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PAPER

Skin disease is prevented but nephritis is accelerated by multiple pregnancies in autoimmune MRL/LPR mice

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The role of pregnancy in the progression of systemic lupus erythematosus (SLE) is still poorly understood. We analysed the effect of repeated pregnancies in MRL/lpr mice, a murine model of SLE. Seven-week old female mice were used: multiparous mice underwent three consecutive pregnancies (M); age-matched virgin mice served as controls (V). Animals were harvested at 20 weeks of age. Skin lesions were characterized as hair loss and scabs in the dorsum of the neck. Virgin skins showed thickened dermis, fibrosis and mononuclear cell infiltrates, which were practically absent in M. This was accompanied by higher IFN-γ and lower IL-10 mRNA expression levels in V compared to M skin. Plasma IFN-γ protein levels were also upregulated in V versus M. However, survival and kidney function were dramatically reduced and accompanied by hypertension after multiple pregnancies. Kidney histology also showed markedly increased renal lesions in M. In contrast to plasma and skin levels, both IL-10 and IFN-γ mRNA were lower in the kidneys of V versus M mice. Concluding our findings, the pathomechanisms of lupus kidney and skin disease may be regulated differently at the organ level during pregnancy. Both IFN-γ and IL-10 may be important regulatory cytokines at the local level. *Lupus* (2007) 00, 1–13

Key words: cytokines; kidney; mouse; skin; systemic lupus erythematosus

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by pathogenic autoantibody overproduction[1] and a significant prevalence.[2] It is a complex disease affecting several organs, primarily the kidneys, skin and joints. The majority of SLE patients are female in fertile age.[3] Thus, understanding the role of pregnancy in SLE is of high relevance and would help to elaborate therapeutic strategies for women suffering from autoimmunity and who have still a desire to procreate.

The MRL/MpJ-*Fas*^{lpr} mouse strain (MRL/lpr) is a widely used model of SLE.[4] MRL/lpr mice spontaneously develop a severe autoimmune disease, which shares most clinical signs and symptoms of human lupus. The lpr mutation affects the Fas gene,[5] resulting in a severe defect of apoptosis and proliferation of abnormal lymphocytes ($CD4^-$, $CD8^-$ double negative (DN), $B220^+$, $CD3^+$ T-lymphocytes[6]) accompanied by abnormal lymphocyte function[7] and autoantibody production[8] leading to severe glomerulonephritis.[9] Skin pathology is characterized by immunoglobulin deposition and cellular infiltration[10] and the lesions mimic those in human lupus.[11]

Cytokines are one of the major regulators of SLE. Murine as well as human lupus is associated with a predominant proinflammatory cytokine response. In MRL/lpr mice elevated IFN-γ and significant reduction in IL-4 and IL-10 cytokine production has been demonstrated.[12] Recent data indicate that human lupus nephritis is also associated with IFN-γ upregulation and IL-4, IL-10 downregulation.[13,14]

IL-10 stimulates B-lymphocyte functions[15] and acts as an anti-inflammatory cytokine inhibiting cellular infiltration.[16] IL-10 overproduction has been demonstrated to be associated with autoantibody production in lupus patients[17] while IL-10 serum level has been correlated disease activity.[18] However, in the MRL/lpr model, IL-10 had suppressive effects against T-lymphocyte driven autoimmunity.[19]

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Pregnancy induces a complex and only a partially understood change in cytokine production. Both murine and human pregnancies are proposed to be associated with a shift towards anti-inflammatory cytokine predominance,[20,21] and decreased proinflammatory cytokine production.[22,23] The influence of pregnancy on systemic autoimmunity is controversial. Pregnancy may exacerbate human SLE according to some reports.[24,25] Some recent reports describe an adverse effect of gestation on disease progression,[26,27] but not according to others.[28,29] To the best of our knowledge, there is no data on cytokine productions during MRL/lpr pregnancy.

We hypothesized that, due to an anti-inflammatory cytokine shift observed in pregnancy of normal mice, repeated pregnancies may delay the progression of skin and kidney disease in MRL/lpr mice.

Materials and methods

Animals

MRL/lpr (MRL/MpJ-*fas*^{Tnfsrlpr}, MHC haplotype: H2:k) female mice (Jackson Laboratories, USA) had access to standard rodent chow (Altromin, Germany) and water ad libitum. Mice were divided randomly into two groups at seven weeks of age. The multiparous (M) group females were mated three times consecutively with NMRI males (Charles River, Hungary) and weaned immediately after parturition to exclude the possible immunmodulatory effects of lactation.[30] NMRI males (MHC haplotype: H2:q) were used for allo-mating. Non-syngeneic matings were performed to model allogenic human pregnancies. Pregnancy duration was 21 days. Females had five days rest after parturition, before the next mating. MRL/lpr females were always paired with the same NMRI male (see Figure 1 for study design). Mice of the second virgin (V) group were housed apart, two virgin females were kept per cage to provide the same social environment in both groups. The experiments were carried out in four parts. The first experiment (n = 12/group) was a survival experiment; to determine time of harvest for tissue collection and to measure proteinuria. Molecular, histological and functional measurements were performed in a second experiment (n = 6/group), which was terminated at 20 weeks of age. The third experiment (n = 6/group) was carried out to increase the number of samples for histological and molecular measurements. The fourth (n = 5/group) experiment was carried out to reconfirm changes in skin pathology and survival. All animal experiments were carried out according to the institutional regulations and the Hungarian law on animal care and protection (1998/XVIII, 243/1998(XII.31)).

Renal functional studies

Twenty-four hour urine samples were collected from all animals in diuresis cages (Tecniplast, Italy) at eight, 12, 16, 20 weeks of age, right after parturition (n = 5–8/group). Blood samples were obtained by retro-orbital puncture and were anticoagulated with heparin. Blood urea nitrogen (mmol/L, n = 4–8/group and preterminal uremic animals) levels were evaluated in a Reflotron Plus laboratory machine (Boehringer Mannheim, Germany). Proteinuria (mg protein/24 hour) was measured photometrically using the Bradford method with Bio-Rad Protein Assay (Bio-Rad, USA) at 595 nm.

