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ORIGINAL ARTICLE

Sex-specific differences in SLE – Significance in the experimental setting of inflammation and kidney damage in MRL-Fas^{lpr} mice

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ABSTRACT

Animal models are an important tool in the research of chronic autoimmune diseases, like systemic lupus erythematosus (SLE). MRL-Fas^{lpr} mice are one of different lupus models that develop spontaneously an SLE-like disease with autoantibodies and immune complex deposition that leads into damage of different organs. In contrast to human SLE, both sexes of MRL-Fas^{lpr} mice develop a similar autoimmune disease. Due to the sex bias in human and the delayed disease progression in male MRL-Fas^{lpr} mice, the majority of studies have been performed in female mice. To determine the suitability of male MRL-Fas^{lpr} mice for SLE research, especially with regard to the 3 R-principle and animal welfare, analyses of phenotype, inflammation and damage with focus on kidney and spleen were performed in mice of both sexes. Female mice developed lymphadenopathy and skin lesions earlier as males. At an age of 3.5 month, more immune cells infiltrated kidney and spleen in females compared to males. At the age of 5 months, however, substantially less sex-specific differences were detected. Since other studies have shown differences between both sexes on other manifestations like autoimmune pancreatitis and Sjögren syndrome in MRL-Fas^{lpr} mice, the use of male mice as part of 3 R-principle and animal welfare must be carefully considered.

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Autoimmunity; SLE; lupus nephritis; sex-specific differences; animal model; MRL-Fas^{lpr}

Introduction

Chronic autoimmune diseases, like systemic lupus erythematosus (SLE), are characterized by a dysregulated immune response with increased inflammatory processes and autoantibody production. In SLE, various organs are affected and damaged as a consequence of inflammation and deposition of immune complexes. Lupus nephritis (LN) is one of the most serious manifestations in SLE and leads to increased mortality. The participation of various cell types, not just immune cells, and the variety of inflammatory mediators as well as autoantibodies make these diseases complex and the therapy difficult. Most human autoimmune diseases, such as SLE, rheumatoid arthritis (RA) and Sjögren syndrome, display a sex bias toward women (SLE: 9:1; RA: 3:1; Sjögren syndrome: 13:1) [1,2]. It has been shown that genes on sex chromosomes and sex hormones play an important role in the development of autoimmune disease [3–5].

Reliable models are necessary in the research of chronic autoimmune diseases and the development of new therapeutic targets and options. Due to the complexity of these diseases, animal models are a typical research tool, especially to analyze complex interrelationships between different cell types. In addition, the effects of hormones or new therapeutic substances can be analyzed in a systemic context and not just on a limited number of cells *in vitro*. Depending on the question to be investigated, different models are suitable and the advantages as well as disadvantages must be carefully weighed up [6]. There are also various mouse models for SLE that can be distinguished between spontaneously diseased (for example NZB/W F1 and MRL-mice) and induced models (for example "pristane induced lupus" and "graft versus host disease induced lupus").

MRL-Fas^{lpr} mice are a popular model for SLE but are also occasionally used in the research of Sjögren´s syndrome and RA. These mice develop spontaneously an autoimmune disease with similarities to human SLE. These include a similar spectrum of autoantibodies, like antinuclear antibodies (ANA), anti-dsDNA- and anti-smith-antibodies, as well as deposition of immune complexes that lead to organ damage. Additionally, immune cells from MRL-Fas^{lpr} mice, like macrophages and T-cells, show the same dysregulation as in human SLE-patients. Lymphadenopathy and splenomegaly are also observed in MRL-Fas^{lpr} mice and are possible symptoms in SLE-patients. In addition, skin lesions show a comparable histopathological pattern between patients and the mouse model [7]. The development of severe, rapidly progressive glomerulonephritis also makes MRL-Fas^{lpr} mice a useful model for chronic

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kidney damage with an autoimmune background, such as LN. In contrast to human autoimmune diseases, male MRL-Fas^{lpr} mice display a delayed disease progression but the percentage of affected males is not reduced compared to MRL-Fas^{lpr} females. The delayed onset is one reason why most studies are performed in female MRL-Fas^{lpr} mice.

Because animal models continue to play an important role in research, animal welfare and the 3 R-principle (Replacement, Reduction, and Refinement) are becoming increasingly important in this field [8]. In the context of "reduction", the question arises whether male MRL-Fas^{lpr} mice, that make 50 % of MRL-Fas^{lpr} offspring, can also be used to investigate autoimmune processes and autoimmune kidney damage. To answer this question, we compared phenotypical symptoms, immune cell composition and inflammation as well damage markers in MRL-Fas^{lpr} mice of both sexes with the focus on kidney and spleen.

Methods

Animal experiments

We used surplus material from female and male MRL-Fas^{lpr} mice (1–6 month) from other studies. All mice were housed in accordance with standard animal care requirements. The material used comes from animal studies approved by the ethical board (G14-1-054 and G17-1-074). The studies were performed in accordance with the German animal protection law and the guidelines for the use of experimental animals as stipulated by the Guide of Care and Use of Laboratory Animals of the National Institutes of Health.

