deficiency in disease development. MRL-*Fas*^{lpr} Stat4$^{+/+}$ mice were littermate controls. IL-12p40-deficient ($^{-/-}$) MRL-*Fas*^{lpr} mice were derived by a series of 10 genetic intercross/backcross matings as previously described.[10]

The use of mice in this study was reviewed and approved by the Standing Committee on Animals at the University of Mainz. All of the animal experiments in this study were performed in Germany. Mice were bred and housed in our pathogen-free facilities at Johannes Gutenberg University in Mainz.

Treatment with oligonucleotides

To investigate the effect of Stat4 blockade in mice with manifest lupus nephritis, we treated MRL-*Fas*^{lpr} mice with AS oligonucleo-tides, MS oligonucleotides (each kindly provided by Professor Neurath, Mainz, Germany) or phosphate-buffered saline (control).[34] MRL-*Fas*^{lpr} mice were purchased from the Jackson Laboratory. We started oligonucleotide treatment at 5 months of age. Oligonucleotides were administered intraperitoneally three times per week for 3 weeks. Each group consisted of 12 mice.

Renal function

Proteinuria and hematuria were measured with a dipstick (Multistix: Bayer Diagnostics, Bridgend, UK). We assessed urinary protein levels and hematuria only semiquantitatively on a monthly basis beginning at 2 months of age. On the days of analysis, dipstick proteinuria measurements were made in individual mouse in the morning and repeated in the evening. If

the measurements were inconsistent, the mouse was reassessed on the following day and in addition, all mice were reassessed twice a week.[33] We measured BUN levels using a colorimetric analysis kit (Infinity, Thermo Electron, Melbourne, Victoria, Australia), and serum creatinine using the creatinine reagent kit and Creatinine Analyzer 2 (Beckman Coulter, Galway, Ireland) according to the manufacturer's instructions.

Cytokine levels and IgG subclasses

Levels of IL-18, INF-γ, IL-12, and IL-1β in serum were quantified using the enzyme-linked immunosorbent assay technique according to the manufacturer's instructions (OpEIA mouse kit, BD Bioscience, San José, CA). IgG subclasses: levels of IgG and its subclasses (IgG1, IgG2a, IgG2b, IgG3) have been determined by enzyme-linked immunosorbent assay technique (Alkaline phosphatase-based enzyme-linked immunosorbent assay, SouthernBiotech, Birmingham, AL)

Body weight, organ weight, lymph nodes

Body weight was measured once a week. After killing, organ weight was measured and the size of lymph nodes was scored (0 none, 0.5 = small, 1 = moderate, 2 = large, 3 = very large).

Histopathology

We fixed kidneys in 10% neutral-buffered formalin for 24 h, and stained paraffin sections (4 μm) with periodic acid-Schiff's reagent. We evaluated kidney pathology as previously described.[33] Briefly, we assessed glomerular pathology by scoring each glomerulus on a semiquantitative scale: 0 = normal (35–40 cells/glomerular cross-sections (gcs)); 1 = mild (glomeruli with few proliferative lesions showing slight 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); 3 = severe (glomeruli with segmental or global sclerosis and/or severe hypercellularity (>60 cells/gcs), necrosis, crescent formation). We scored 20 gcs per kidney. Interstitial/tubular pathology was assessed semiquantitatively on a scale of 0–3 in 10 randomly selected high-power fields (hpf). 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 (score: 0 = none, 1 = <5 cell layers, 2 = 5–10 cell layers, 3 = >10 cell layers).

Immunostaining

Kidney tissue was processed and stained for the presence of F4/80, CD4, CD8, and B220, as previously described for the kidney.[35] We determined the number of positive cells in 10 randomly selected hpf.