Blood pressure

To assess blood pressure at the time of harvest (V: n = 6, M: n = 4), a tail cuff blood pressure system (IITC Life Science, USA) was used. For optimal

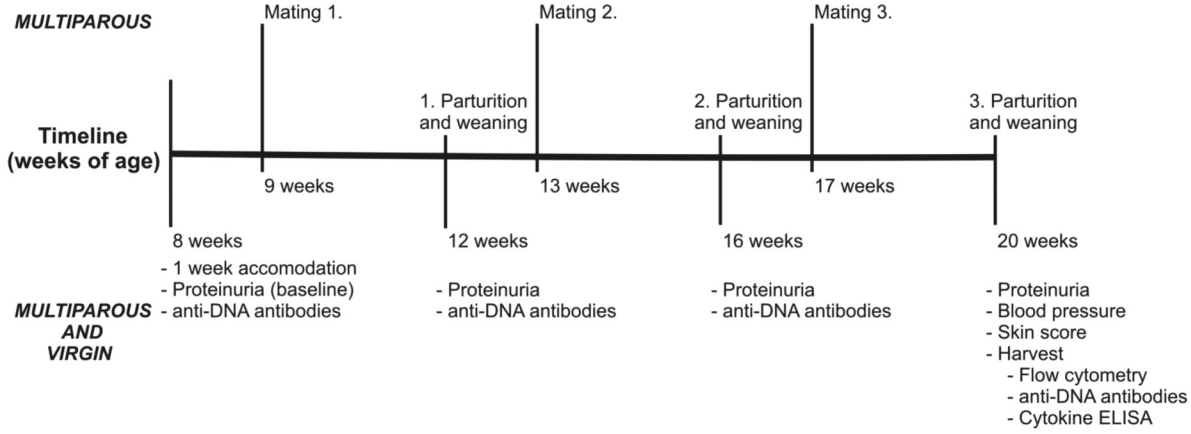

Figure 1 Study design and timeline of the experiments. Upper half: interventions on multiparous mice only; lower half: interventions on both multiparous and virgin mice.

results, the environmental temperature was set to 30°C and the animals were habituated for the procedure three times before measurement. All values were determined as mean value of three consecutive measurements.

Flow cytometry

Blood lymphocytes were used to evaluate the immune state of the animals by analysing the percentage of $CD4^-$, $CD8^-$ $CD3^+$, $B220^+$ (DN $B220^+$) cells by flow cytometry (V: $n = 9$, M: $n = 5$). The following anti-mouse mAbs were purchased from BD PharMingen (Soft-Flow, Hungary): PE-conjugated anti-CD3-complex (17A2), FITC-conjugated anti-CD4 (GK 1,5), APC-conjugated anti-CD8 (53–6.72), PerCP-conjugated anti-CD45/B220 (RA3-6B2), and anti-$CD19^-$. For biotinylated antibodies, a streptavidin-APC complex (BD PharMingen) was used as second step reagent. Briefly, 30 μL peripheral blood sample was incubated with mAbs in the dark at room temperature for 20 min and then erythrocytes were lysed. Samples were washed in 2 mL PBS, then fixed in 350 μL cold 2% paraformaldehyde, resuspended in FACS buffer and analysed using a BD FACSCalibur® flow cytometer (Becton Dickinson Immunocytometry, Mountain View, CA). Data were evaluated with CellQest software (BD).

ELISA of plasma INF-γ and anti-dsDNA autoantibody levels

Plasma IFN-γ levels were measured using a supersensitive IFN-γ ELISA kit (Bender MedSystems, Austria) following the manufacturer's instructions ($n = 4$/group).

The levels of anti-dsDNA antibodies in plasma samples at specific time points (eight, 12, 16, 20 weeks of age, $n = 10$/group) were evaluated by a modified anti-dsDNA ELISA[31] using goat anti-mouse IgG (Sigma) as conjugate antibody.

Skin pathology

Evaluation of cutaneous lupus on the backside of the neck, the ears and the nose was carried out both macroscopically (V: $n = 20$, M: $n = 23$) and microscopically at the time of harvest. A macroscopic score was established (range from 0 to 2): 0 meaning no signs of cutan lesions, 1 meaning a mild scab without hair loss and 2 meaning severe lesions.

Histology

Kidney and neck cutaneous samples were fixed in 10% buffered formalin. Four μm paraffin sections were cut and stained with H&E, periodic-acid Schiff (PAS) or Crossman's tri-chrome. Samples were all evaluated in a blinded manner. Severity of skin disease ($n = 9$/group) was graded semiquantitatively (Table 1). For kidney lesions ($n = 9$/group), the glomerulosclerosis index and the tubular, interstitial and vascular damage scores were assessed on PAS stained sections as described elsewhere.[32] Perivascular cell accumulation was determined semiquantitatively at 400× magnification by scoring the number of cell layers surrounding the medium-sized vessels as follows: 0) none; 1) <5 cell layers; 2) 5–10 cell layers; 3) >10 cell layers. Periglomerular infiltration was scored in a similar way, counting the infiltrating cell layers around the glomeruli: 0) none; 1) 1–3 cell layers; 2) 4 or more cell layers.

Renal IgG and C3 deposition, and $CD3^+$ infiltration

Kidney samples ($n = 4$/group) were evaluated for IgG deposition by direct immunofluorescence staining. Briefly, 8 μm cryostat sections were attached to SuperFrost slides at room temperature and fixed in cold acetone. Air-dried slides were then washed in PBS, blocked with 20% normal goat serum then stained with FITC-conjugated goat anti-mouse antibody (Sigma) in 1 : 100 dilution and incubated in a dark chamber at 37°C for 60 min. Samples were coated using Fluorescent Mounting Medium (DAKO, Germany) and analysed in a fluorescent microscope (Leica DMR-HC, Leica, Germany) using ImageJ software (NIH, Bethesda, Maryland, USA).

Complement-3 deposition was evaluated in cryostat kidney sections ($n = 4$/group) using immunohistochemistry as described elsewhere.[33] Glomerular staining was evaluated in a blinded manner counting 30 glomeruli per sample, with a semiquantitative score system: 0) no staining, 1) weak staining, 2) moderate staining in ~50% of glomerular tuft, 3) moderate staining in >50% of glomerular tuft, 4) strong staining of the entire glomerulus.