Phenotyping

In connection with an animal study, the female ($n = 27$) and male ($n = 18$) MRL-Fas^{lpr} mice were phenotyped over a period of five month every week. Therefore, the lymph node swelling on neck, forelegs and hind legs were scored as described before [9]. The severity of visible skin lesions were scored as follows: 0 = none; 1 = mild (snout and ears); 2 = moderate (<2 cm; snout, ears, intrascapular); 3 = severe (>2 cm; nout, ears, intrascapular). In addition, spontaneous urine was collected at this time. When taking the tissue samples with three and five month of age, the mice, kidney, spleen and lymph nodes were weighed, to calculate the organ to body weight ratio.

ELISA

The albuminuria of the collected spontaneous spot urine from up to 23 female and 18 male MRL-Fas^{lpr} mice for each time point was measured with mouse Albumin ELISA Quantitation Kit (Bethyl Laboratories) according to the manufacturer's instructions.

Renal histopathology

Histopathology of kidney sections from 11 female and 8 male MRL Fas^{lpr} mice was assessed as described before [9]. Quickly, kidneys were fixed in 10 % neutral buffered formalin for 24 h before embedded in paraffin. Paraffin sections (4 µm) were stained with periodic acid-Schiff reagent as well as hematoxylin and eosin. The histopathological score was evaluated using the following parameters. Glomerular pathology was assessed by examining 20 glomerular cross-sections (gcs) per kidney and scoring each glomerulus on a semiquantitative scale: 0 = normal (35–40 cells/gcs); 1 = mild (glomeruli with few lesions showing slight proliferative changes, mild hypercellularity (41–50 cells/gcs), and/or minor exudation); 2 = moderate (glomeruli with moderate hypercellularity (51–60 cells/gcs), including segmental and/or diffuse proliferative changes, hyalinosis, and moderate exudates); and 3 = severe (glomeruli with segmental or global sclerosis and/or severe hypercellularity (>60 cells/gcs), necrosis, crescent formation, and heavy exudation). Interstitial/tubular pathology was assessed semiquantitatively on a scale of 0–3 in 10 randomly selected high power fields (×400). We determined the largest and average number of infiltrates and damaged tubules and adjusted the grading system accordingly: 0 = normal, 1 = mild, 2 = moderate, and 3 = maximum.

Flow cytometry

Single-cell suspension from kidney, spleen and blood of 3.5-month-old female ($n = 4$–9) and male ($n = 4$–9) MRL-Fas^{lpr} mice were prepared and stained as previously described [10]. Antibodies used are listed in Table 1. 0.5 to 1.0 x 10^6 cells per sample were collected and analyzed with a FACS Calibur (Becton Dickinson) and Flowjo software (Tree Star). The flow cytometry slides were analyzed as follows: CD45$^+$ cells were primarily differentiated from other cell types like tubular epithelia cells or dead cells. Thus, a gate was set for CD45$^+$ cells, and in this population, the gate was set to CD68$^+$ or CD4$^+$ cells, respectively. The resulting subpopulations of CD68$^+$ or CD4$^+$ cells were then evaluated and analyzed for the activity markers shown.

mRNA-expression analysis in kidney, spleen and lymph nodes of female and male MRL-Fas^{lpr} mice

The innuPREP RNA Mini Kit 2.0 (Analytik Jena) was used for total RNA isolation from homogenized tissue (kidney, spleen, lymph nodes) according to the instructions for use as previously described [11]. mRNA-Expression was quantified from 1, 3.5 and 6 months old female ($n = 5$) and male ($n = 5$) MRL Fas^{lpr} mice with a two-step real-time RT-PCR

Table 1. Antibodies for flow cytometry.

Antibody	Clone no.	Company (Catalog#)
CD68	FA-11	Biolegend (137013)
CD4	RM4-5	BD Biosciences (560758)
CD69	H1.2F3	eBioscience (45-0691-82)
Ly6c	HK 1.4	Biolegend (128028)
CD86	GL1	eBioscience (17-0862-81)
IFNγ	XMG1.2	BD Biosciences (560758)
TNFα	MP6-XT22	eBioscience (12-7321-81)
IL-4	TC11-18H10.1	BD Biosciences (560758)
IL-17	11B11	BD Biosciences (560758)

(qRT-PCR). Reverse transcription was performed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and qRT-PCR analysis with PowerTrack™SYBR Green Mastermix (Applied Biosystems) according to the manufacturer's instructions. qRT-PCR was performed in a qTOWER3G cycler (Analytik Jena) with the qRT-PCR program: initial denaturation by 95 °C 2 min and 45 cycles with 95 °C 5 sec. denaturation and 60 °C 30 sec. hybridization and elongation. Oligonucleotides used are listed in Table 2. Specific gene expression was normalized to β-actin mRNA-expression and the relative mRNA-expression was calculated with 2(ΔΔC(T)) method [12].

Statistics

All data represent means ± SEM and were analyzed by GraphPad Prism 9.5.1. Statistical differences were determined using classical t-test analyses. In case of more than two means, one-way or two-way ANOVA analysis was used, followed by Boneferroni's multiple comparisons test. Repeated measurements were analyzed with Mixed-effects analysis followed by Boneferroni's multiple comparisons test.

Results

Female MRL-Fas^{lpr} mice show strong phenotypic changes earlier than male mice

MRL-Fas^{lpr} mice develop an autoimmune disease similar to human SLE, inclusive a rapid progressive nephritis, comparable to LN class III and IV in SLE patients. Different to human SLE MRL-Fas^{lpr} mice of both sexes develop the autoimmune disease. It has been described that female mice have a more rapid and severe disease progression than male mice [13], therefore predominantly female MRL-Fas^{lpr} mice are used in experiments. For a first assessment of the differences between female and male MRL-Fas^{lpr} mice, we compared survival, lymphadenopathy, skin lesions, and albuminuria over time.