In vitro culture

To check the specificity and efficacy of the oligonucleotides used in the in vivo treatment, splenocytes of BALB/c and MRL-*Fas*^{lpr} mice were isolated as previously descried[26] and stimulated with IL-12, IL-18 (R&D Systems, Minneapolis, MN), goat anti-IL-18 (Santa Cruz Biotechnologies, Santa Cruz, CA), with or without the addition of either Stat4 AS or Stat4 MS oligonucleotides (kindly provided by Professor Dr Neurath, Mainz, Germany, and modified by Carl Roth GmbH, Karlsruhe, Germany).[34]

Retrovirus-mediated cytokine gene transfer into cultured TECs

We isolated and cultured TECs derived from MRL-*Fas*^{lpr} mice at 2–3 months of age, as previously reported.[16] For the retroviral gene transfer, we used a helper-free retrovirus packaging cell line (PT67; Clontech Laboratories, Mountain View, CA). The complete IL-18 precursor complementary DNA was kindly provided by Professor Dr Neurath (Johannes Gutenberg University of Mainz, Germany). This recombination-incompetent retroviral vector (pLEGFP-N1, Clontech Laboratories Inc.) containing the murine *IL*-18 gene was introduced into PT67 cells containing proviral sequences necessary for the 'packing' (for example, encapsidation) of the virus.[36] Using the supernatant of PT67-IL-18 cells, we infected TECs with the recombinant retrovirus in the presence of polybrene. To verify gene transfer into TECs, (1) vector-containing cells were detected by fluorescence (green fluorescent protein, part of the vector) (2) neomycin-resistant TECs were selected using G418 (Sigma, St Louis, MO; 800 μg/ml), and (3) IL-18 was measured in supernatants collected 5 days after infection by enzyme-linked immunosorbent assay. For control experiments, TECs were infected with the pLEGFP-N1 retroviral vector without the gene of interest. TECs genetically modified to express a gene are termed 'carrier cells', for example, TEC-producing IL-18 as 'IL-18-carrier cells'; TECs containing the vector without insert as 'carrier cells'.

Delivery of TECs under the renal capsule

We placed 'carrier cells' under the renal capsule of MRL-*Fas*^{lpr} IL-12/IL-23$^{-/-}$MRL-*Fas*^{lpr} and MRL$^{+/+}$ mice at 2 months of age. We tested the viability of the TECs immediately before implantation using trypan blue (Invitrogen, Carlsbad, CA) exclusion staining, viability was 490%. The animals were anesthetized and the left kidney was exposed through a left flank incision. 'Carrier cells' (1×10^6 in 50 μl of Hanks balanced salt solution w/o Ca) (Sigma) were micropipetted under the capsule of the dorsal surface of the left kidney.

Statistical analysis

Data were compared using SPSS 14 (Chicago, IL). The significance was determined by Student's unpaired *t*-test or Mann–Whitney *U*-test. *P*-values <0.05 were considered significant.

Supplementary Material

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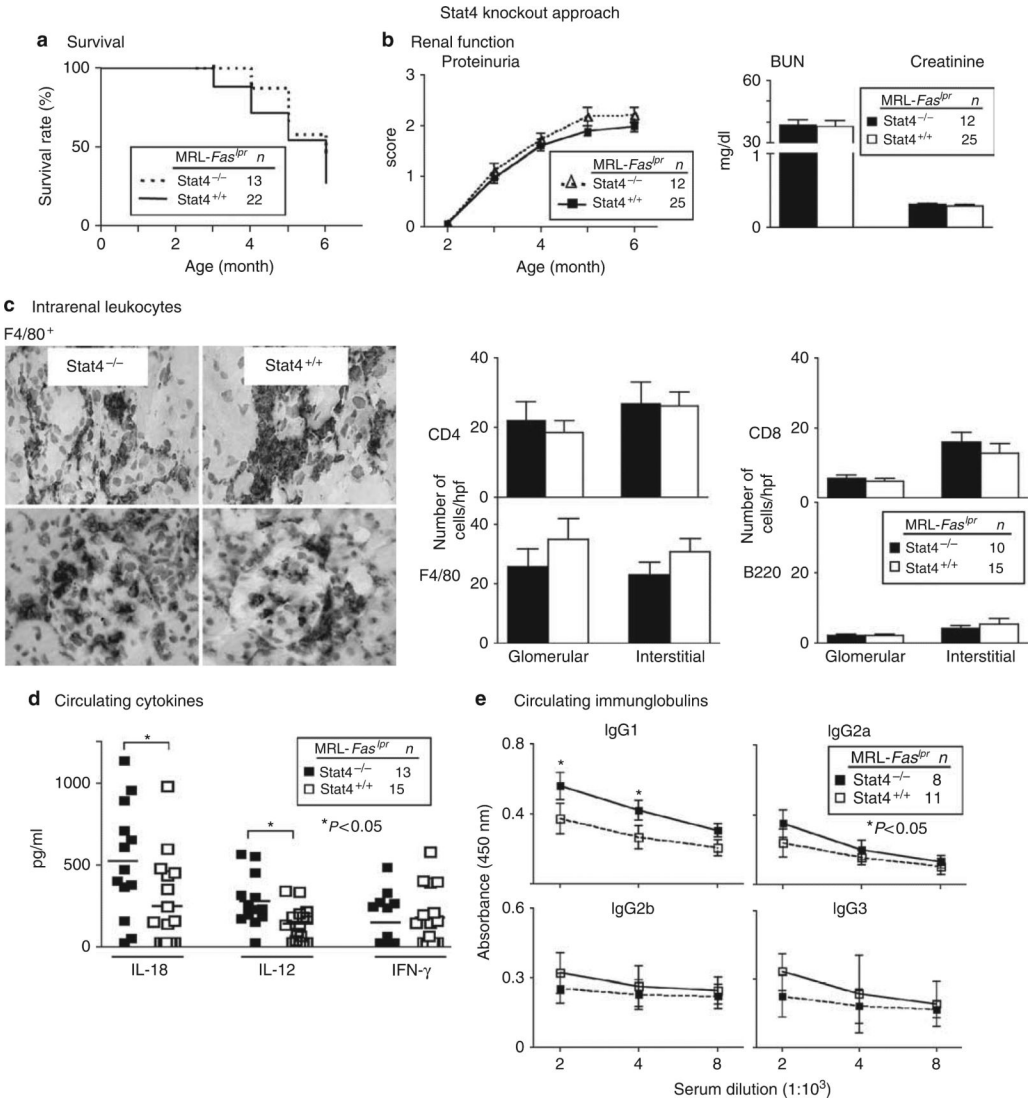