CD3 infiltration (mouse monoclonal anti-CD3, 1 : 50, DAKO) was determined on paraffin sections ($n = 8$/group) with a commercial mouse-on-mouse kit (Innogenex, Biogenex, Germany). Antigen retrieval was performed in citrtic buffer pH 6.0 for 20 min.

Table 1 Scoring system used to evaluate the skin pathology in H&E stained paraffin sections

Score	*Acanthosis*[a]	*Hyperkeratosis*[b]	*Inflammation*[c]	*Fibrosis*[d]
0	None	None	None	None
1	Mild	Mild	Slight	Slight
2	Moderate	Moderate	Heavy	Heavy
3	Marked	Marked	—	—

[a]Thickening of the dermis; [b]increased amount of keratin; [c]mononuclear cell infiltrates; [d]increased dermal cellularity and thickening.

Slides were blocked with 5% goat serum for 15 min, followed by the incubation with the primary antibody mixture overnight at 4°C. Streptavidin-conjugated alkaline phosphatase (Biogenex) was applied for 20 min. Samples were washed again and Fast Red substrate (DAKO) was added. Slides were counterstained with Mayer's haemalaun and coated. Positive cells were counted in 10 randomly selected fields at 400× magnification using a 10 × 10 points Zeiss eyepiece (Integration-plate II; Zeiss Co., Germany) and expressed as $CD3^+$ cells per mm.^{2}

Real-time RT-PCR

The RNA from kidney samples (n = 7–11/group) was extracted with phenol-chlorophorm method using Trizol (Gibco, Life Technologies, Germany). Skin mRNA was extracted using the Stratagene Absolutely RNA Miniprep kit (Stratagene, La Jolla, CA, USA). Reverse transcription and amplification reactions for IFN-γ, IL-10, IL-4 and β-actin were performed as described elsewhere.[34] All samples were normalized to their β-actin content. Expression levels were calculated using the formula $2^{-\Delta Ct}$, where ΔCt is the difference of mean cytokine threshold cycle and mean β-actin threshold cycle of triplicate measures. All reactions were performed on an ABI Prism 7700 Sequence Detection System (Perkin Elmer Applied Biosystems). Primer and probe sequences are available upon request.

Statistics

Results are shown as medians ±75% quartiles (for non-parametric data) or means ± SD (for parametric data). Data were analysed statistically using SPSS for Windows (SPSS Inc.). Kaplan–Meier and log-rank test were made for survival analysis. The Kolmogorov–Smirnov (KS) test was performed to analyze distribution normality. Mann–Whitney *U*-test was applied to analyse all non-parametric data (skin macroscopy scores, skin and kidney histology scores, IgG and C3 depositions, proteinuria). For normally distributed values of blood pressure, Student's *t*-test was employed.

Results

Dramatic improvement in skin pathology after repeated pregnancies

Pregnancy modifies the systemic cytokine expression favouring the production of anti-inflammatory cytokines,[23] which could have a beneficial effect on dermatitis. The autoimmune skin disease was dramatically ameliorated in multiparous mice. Only three out of 23 multiparous mice (13%) had macroscopic skin lesions (hair loss and mild scab formation) compared to 15 out of 20 (75%) severe skin lesions of virgin mice (Figure 2a–c, V: n = 20, M: n = 23, P = 0.0005, Mann–Whitney test). Lesions were found in the dorsal neck interscapular region and on the ears. None of the animals lost due to terrminal renal failure during the follow up period showed any macroscopic skin disease in the multiparous group. Microscopically, lesions of virgin mice were characterized by ulceration, hyperkeratosis, thickening of the dermis, fibrosis and mononuclear cell infiltrates (Figure 2d,e, n = 9/group, P = 0.0078, Mann–Whitney test). Some virgin mice had extremely high amount of infiltrates in the skin (Figure 2g). Multiparous skins without macroscopic alteration showed normal histology (Figure 2f). The few multiparous animals having mild scabs showed only moderate mononuclear infiltration and fibrosis while hyperkeratosis, ulceration and dermal thickening were completely absent (Figure 2h).

Survival and kidney function was dramatically reduced and accompanied by hypertension after multiple pregnancies

As in murine lupus IFN-γ is thought to be a main contributor of the disease,[12,35] we expected an improvement of renal pathology and survival in multiparous animals due to a continous shift towards anti-inflammatory cytokine production as a consequence of repeated pregnancies.[20] In contrast to the ameliorated autoimmune skin disease, and contrary to our expectations, the life span of multiparous females was significantly shortened to 20 weeks. Only 30% of M mice were alive at the age of 20 weeks compared to 78% of V mice (Figure 3, P = 0.038, V versus M). In this first study, 60% of M animals showed uremic symptoms (such as: subcutaneous water retention, fuzzy hair, tremors and severely reduced activity) preterminally, at 20 weeks of age, with blood urea levels exceeding 20 mmol/L. Proteinuria levels started to increase at 12 weeks of age (median 0.22 ± 0.07 mg/24 hour versus 0.38 ± 0.2, V versus M, P = 0.029, n = 5–8/group) and reached 2.0 mg/day at week 20 in multiparous animals (n = 5), while virgin mice (n = 8) showed only mild increase in proteinuria (Figure 4a). Proteinuria of multiparous mice after the first pregnancy was significantly higher than before pregnancy (P = 0.029, compared to eight weeks of age, n = 5–8/group). In addition, surviving multiparous animals showed significantly worsened kidney function and 70% of M mice died with uremic symptoms during follow-up. BUN levels closely paralleled proteinuria.