For survival, we observed 11 female and 10 male MRL-Fas^{lpr} mice over 6 month (Figure 1(a)). The first female mice died between 3 and 4 month and after the 6th month less than 50 % female mice survived. In contrast, the first male MRL-Fas^{lpr} mice died between 4 and 5 month and 70 % of the male mice survived after the observation period. Albuminuria was measured in spontaneous urine over a time of 5 months and showed an increase in both sexes (Figure 1(b)). After 3 month, albuminuria was a bit more severe in female than in male mice. The skin lesions and the lymph node size differed in female and male mice from the age of 3 months, with the female animals showing a more rapid increase in both parameters (Figure 1(c)). Due to a greater variance in the development of skin lesions and albuminuria, we only detected at 4 month of age a significant difference between female and male mice. The increase of lymph node size was more homogenous between female and male MRL-Fas^{lpr} mice. Female mice had significant bigger lymph nodes at an age of 3 month. At the age of 5 months, the scores of the lymph nodes converge again.

In addition to the described observations, the organ to body weight ratio of kidney, spleen and lymph nodes from female and male MRL-Fas^{lpr} mice with 3 and 5 month were collected (Figure 1(d)). The calculated ratio of kidney weight showed no sex-specific differences at both ages. With regard to kidney damage, the histopathology showed no significant differences between the two sexes analyzed at 3 and 5 month of age (Figure 1(e)). The organ to body weight ratio of spleen and lymph nodes was higher in female mice than in male mice at 3 and 5 month of age and reaching statistical significance for spleen and lymph nodes at 3 and 5 months of age, respectively. These differences were statistically significant at 3 month of age for spleen and 5 month of age for lymph nodes.

Increased ratio of CD4$^+$ and CD68$^+$ cells in kidney and spleen of 3.5-month-old female MRL-fas^{lpr} mice compared to male mice

Immune cells play an important role in the pathogeneses of SLE. Monocytes and macrophages are key factors of the innate immune system, they are important regulators of the adaptive immune system and are highly involved in SLE [14,15]. Characteristic for these cells in SLE is the increased production of inflammatory mediators and the reduced phagocytic activity. For this reason, we characterized the monocytes and macrophages in kidneys, spleen and blood of 3.5-month-old female and male MRL-Fas^{lpr} mice by FACS analysis (Figure 2(a)). Therefore, we used the markers CD68$^+$, CD68$^+$CD69$^+$, CD68$^+$Ly6c$^+$, CD68$^+$CD86$^+$, CD68$^+$IFNγ$^+$ and CD68$^+$TNFα$^+$ and calculated the percentage of positive cells.

Table 2. Oligonucleotides used for qRT-PCR.

Oligo (murin)	5′-primer	3′-primer	Genbank accession Nr.
β-Actin	Qiagen-Mm_Actb_2_SG		
TNFα	CATCTTCTCAAAATTCGAGTGACA	TGGGAGTAGACAAGGTACAACCC	NM013693.3
IFNγ	Qiagen-Mm_Ifng_1_SG		
IFNα	TGCTGGCTGTGAGGACATAC	TCCTCTCCACACTTTGTCTCAG	NM010504.3
IL-6	Qiagen-Mm_IL6_1_SG		
S100A8	CTCCGTCTTCAAGACATCGTTTG	TCATTCTTGTAGAGGGCATGG TG	NM013650.2
IL-34	Qiagen-Mm_IL34_1_SG		
Vimentin	TCC AGA GAG AGG AAG CCG AA	AAG GTC AAG TGC CAG AG	NM011701.4
MMP3	TGGAGATGCTCACTTTGACG	ATGGAAACGGGACAAGTCTG	NM010809.2
TGFβ	TGACGTGACTGGAGTTGTACGG	GGTTCATGTCATGGATGGTGC	NM011577.2
CSF1	Qiagen-Mm_Csf1_2_SG		
KIM-1	ATGCCCATCTTCTGCTTGTC	GTGTGGGAATCTCTGGTTTAACTT	NM134248.2