Figure 1. Nephritis, systemic disease, and survival are not altered in MRL/MpJ-*Fas*^{lpr}/*Fas*^{lpr} (MRL-*Fas*^{lpr}) Stat4$^{-/-}$ mice

(a) There are no differences in survival between MRL-*Fas*^{lpr}Stat4$^{+/+}$ and Stat4$^{-/-}$ mice. **(b)** Proteinuria, blood urea nitrogen (BUN), and creatinine did not show differences between MRL-*Fas*^{lpr}Stat4$^{+/+}$ and Stat4$^{-/-}$ mice. **(c)** Interstitial infiltration is similar in MRL-*Fas*^{lpr}Stat4$^{+/+}$ and Stat4$^{-/-}$ at 5 months of age. Representative photomicrograph of F4/80^{+} cells; top panel: interstitial, bottom panel: glomeruli (magnification × 40). **(d)** Serum levels of interleukin (IL)-18 and IL-12 are increased in MRL-*Fas*^{lpr}Stat4$^{-/-}$ compared with Stat4$^{+/+}$ mice. **(e)** MRL-*Fas*^{lpr}Stat4$^{-/-}$ mice showed significantly higher levels of immunoglobulin (Ig) G1 compared with Stat4$^{+/+}$ mice. Values are mean±s.e.m.

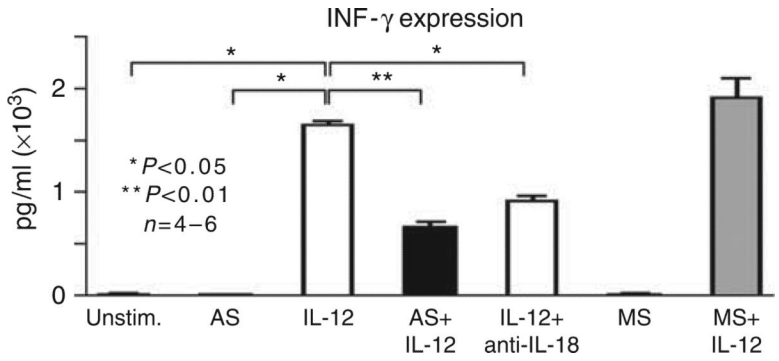

Figure 2. Antisense (AS) oligonucleotides inhibit the interferon (IFN)-γ expression in splenocytes of BALB/c Stat4$^{+/+}$ mice

Interleukin (IL)-12 induces INF-γ production in splenocytes while AS oligonucleotides block IL-12-induced INF-γ production sufficiently. Missense (MS) oligonucleotide treatment had no effect, but incubation of IL-12 with anti-IL-18 resulted in decreased INF-γ expression of splenocytes. Values are mean±s.e.m. Unstim., unstimulated.