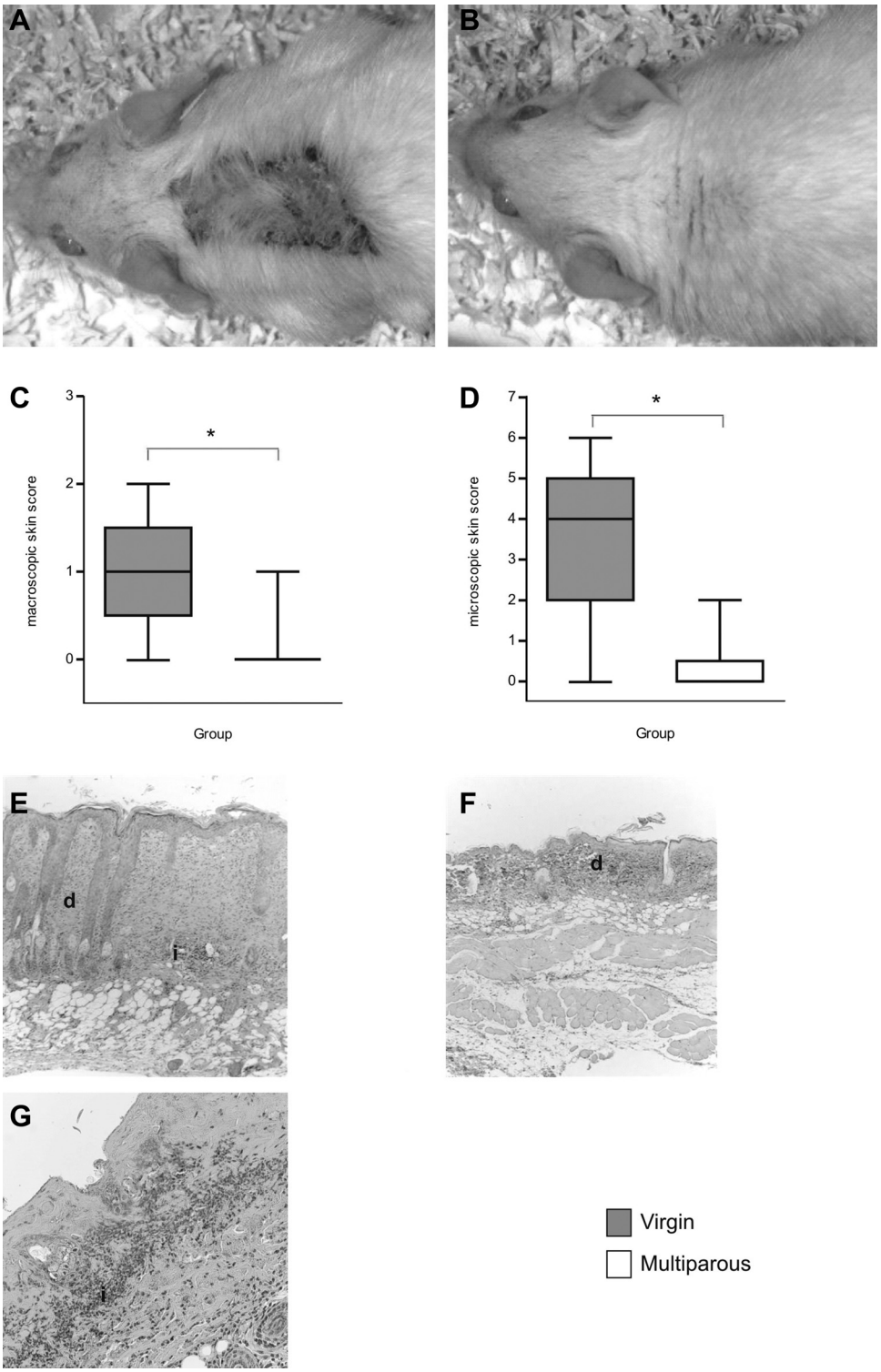

Figure 2 Macroscopy and histology of skin lesions. Macroscopic skin lesions were observed in 75% of virgin mice (A) compared to 13% in multiparous mice (B), multiparous mice had lower macroscopic score (C, $P < 0.001$, V: $n = 20$, M: $n = 23$, Mann–Whitney *U*-test). Histological evaluation showed similar results (D, P = 0.0078, $n = 9$/group, Mann–Whitney–*U*-test). Lesions of virgin mice (E, G) showed thickened dermis (d) and mononuclear infiltrates (i) while multiparous animals showed no histological alteration (F) or had only mild fibrosis and/or mild inflammation without hyperkeratosis or ulceration (H).

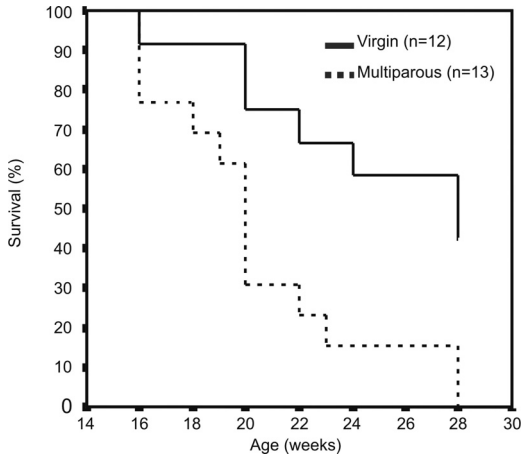

Figure 3 Survival curves. Life span of multiparous mice (dotted line) was significantly shortened compared to virgin animals ($P = 0.038$, Kaplan–Meier, Log rank test, V versus M).

The severe kidney dysfunction was accompanied by elevated mean arterial blood pressure (Figure 4b) in multiparous mice compared to virgin animals.

Histological evaluation of the kidneys showed that renal lesions were markedly increased in multiparous mice according to the functional data. Both glomerulosclerosis and tubulointerstitial damage indices (Figure 4c) as well as perivascular infiltration (Figure 4d) were more severe in the multiparous group (Figure 4e–h). However, there was no difference in the extent of periglomerular infiltration (median score: V: 0.76 versus M: 0.74, ns, $n = 9$/group) and vascular damage indices between the groups (median score: V: 1.49 versus M: 1.71, ns, $n = 9$/group). The number of $CD3^+$ infiltrating cells in the kidneys did not differ between the two groups (median: V: 320 versus M: 384 cells/mm^2, ns, $n = 8$/group). Evaluation of IgG (Figure 5a,b) and C3 (Figure 5c,d) in cryostat kidney sections showed an increased deposition of both IgG (median fluorescence intensity: V: 31.1 versus M: 47.1, $P = 0.02$, $n = 4$/group) and C3 (median score: V: 2.04 versus M: 2.76, $P = 0.033$, $n = 4$/group) in multiparous mice.