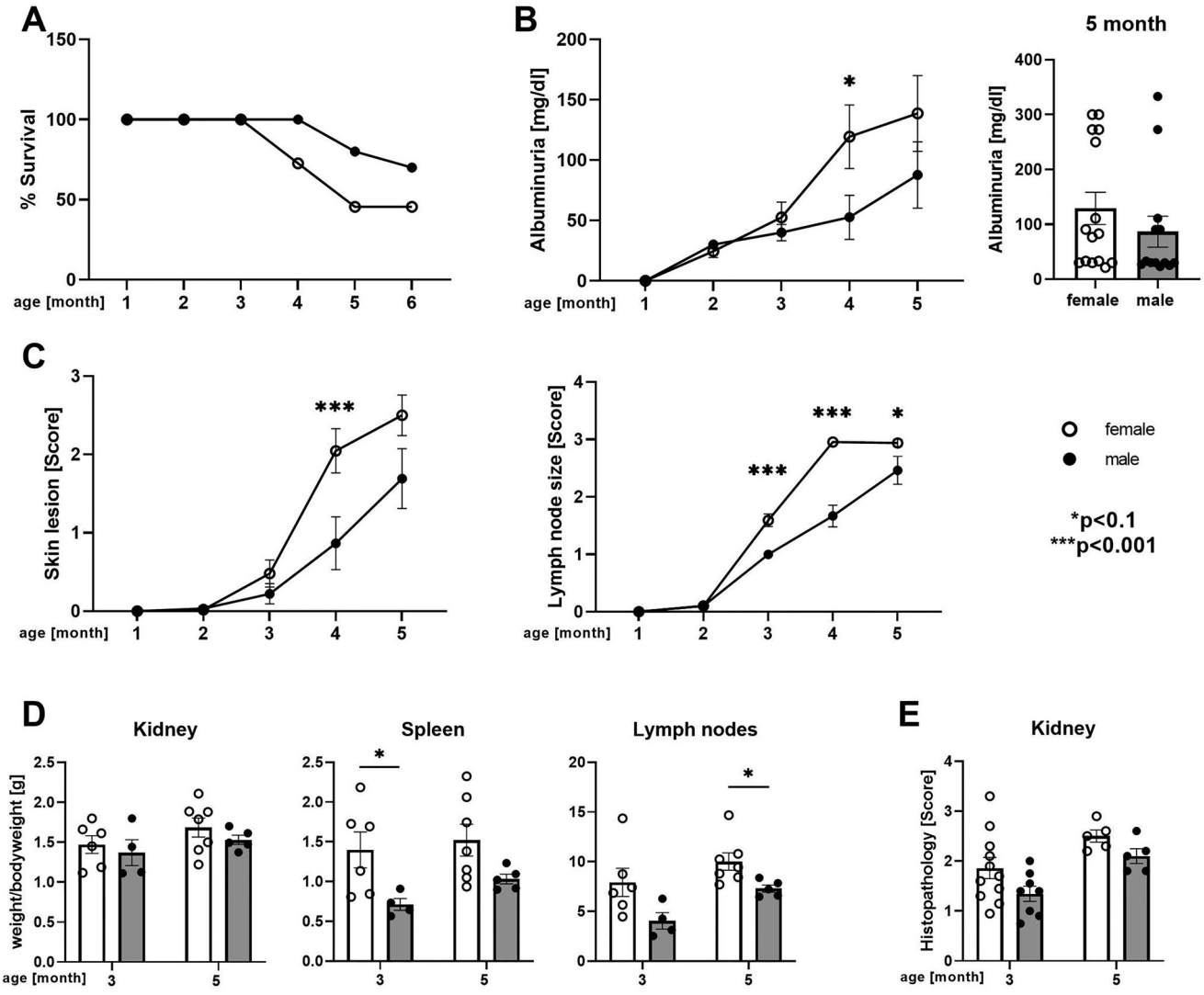

Figure 1. Female MRL-Fas^{lpr} mice show strong phenotypic changes earlier than male mice. (a) Survival curve of female ($n = 11$) and male ($n = 10$) MRL-Fas^{lpr} mice over 6 month. (b) Albuminuria of female and male MRL-Fas^{lpr} mice. Measured over 5 months with spontaneous urine. (c) Scores of skin lesions and lymph node size in MRL-Fas^{lpr} mice of both sexes over 5 months. Skin lesions were scored on face and neck as well as lymph node size on neck, foreleg and hind leg of each mice. (d) Organ to body weight ratio of kidney, spleen and lymph nodes from female and male MRL-Fas^{lpr} mice with 3 and 5 month were calculated. (e) Renal histopathology of female and male MRL-Fas^{lpr} mice were assessed by hematoxylin-eosin (HE) and periodic acid-Schiff (PAS) staining and were scored blinded in a semi-quantitative manner. All data are represented as mean (± SEM). (*: $p < 0.1$; ***: $p < 0{,}001$, mixed-effects analysis (A-C), t-test (D + E)).

In female kidneys, we measured significant more monocytes and macrophages (CD68$^+$) as well as significant increased percentage of CD68$^+$CD69$^+$ activated monocytes/macrophages and CD68$^+$CD86$^+$ macrophages compared with the cells from male mice. The total CD68$^+$ monocyte and macrophage population in the spleen showed no sex-specific differences, but in female spleens the frequency of all measured activated subpopulations of these cells were significantly higher. In the blood of the MRL-Fas^{lpr} mice only CD68$^+$CD86$^+$ macrophages showed a significant sex-specific bias toward female mice (Figure 2(a)).

In addition to monocytes and macrophages, T-cells are also important in the pathogeneses of SLE. They play a dominant role in cell-differentiation, –activation and produce inflammatory mediators. In autoimmune diseases, like SLE, the T-cell regulatory mechanisms are dysregulated, which leads to an enhanced T-cell activation. For that reason, we analyzed the sex-specific differences in T-cell populations in these mice as well (Figure 2(b)).