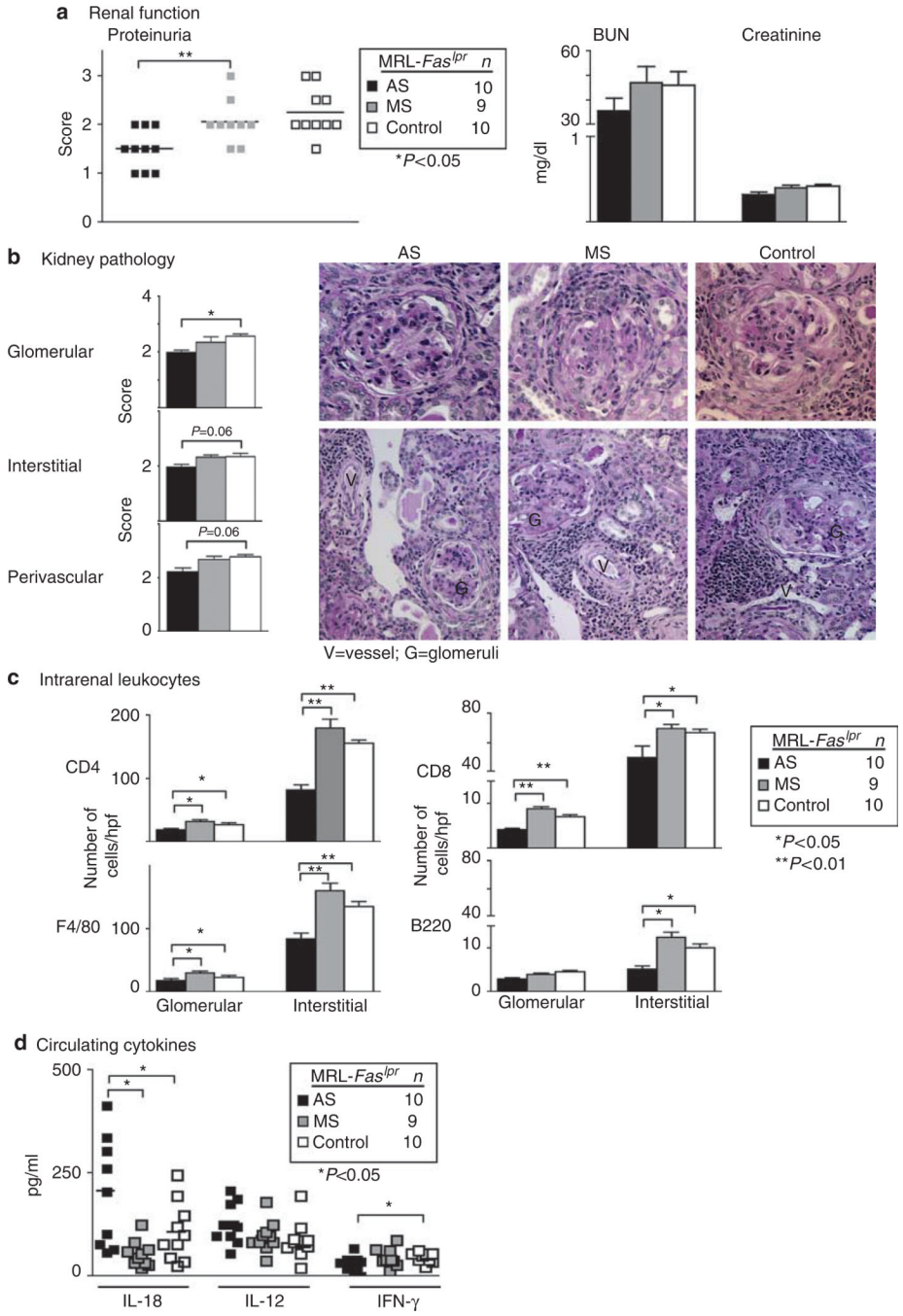

Figure 3. Antisense (AS) treatment for 3 weeks in MRL/MpJ-*Fas*^{lpr}/*Fas*^{lpr} (MRL-*Fas*^{lpr}) mice with advanced renal disease ameliorate kidney disease temporarily while interleukin (IL)-18 is compensatorily upregulated