Neither lymphoproliferation nor plasma anti-dsDNA levels were affected by repeated pregnancies

We hypothesized that the systemic change in cytokine balance during pregnancy can possibly affect lymphoproliferation, B-cell activity and/or autoantibody levels, both systemic characteristics of SLE in MRL/lpr mice. Despite the fact that the proportion of $CD8^+$ T-cells was consistently lower in blood samples of multiparous mice (V: 6.48 ± 0.94 versus M: 4.8 ± 1.16, mean percent of total lymphocytes $\pm$ SD), there was no difference in the percentage of $CD4^-$ $CD8^-$ $B220^+$ DN cells (V: 67 ± 9.3 versus M: 62.1 ± 18.3) or $CD19^+$ B-cells (V: 1.86 ± 1.48 versus M: 1.43 ± 1.15) between the groups. Anti-dsDNA antibody levels in the plasma of multiparous animals were similar to those of virgin mice (median OD_{450} at 12 weeks: V: 0.226 versus M: 0.232; at 20 weeks: V: 0.317 versus M: 0.294, ns, $n = 10$/group). There was no significant difference in spleen or lymph node enlargement between the groups (data not shown).

Marked and inverse changes in IFN-γ production in the blood and the kidney

We observed significant diminished plasma IFN-γ levels in multiparous mice when compared to virgin animals at the time of harvest (Figure 6a), supporting the idea that pregnancy is accompanied by a diminished systemic production of proinflammatory cytokines.[23] To evaluate whether altered cytokine production at the organ level could contribute to the changes seen in the kidney and skin, IFN-γ and IL-10 mRNA levels were measured in these organs, accompanied by IL-4 mRNA measurements in kidneys. Interestingly, in the kidneys of multiparous mice, expression of IFN-γ, IL-10 and IL-4 were significantly higher compared to virgins (Figure 6b,c). However in skin samples, we found that the expression of IL-10 mRNA was higher, but IFN-γ mRNA levels were markedly reduced after repeated pregnancies (Figure 7a,b). These data suggest that lupus may be regulated differently at the organ level during pregnancy in this model.

Discussion

We report in the present study that skin disease dramatically disappeared after multiple pregnancies compared to severe skin disease in virgin MRL/lpr animals, despite a more severe nephritis manifested as shortened survival and higher blood pressure. In the background of ameliorated skin pathology, we observed decreased local IFN-γ and elevated IL-10 mRNA expression levels in the skin of multiparous mice accompanied by lower plasma IFN-γ protein levels. Despite the reduction in skin and plasma IFN-γ values, IFN-γ as well as IL-10 mRNA levels were elevated in the kidneys of multiparous mice. Our findings suggest that regulation of disease in MRL/lpr mice during pregnancy may involve different mechanisms at the organ level.