In kidneys and spleens of female MRL-Fas^{lpr} mice, we found significant more CD45$^+$ and CD4$^+$ T-cells compared to the age-matched male animals. The CD4$^+$ CD69$^+$ (activated T-cells), CD4$^+$ CD86$^+$ (proliferating T-cells), CD4$^+$ IFNγ$^+$ (Th1-cells) and CD4$^+$ IL-17$^+$ (Th17-cells) T-cell subpopulations were also significant higher in kidneys of female MRL-Fas^{lpr} mice. Otherwise, in male kidneys significantly more CD4$^+$ IL-4$^+$ Th2-cells were found as in female ones. Only the CD4$^+$ TNFα$^+$ T-cell subpopulation had no sex-specific differences in kidneys of these mice. Similar to the situation in the kidney significantly more CD4$^+$, CD4$^+$ CD69$^+$ and CD4$^+$ IFNγ$^+$ cells were detected in spleens of female MRL-Fas^{lpr} mice compared to the spleens of male mice. All other measured subpopulations showed no sex-specific differences in spleens of MRL-Fas^{lpr} mice. Additionally, in the blood of MRL-Fas^{lpr} mice no sex-specific

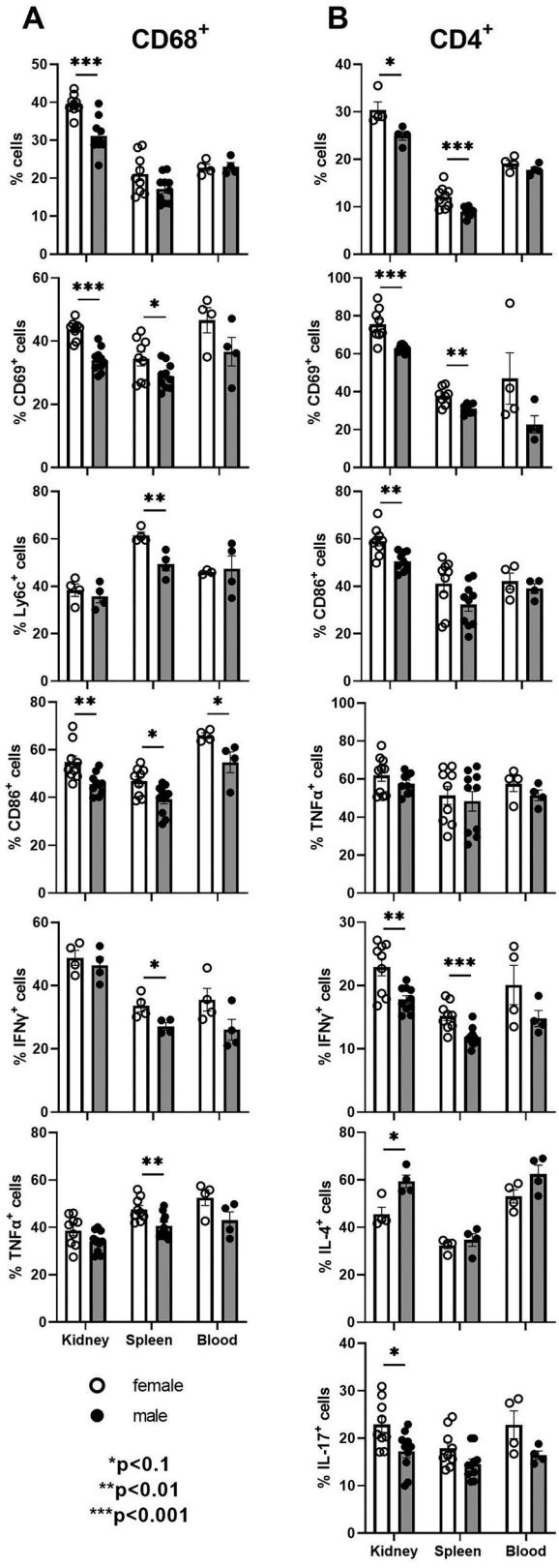

Figure 2. Increased ratio of $CD4^+$ and $CD68^+$ cells in kidney and spleen of 3.5-month-old female MRL-Fas^{lpr} mice compared to male mice. Cells of kidney, spleen and blood were isolated from 3.5-month-old MRL-Fas^{lpr} mice of both sexes. Flow cytometry was used to analyze (a) $CD68^+$ (monocytes and macrophages), $CD68^+CD69^+$ (activated monocytes/macrophages), $CD68^+Ly6c^+$ (macrophages), $CD68^+CD86^+$/$CD68^+IFN\gamma^+$ (classic activated "M1" macrophages) and $CD68^+TNF\alpha^+$ (activated macrophages) as well as (b) $CD4^+$ (T-cells), $CD4^+CD69^+$ (activated T-cells), $CD4^+CD86^+$ (proliferating T-cells), $CD4^+TNF\alpha^+$ (pro-inflammatory T-cells), $CD4^+IFN\gamma^+$ (Th1-cells), $CD4^+IL\text{-}4^+$ (Th2-cells) and $CD4^+IL\text{-}17^+$ (Th17-cells). All data are presented as mean ± SEM. (*: $p < 0.1$; **: $p < 0.01$; ***: $p < 0.001$, t-test; $n = 4$–9).

differences in the cell distribution of $CD4^+$ subpopulations were detected.

Expression of inflammation, fibrosis and disease markers in kidney and spleen of one to 6-month-old MRL-Fas^{lpr} mice of both sexes

For an overview about sex-specific differences in inflammation, fibrosis and disease marker expression in kidney, spleen (Figure 3) as well as lymph nodes (data not shown) we compared mRNA-expression of female and male MRL-Fas^{lpr} mice at 1, 3.5 and 5 month of age. We used a selection of known markers characteristic for SLE (Tumour necrosis factor α (TNFα), S100 calcium-binding protein A8 (S100A8), Interleukin (IL)-6, IL-34, Interferon (IFN) α and IFNγ) and kidney damage (Transforming growth factor β (TGFβ), Vimentin, Matrix metalloproteinase 3 (MMP3), Colony stimulation factor 1 (CSF1) and Kidney injury molecule 1 (KIM1)) (Figure 3).