(a) Mice treated with AS oligonucleotides showed less proteinuria and tended towards decreased serum blood urea nitrogen (BUN) and creatinine levels compared with control (phosphate-buffered saline treated) and missense (MS)-treated mice. **(b)** Mice treated with AS oligonucleotides showed reduced glomerular pathology. Representative photomicrographs of pathology are shown for glomerular, interstitial, and perivascular area. **(c)** AS treatment led to a reduced number of glomerular $CD4^+$, $CD8^+$, or $F4/80^+$ cells (intraglomerular and periglomerular) and interstitially compared with MS and control-

treated mice. **(d)** Serum levels of interferon (INF)-γ were significantly lower in mice treated with AS oligonucleotides compared with controls. Serum levels of IL-18 were significantly higher in mice treated with AS oligonucleotides compared with MS oligonucleotides and controls, while IL-12 levels were unaffected. Values are mean±s.e.m.

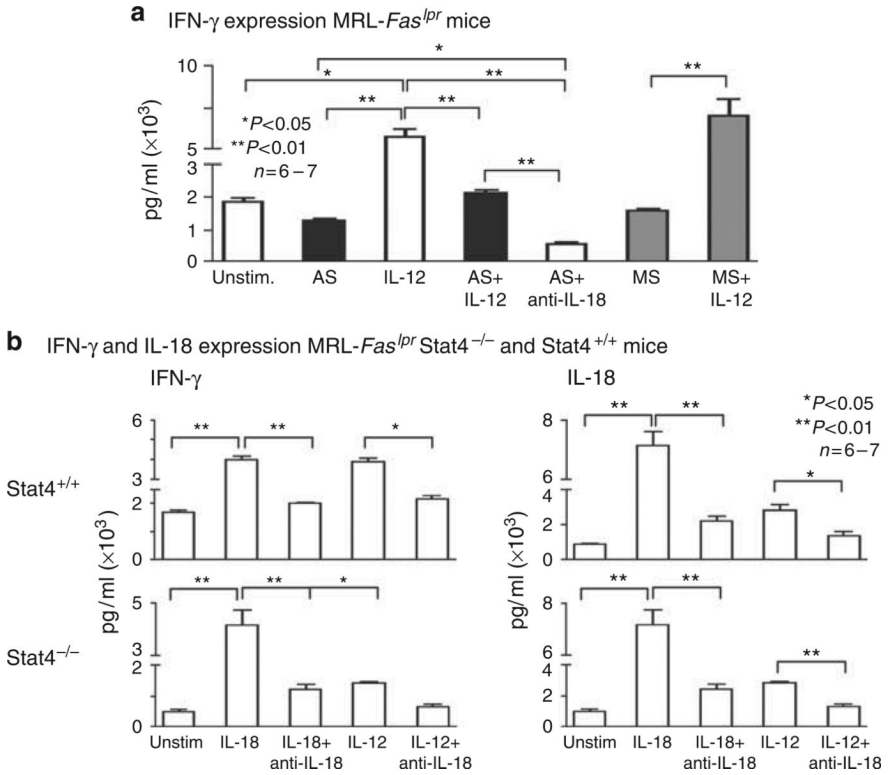

Figure 4. Stat4, interleukin (IL)-12, and IL-18 are potent inducers of interferon (IFN)-γ in splenocytes of MRL/MpJ-*Fas*^{lpr}/*Fas*^{lpr} (MRL-*Fas*^{lpr}) Stat4$^{+/+}$ and Stat4$^{-/-}$ mice

(a) Following stimulation with IL-12, administration of antisense (AS) oligonucleotides was effective in blocking INF-γ production. Unstimulated splenocytes of MRL-*Fas*^{lpr} mice showed a high baseline production of IFN-γ. This baseline production is reduced by the administration of AS oligonucleotides and can be reduced even further by co-incubation with AS oligonucleotides and anti-IL-18. **(b)** Moreover, IFN-γ and IL-18 production in MRL-*Fas*^{lpr}Stat4$^{+/+}$ were comparable to levels in MRL-*Fas*^{lpr}Stat4$^{-/-}$ mice. Values are mean±s.e.m.