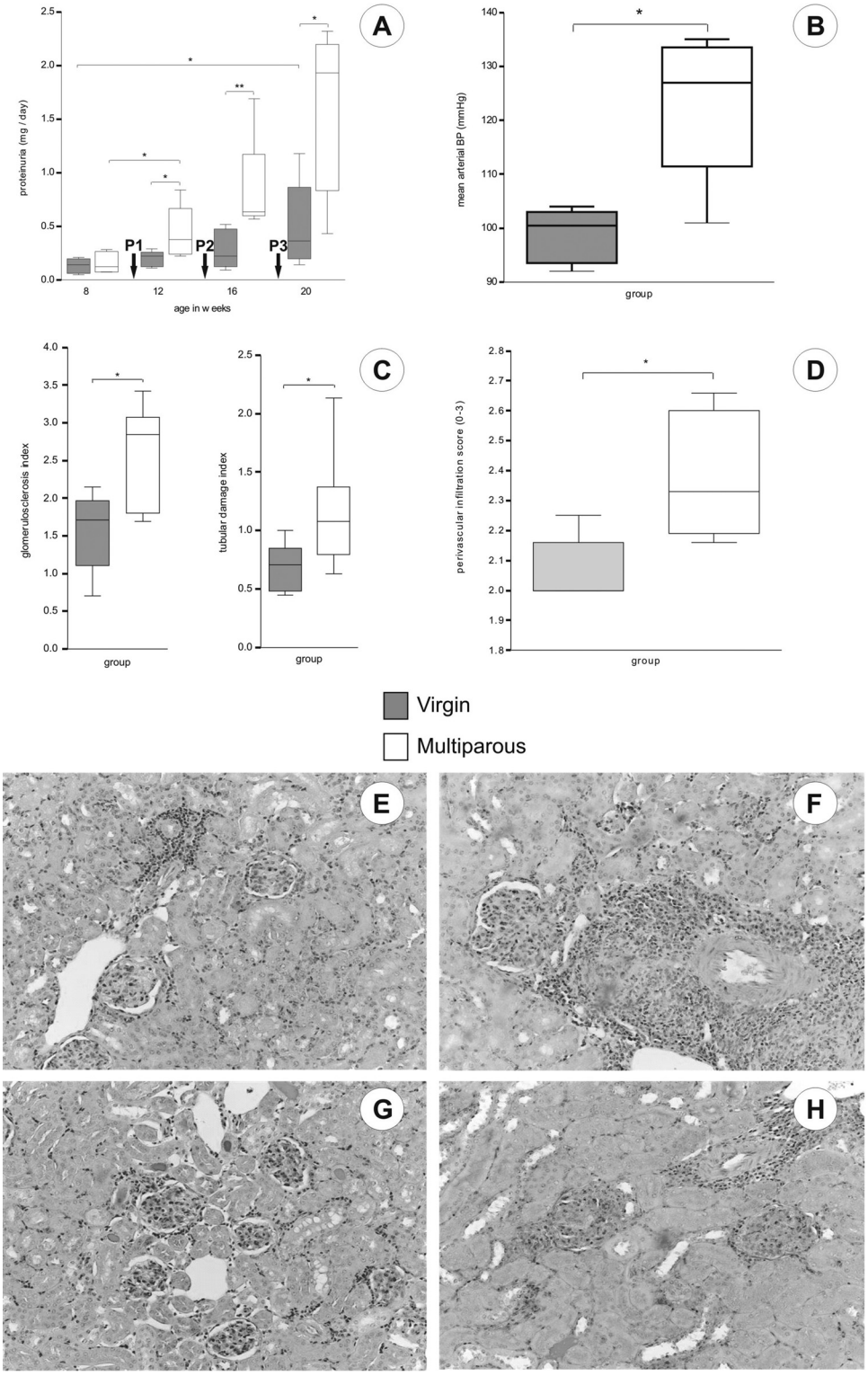

Figure 4 Kidney function and histology. A: Daily urinary protein excretion (n = 5–8/group, Mann–Whitney U test) B: Mean arterial blood pressure at time of harvest (t-test, $P = 0.019$, M: $n = 4$, V: $n = 6$). C: Glomerulosclerosis (V versus M $P = 0.037$) and tubular damage (V versus M $P = 0.028$) indices (n = 9/group, Mann–Whitney U-test). D: Perivascular infiltration index (n = 9/group, V versus M $P = 0.01$, Mann–Whitney U-test). Vasculitis (E) and normal glomerular structure (E,G) of virgin kidneys, multiparous kidney with massive mononuclear infiltrate (F) and sclerotic glomeruli (H). Data are presented as median ±75% quartiles, *: $P < 0.05$ V versus M, **: $P < 0.05$ M 12 weeks versus M eight weeks. (E,G: H&E stain; F,H: PAS stain).

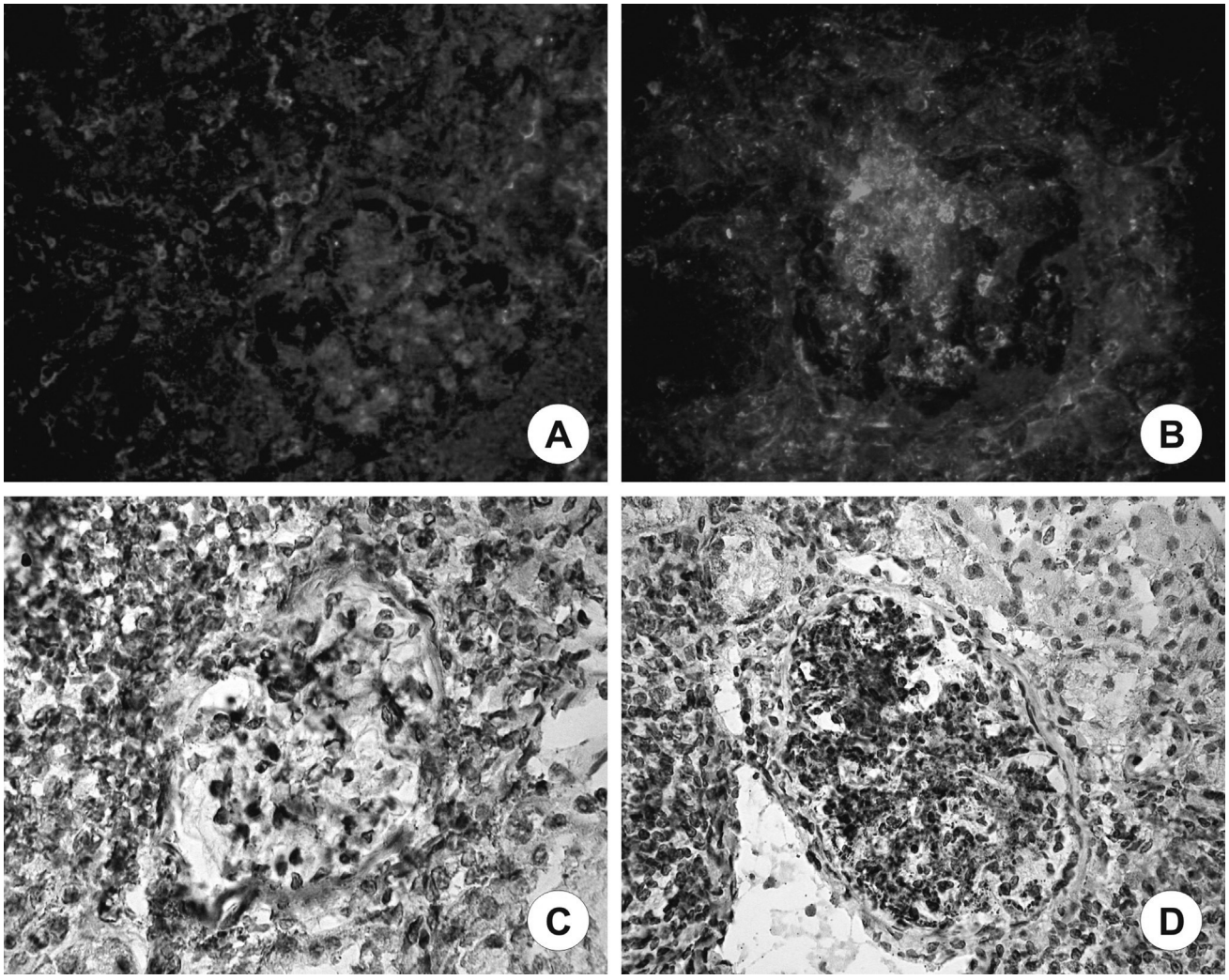

Figure 5 Representative photomicrographs of IgG and C3 deposition in the kidneys. A,B: Direct immunofluorescence of IgG deposits, 400× magnification (A: virgin, B: multiparous). C,D: immunohistochemistry of C3, 400× magnification (C: virgin, D: multiparous).

The typical histological feature of MRL/lpr skin lesions is inflammatory cell infiltration.[11] Although there is IgG deposition at the dermo-epidermal border,[10] skin lupus is probably not a B-cell dependent disease, as in complement receptor knockout MRL/lpr mice ear necrosis was not influenced.[36] However, several reports suggest that IFN-γ plays an important role in the development of skin lesions in SLE. The genetic deficiency of IL-12 (a cytokine promoting IFN-γ production) ameliorated skin disease of transgenic MRL/lpr mice.[37] Moreover, mice with normal background overexpressing IFN-γ in the epidermis developed inflammatory skin disease, similar to lupus.[38]

In our experiments, the severe lesions (**10**) found in 75% of virgin mice were practically absent in the multiparous group. In the background we detected decreased IFN-γ mRNA in skin samples of multiparous mice, supporting the central role of IFN-γ in the local regulation of skin lupus. On the other hand, IL-10 expression was enhanced, compared to virgin mice, possibly due to the systemic cytokine shift generally observed in pregnancy.[22] Whether IL-10 and IL-4 are beneficial or deleterious in lupus is still a point of discussion. IL-10 knock-out MRL/lpr mice had earlier onset of skin lesions, compared to wild type MRL/lpr and recombinant IL-10 administration to MRL/lpr mice ameliorated the disease.[19] In our study, IL-10 appeared to be important for the skin protection.