The mRNA-expression of the general inflammation marker TNFα showed no significant difference between female and male organs to all analyzed time points. However, in the spleen we observed a reduction of TNFα-mRNA-expression over the time in both sexes. In contrast, the mRNA-expression of IFNγ increased over the time in kidney and spleen in female as well as male organs but without reaching significance. mRNA-expression of IFNα showed a significant increase in female kidneys compared to males at 6 month of age. In spleens, the IFNα-expression was in 1-month-old male mice significantly higher. In addition, we observed a similar reduction of IFNα mRNA-expression in both sexes over the time as comparable to TNFα-expression (Figure 3(a)).

Sex-specific differences of IL-6 mRNA-expression were detected in kidneys of 1-month-old MRL-Fas^{lpr} mice, here the males had a significant higher expression. With 3.5 and 6 months, in both sexes the IL-6 expression was highly increased in the kidneys as well as in spleens, but no significant sex-specific bias was detected (Figure 3(a)). The expression of the inflammation marker S100A8 was significantly higher in the lymph nodes from one-month-old male mice compared to females at the same age (data not shown). For IL-34 expression, we observed the most sex-specific differences in kidneys and spleens in MRL-Fas^{lpr} mice. In 1-month-old mice a significant higher IL-34 expression in male kidneys as well as spleens compared to females was detected. These significant differences were also observed in kidneys of 6-month-old mice. In contrast, the IL-34 mRNA-expression in spleens of female mice at 3.5 month of age was significant higher than in males (Figure 3(a)).

mRNA-expression of the fibrosis and disease markers TGFβ, vimentin, MMP3, CSF1 and KIM1 was quantified in kidneys of these mice (Figure 3(b)). The mRNA-expression of TGFβ was higher in female MRL-Fas^{lpr} mice at 6 month of age compared to male mice. Overall, in both sexes the TGFβ-, vimentin-, MMP3- and CSF1-expression were increased over the time, while the KIM1-expression increased only between 1 and 3.5 month of age.