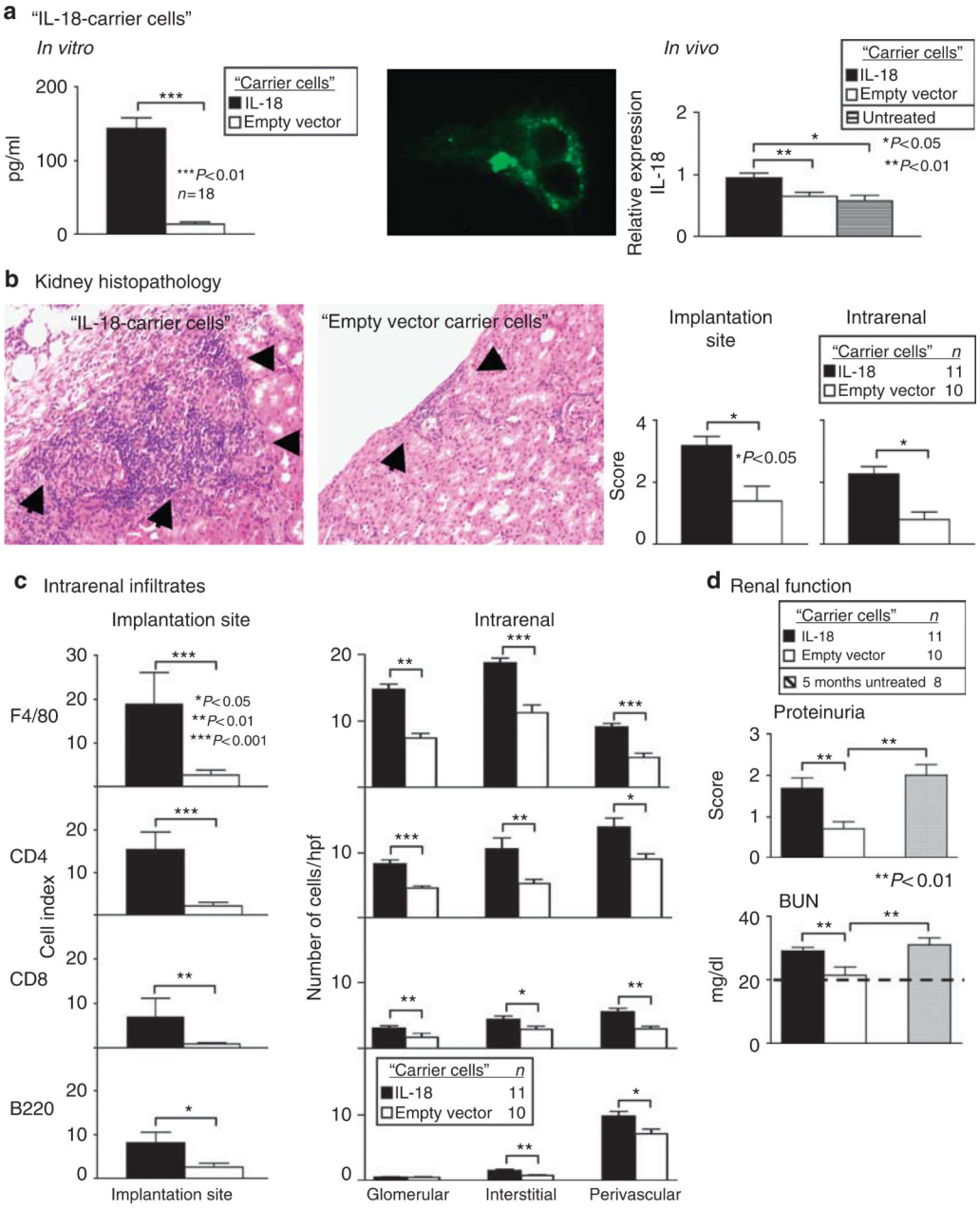

Figure 5. Intrarenal overexpression of interleukin (IL)-18 incites nephritis in MRL/MpJ-*Fas*^{lpr}/*Fas*^{lpr} (MRL-*Fas*^{lpr}) mice