In both murine and in normal human pregnancy, there is a systemic cytokine profile change during gestation including increased production of IL-4 and IL-10[20,22,23] accompanied by decreased IFN-γ expression.[39] However, in pregnant women with SLE, elevated IFN-γ, and markedly increased IL-10 expression was reported compared to healthy women.[39] This systemic change in cytokine levels could explain the

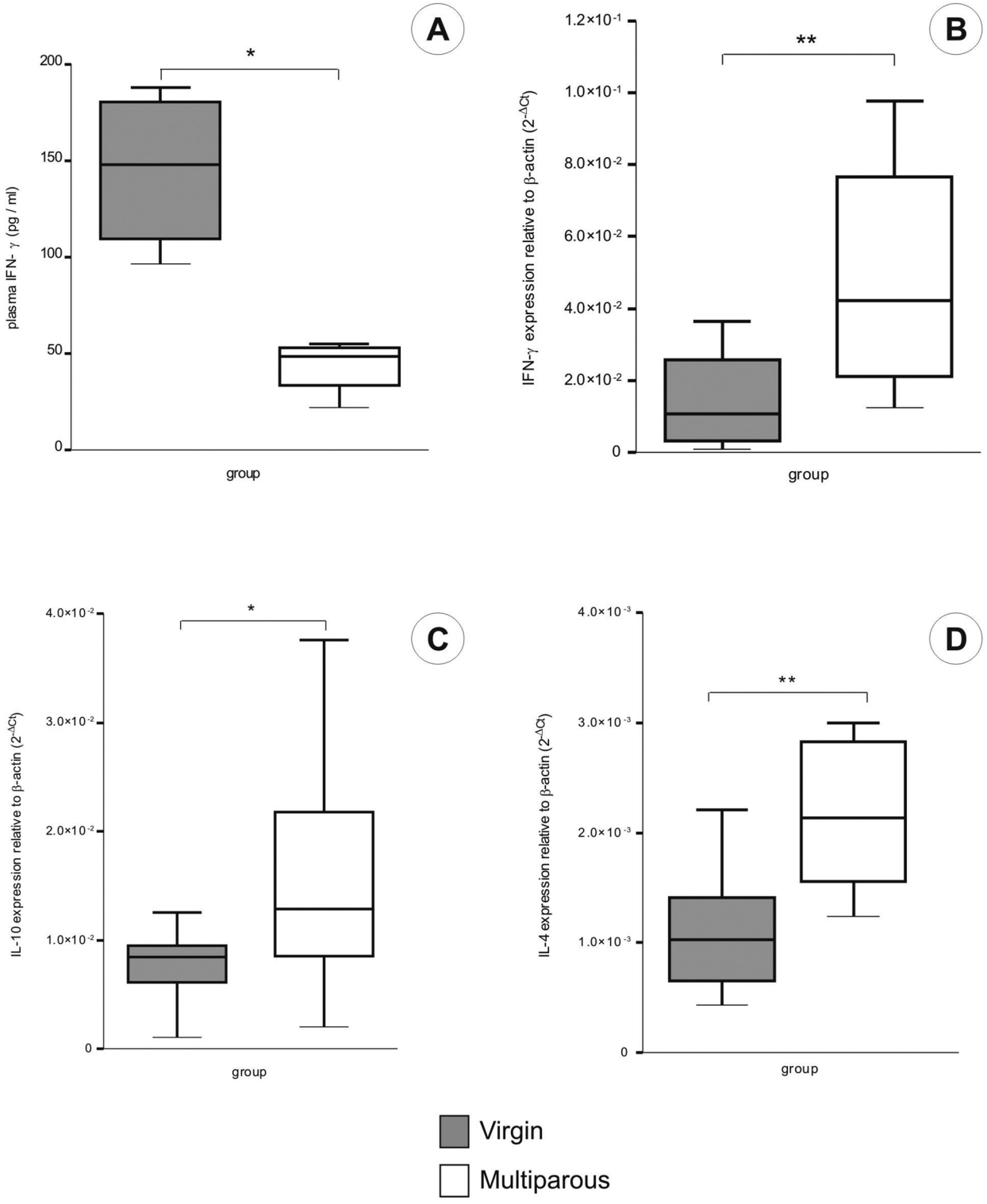

Figure 6 Cytokine expression in blood and kidneys. A: Plasma IFN-γ levels were significantly lower in multiparous mice at the time of harvest, compared to virgins ($P = 0.028$, $n = 4$/group, Mann–Whitney *U*-test). B–D: Renal cytokine mRNA expression analysis showed upregulation of both IFN-γ (B, $P = 0.005$, V: $n = 11$, M: $n = 9$), IL-10 (C, $P = 0.033$, V: $n = 11$, M: $n = 9$) and IL-4 (D, $P = 0.009$, V: $n = 8$, M: $n = 7$) in multiparous animals. Data are presented as medians ± 75% quartiles, *: $P < 0.05$, **: $P < 0.01$ V versus M, Mann–Whitney *U*-test.