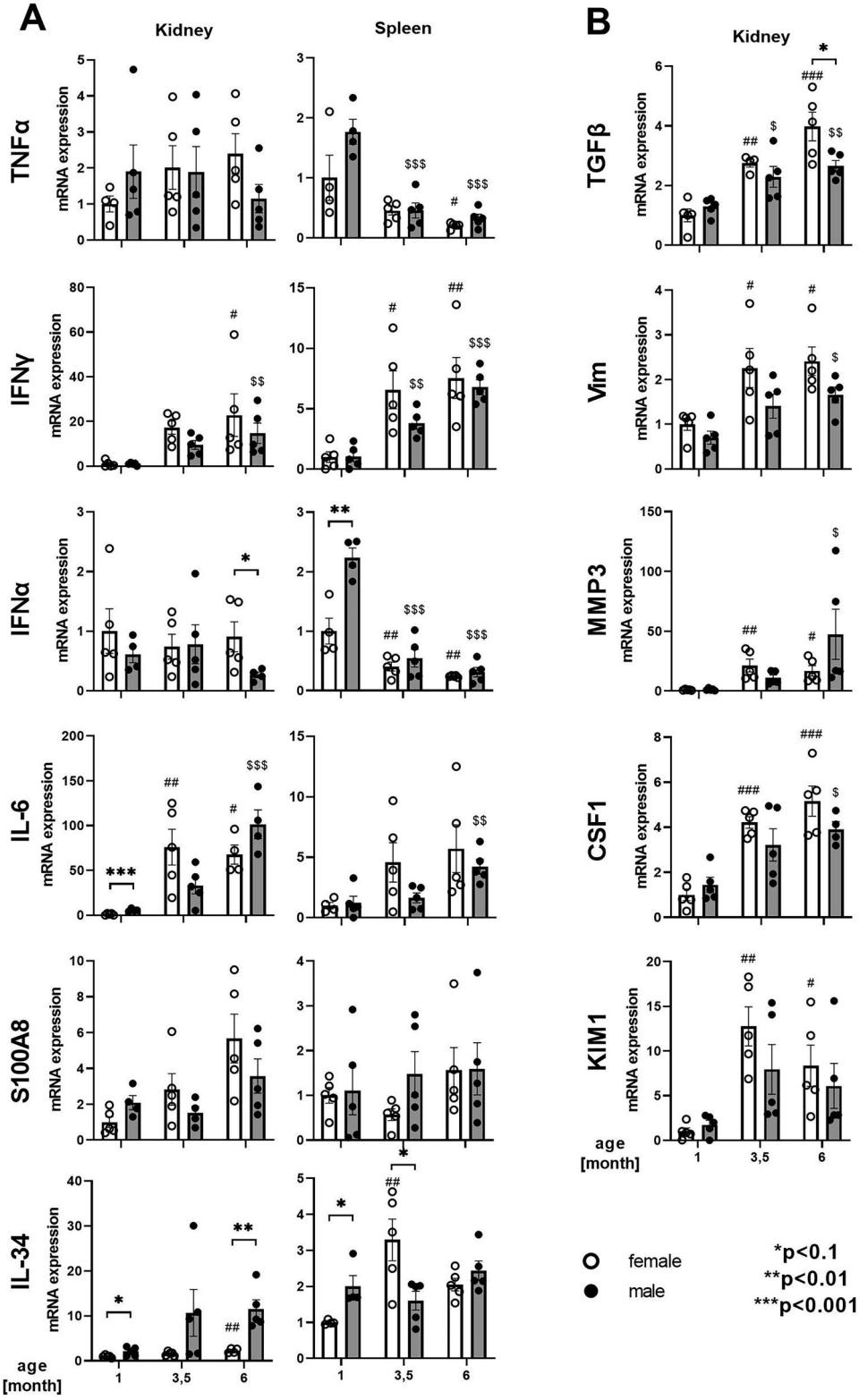

Figure 3. Expression of inflammation, fibrosis and disease marker in kidney and spleen of one to 6-month-old MRL-Fas^{lpr} mice of both sexes. mRNA-expression analysis were performed with total RNA from kidney and spleen of 1, 3.5 and 6-month-old female and male MRL-Fas^{lpr} of (a) inflammation marker (TNFα, IFNγ, IFNα, IL-6, S100A8 and IL-34) as well as (b) fibrosis and disease marker (TGFβ, vim, MMP3, CSF1 and KIM1). Data were normalized to the housekeeping gene β-Actin. All data are shown as mean ± SEM compared to one-month-old females. (differences between both sexes: *: $p < 0.1$; **: $p < 0.01$; ***: $p < 0.001$, t-test; mRNA-expression over the time in females, compared to one-month-old females: #: $p < 0.1$; ##: $p < 0.01$; ###: $p < 0.001$, one-way ANOVA; mRNA-expression over the time in males, compared to one-month-old males: $: $p < 0.1$; $$: $p < 0.01$; $$$: $p < 0.001$, one-way ANOVA; $n = 5$).

Discussion

Despite the increasingly present animal welfare and the 3 R-principle (Replacement, Reduction, and Refinement) as well as the increase of complex *in vitro* models, animal models continue to play an important role in research. There are currently no adequate alternatives to animal

testing in different parts of the research such as in the investigation of interactions of many different cell types and the development of new therapeutic options [16–18]. In the research of SLE various lupus models were used to study different aspects of the diseases [6]. In this study, we evaluated MRL-Fas^{lpr} mice that spontaneously develop a lupus-like disease. The symptoms of these mice show many parallels to human SLE, including autoimmune processes with inflammation, autoantibody production and immune complex deposition. Similar organs are affected in murine lupus and human SLE, like damage of the kidney (LN), heart, lung, central nervous system and skin (skin lesions). One major difference between MRL-Fas^{lpr} mice and human SLE is the strict female sex-bias of 9:1 in human SLE, whereas in the mouse model the disease develops similarly in both sexes. It has been described that male MRL-Fas^{lpr} mice show a weaker and slower disease progression compared to female mice [13]. Because of the strong sex-bias in human SLE and the delayed disease development in male MRL-Fas^{lpr} mice, female MRL-Fas^{lpr} mice are preferentially used in current research. Additionally, it has been described, that sex hormones and sex chromosomes have influence on autoimmune disease in humans as well as mice [3–5]. In consequence, this leads to many excessive male MRL-Fas^{lpr} mice that cannot be used in animal experiments. Even if there is a strong sex bias toward females in human SLE, it has been shown that occurrence of SLE is not sex-specific [19]. Therefore, we analyzed male and female MRL-Fas^{lpr} mice for phenotypic symptoms, immune cell composition and inflammation as well as disease marker with a focus on kidneys and spleen to see whether male mice can also be used in investigation of pathogenesis of SLE and LN.

As described before by Andrews et al. in 1978 [13], we observed that male MRL-Fas^{lpr} mice exhibit delayed disease development (Figure 1). Especially between 3 and 4 months, female MRL-Fas^{lpr} mice develop more severe symptoms such as swollen lymph nodes and skin lesions (Figure 1(c)). With 5 month, the phenotypic differences between males and females hardly exist anymore. Andrew et al. showed also that the autoantibody concentration between both sexes is different between 3 and 4 month but adjusts at an age of 5 to 6 month [13]. Other studies showed also no sex-specific differences in autoantibody and immune complexes concentrations in 5-month-old MRL-Fas^{lpr} mice [13,20,21]. In accordance, we observed a substantially reduced sex-bias of phenotypical symptoms as well as autoantibody and immune complexes in a late stage of disease in this mouse model compared to an early stage.

In accordance with the sex-dependent differences in phenotypic symptoms of MRL-Fas^{lpr} mice at 3 to 4 months, we were also able to detect differences in immune cell composition at the same age. Overall, there was an increased percentage of CD68$^+$ monocytes and macrophages as well as CD4$^+$ T-cells in the kidneys and spleen of female mice compared to the males (Figure 2). This highlights the delayed disease development in male MRL-Fas^{lpr} mice. Furthermore, the significantly increased CD4$^+$ IL-4$^+$ Th2-cells in male MRL-Fas^{lpr} mice could contribute to the delayed disease development in the male mice. Santiago et al. showed that a constitutive expression of IL-4 in transgenic lupus mice protects these mice from lethal lupus-like glomerulonephritis [22]. In addition, it has been discussed that Th2 cytokines may help to suppress inflammation in early RA [23].