(a) Left panel: successful gene transfer of IL-18 using a retroviral expression system. IL-18 concentrations in the supernatant of 'IL-18-carrier cells'/'carrier cells.' Middle panel: fluorescence of the EGFP-IL-18 fusion gene in vitro, showing successful gene transfer. Right panel: relative expression of IL-18 in the kidney following 'carrier cell' implantation compared with untreated mice at 3 months of age using quantitative real-time PCR. **(b)** Renal histopathology is increased following 'IL-18-carrier cell' compared with 'empty vector carrier cell' at day 28 post implantation. We evaluated the enhanced accumulation of infiltrating leukocytes extending from the subcapsular site to the renal cortex and the intrarenal pathology. Representative microphotographs of the implantation site are shown, magnification × 20. **(c)** Leukocytes are increased at the site of implantation and intrarenal

following ‘IL-18-carrier cell’ compared with ‘carrier cell’ implantation, evaluated by immunostaining for T cells ($CD4^+$, $CD8^+$, and $B220^+$) and Mø ($F4/80^+$). **(d)** Loss of renal function is accelerated following ‘IL-18-carrier cell’ in comparison with ‘empty vector carrier cell’ implantation in MRL-*Fas*^{lpr} mice. We evaluated renal function by measuring (1) proteinuria semi-quantitatively, (2) serum blood urea nitrogen (BUN); dotted line is BUN levels in MRL-*Fas*^{lpr} (5 months of age) controls. Data are the mean±s.e.m.

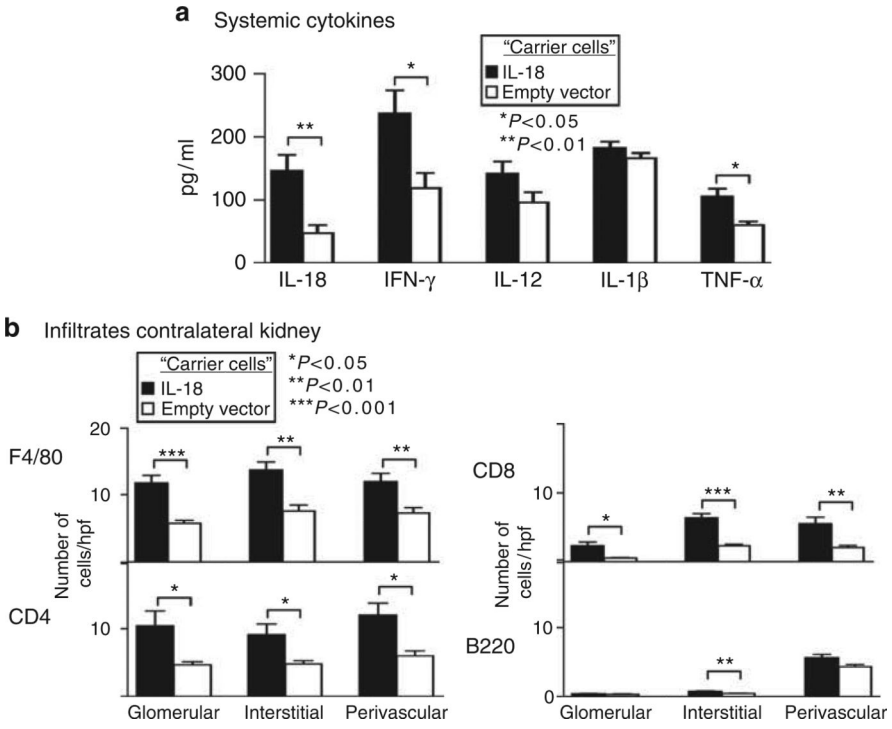

Figure 6. Intrarenal overexpression of interleukin (IL)-18 initiates systemic disease in MRL/MpJ-*Fas*^{lpr}/*Fas*^{lpr} (MRL-*Fas*^{lpr}) mice

(a) Serum IL-18, interferon (IFN)-γ, IL-12, IL-1β, and tumor necrosis factor (TNF)-α levels in MRL-*Fas*^{lpr} following 'IL-18-carrier cell' compared with 'carrier cell' implantation determined by enzyme-linked immunosorbent assay. **(b)** We evaluated the number of intrarenal Mø (F4/80^{+}) and T cells (CD4^{+}, CD8^{+}, B220^{+}) in MRL-*Fas*^{lpr} following 'IL-18-carrier cell' compared with 'carrier cell' implantation at 3 months of age. Values are mean ±s.e.m.