The excessive immune reaction and the resulting inflammation is the underlying cause of SLE, which secondarily leads to organ damage. To obtain an overview of inflammation in the kidney, spleen and lymph nodes in MRL-Fas^{lpr} mice of both sexes over the time, the mRNA-expression of various disease-associated mediators (TNFα, S100A8, IL-6, IL-34, IFNα and IFNγ) was quantified (Figure 3(a)). Sex-specific differences in the expression of these genes were mostly observed in 1-month-old mice. In contrast to our expectations, the expression of some genes was significantly increased in male MRL-Fas^{lpr} mice compared with age-matched females (IL-6, IL-34 in kidneys; IFNα, IL-34 in spleen; S100A8 in lymph nodes). Especially the differentially expressed interleukins and interferons are described as driving factors of SLE in the current literature [24–26]. Even the S100A8 expression has been discussed as marker for disease activity [27]. Why 4-week-old male MRL-Fas^{lpr} mice show higher expression of some mediators than females, while male mice show delayed disease development, is unclear. In current literature, there are no comparable analysis in such young MRL-Fas^{lpr} mice or in SLE patients before diagnosis. However, these observed sex-specific differences were largely lost in the development of lupus-like disease in the MRL-Fas^{lpr} mice. Exclusively IL-34 expression was higher in male MRL-Fas^{lpr} mice than in females at each time point measured. However, an increase in expression, as already described [24], can be observed over time in both sexes. Rezaei et al. discussed a possible sex-specific expression of IL-34 in the study on chronic inflammatory demyelinating polyneuropathies, although further studies are still missing [28].

The mRNA-expression of different fibrosis and kidney damage markers in the kidney of MRL-Fas^{lpr} mice (Figure 3(b)) largely showed the expected increase in both sexes over the observed time of disease development (CSF1, MMP3, Vimentin and TGFβ). Significant sex-specific differences of these makers were only observed for TGFβ expression in kidneys of 6-month-old mice, although the expression increases over time in both sexes.

MRL-Fas^{lpr} mice not only developed marked renal pathology resembling human LN, but also lymphadenopathy and splenomegaly. In addition, the mouse model is also used to investigate other manifestations of SLE. This includes damage of organs like skin, lung, pancreas as well as salivary gland. Some studies that analyze parts of these characteristics of SLE, examined both sexes of MRL-Fas^{lpr} mice and found some sex-specific differences. Differences were found at pharmacokinetics [29], wound healing [30,31], lung pathology [32], lacrimal glands [33,34] and the development of autoimmune pancreatitis [35]. Additionally, female MRL-Fas^{lpr} mice have a greater hearing loss [21] and an earlier manifestation of depression [36] compared to age-matched male mice. At the same time, sex-independent parameters could be identified in some of these studies. In addition to the sex-independent manifestations already

described in this study (lymphadenopathy, splenomegaly, glomerulonephritis and albuminuria), no differences were found in autoantibody production (ANA, anti-dsDNA), immune complexes, hematocrit, inflammation in submandibular tissue and heart pathology of MRL-Fas^{lpr} mice at an age of ≥5 months [13,20,21,34]. During this period (5–6 month) the number of deceased females is also higher than that of males, which can be attributed to the faster progression of disease in female mice. This can lead to a distortion of the data, as the animals with particularly severe symptoms have already died, and this must be taken into account in the analyses.

Overall, we were able to show that sex-specific differences in the phenotypic expression of the lupus-like disease and the mRNA-expression of disease- and damage-associated markers in kidney and spleen of MRL-Fas^{lpr} mice occur primarily in young and middle aged mice (1 to 4 month). After the onset of disease in both sexes at around 5 month, the differences between males and females in the examined parameters decreased, so there were substantially less significant differences. Reasons for the described sex-dependent differences in other organs of MRL-Fas^{lpr} mice are maybe due to the influences of sex hormones. Blankenhorn et al. were able to show that wound healing in MRL-Fas^{lpr} mice depends on testosterone level [30]. In this study, castrated male mice showed the same improved wound healing as female MRL-Fas^{lpr} mice. Whether all other sex-specific differences described before, can also be attributed to sex hormones needs to be further investigated. In addition to the sex hormones, also sex chromosomes play an important role for the sex-bias in autoimmune diseases such as SLE [37]. Observations suggest that the number of X-chromosomes may be an important factor for the sex-bias in SLE, since men with Klinefelter syndrome (47, XXY) have the same probability of developing SLE as women (46, XX) [38,39]. One of the reasons for this seems to be (tissue-specific) incomplete inactivation of the second X-chromosome [37]. Many immune-related genes (like toll-like receptor 7 and CD40 ligand) located on the X-chromosome are increasingly expressed in autoimmune diseases with a sex-bias, most probably due to incomplete inactivation of the X-chromosome [40]. Jiwrajka et al. shows an impaired X-chromosome inactivation in T-cells from spontaneous lupus-mouse models and a female-biased upregulation of immune-related genes in activated T-cells of these mice [41]. The extent to which this effect plays a role in the sex-related differences described above requires further investigations.

In summary, as part of 3 R-principle and animal welfare male MRL-Fas^{lpr} mice can be used as independent groups in studies, depending on the scientific question. Sex-dependent differences of phenotypic symptoms, autoantibodies and immune complexes as well as kidney damage were not observed in MRL-Fas^{lpr} mice older than 5 months. However, the suitability of male MRL-Fas^{lpr} mice for studies must be carefully checked beforehand and, if necessary, small comparative analyses should be carried out.

Disclosure statement

The authors report there are no competing interests to declare.

Ethics statement

The animal study was reviewed and approved by the Landesuntersuchungsamt Rheinland-Pfalz, Referat 23, Mainzer Straße 112, 56068 Koblenz.

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