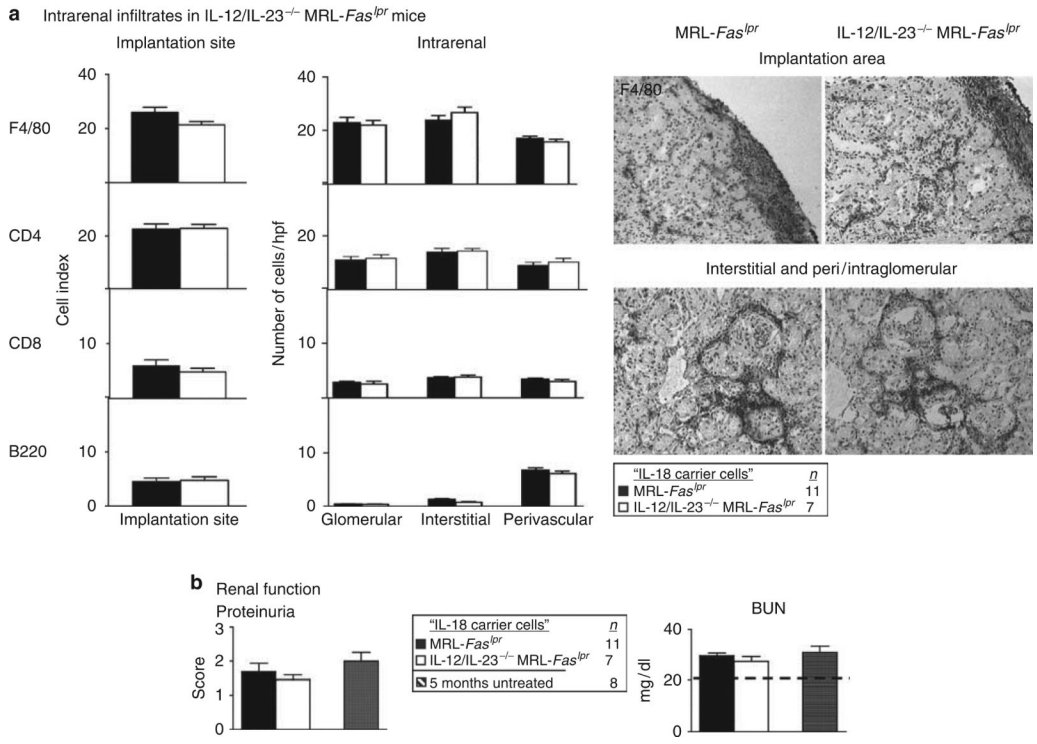

Figure 7. Interleukin (IL)-18 elicits renal injury independent of IL-12 and IL-23 in MRL/MpJ-*Fas*^{lpr}/*Fas*^{lpr} (MRL-*Fas*^{lpr}) mice

(a) Leukocytes are increased at the site of implantation and in the kidney following 'IL-18-carrier cell' implantation in IL-12/IL-23$^{-/-}$ and IL-12p40$^{+/+}$MRL-*Fas*^{lpr} mice, evaluated by immunostaining for T cells (CD4^{+}, CD8^{+}, and B220^{+}) and Mø (F4/80^{+}). We evaluated the accumulation of infiltrating leukocytes extending from the subcapsular site to the renal cortex and the infiltrating intrarenal leukocytes glomerular, interstitial, and perivascular. Representative microphotographs of the implantation site and the interstitial/glomerular intrarenal area are shown, magnification × 20. **(b)** Loss of renal function is accelerated following 'IL-18-carrier cell' implantation in IL-12p40$^{-/-}$ and IL-12p40$^{+/+}$ MRL-*Fas*^{lpr} mice comparable to loss of renal function of a MRL-*Fas*^{lpr} mouse at 5 months of age. We evaluated renal function by measuring (1) proteinuria semi-quantitatively, (2) serum blood urea nitrogen (BUN); dotted line is BUN levels in healthy MRL-*Fas*^{lpr} (2 months of age) controls. Data are the mean±s.e.m.