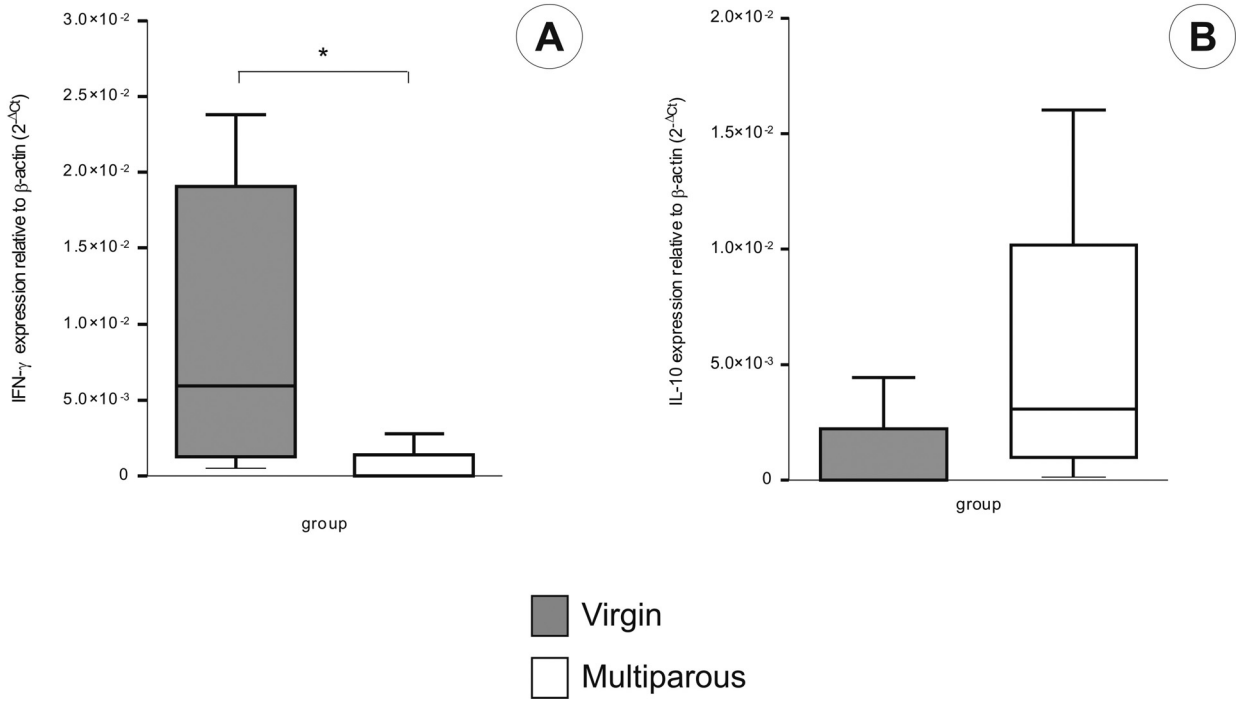

Figure 7 Cytokine expression in the skin. Cytokine mRNA expression analysis of skin samples showed significantly lower IFN-γ (A, Mann–Whitney U, $P = 0.0087$, $n = 6$/group) levels and consistently higher IL-10 expression (B, $P = 0.06$, $n = 6$/group) in multiparous animals compared to virgins. Data are shown as medians ±75% quartiles, *: $P < 0.05$ V versus M.

clinical reports of worsened nephritis in pregnant SLE patients.[25,27] Our results further support these clinical findings, suggesting an adverse effect of pregnancy on lupus nephritis.

Lupus nephritis in the MRL/lpr mice and other murine lupus models,[12,40] as well as in humans, has been strongly associated with IFN-γ overproduction.[41,42] Decreased intrarenal or systemic IFN-γ production[40,43] dramatically ameliorated nephritis[40] and improved survival, whereas the induction of IFN-γ production by IL-12 worsened the progression of glomerulonephritis[44] in MRL/lpr mice. Lupus patients with diffuse proliferative nephritis showed increased IFN-γ expression levels both in blood and kidney biopsy samples.[41,45] In summary, IFN-γ seems to be a key exacerbating cytokine in lupus nephritis. These data support our observation that, in renal samples of the M group with more severe nephritis, IFN-γ production was enhanced, despite lower serum IFN-γ levels in these animals.

Surprisingly, not only IFN-γ but IL-10 and IL-4 were also augmented in multiparous kidneys. However, treatment of MRL/lpr mice with both anti-IL-4[46] and anti-IL-10[47] antibodies ameliorated lupus nephritis. Moreover, genetic disruption of IL-10 accelerated glomerulonephritis and shortened survival,[19] thus supporting our hypothesis that high local IL-10 levels in the kidney may worsen nephritis leading to increased glomerular IgG deposition, without significantly affecting the circulating levels of autoantibodies. In a recent study, Enghard *et al.* described a positive correlation between both IL-4 and IL-10 production and kidney damage of lupus prone NZB/W F1 mice, supporting our results.[48]

In our experiment, a faster deterioration of kidney function was manifested in elevated blood pressure and a shortened survival period. Although seven month (28 weeks) 50% survival in control MRL/lpr mice suggests an unusual mild disease, similar results with even longer (33 weeks) 50% survival had been observed by Robey *et al.*[49] A putative factor contributing to longer survival in our experiments might be the individually ventilated cage (IVC) system wherein our animals were housed. This protects them from pathogens – a possible exacerbating factor for the autoimmune pathology.

During pregnancy, renal blood flow increases by 40–65% leading to elevated intraglomerular pressure and proteinuria. We found mild proteinuria in virgin mice, significantly elevated in multiparous animals right after the first pregnancy. Thus, hemodynamic changes could have contributed to the high local IFN-γ expression despite high IL-10 levels in the kidneys of multiparous mice.

Pre-eclampsia is an important issue in human SLE pregnancy, and it is hard to distinguish the clinical symptoms of renal flare and pre-eclampsia (PE). Although multiparous mice had hypertension, blood pressure did not reach the levels of the murine PE model,[50] and the glomerular lesions were not typical for murine PE.[50] Furthermore, the timing of death in the multiparous group was not associated with third trimester of pregnancy, but death was always accompanied by symptoms of renal failure. These observations suggests that pregnancy itself did not lead to pre-eclampsia in MRL/lpr mice. Our findings suggest an important role of local immune regulation of lupus nephritis during pregnancy, independent of systemic changes in the cytokine profile but possibly influenced by renal hemodynamic changes of pregnancy.

Hormonal changes during gestation are further issues that possibly affect the progression of SLE during pregnancy. Estrogens and progesterone have immunomodulatory effects. Estrogens markedly enhance IL-10 production in human Th cells,[51] raising the possibility to exacerbate lupus nephritis during pregnancy. Indeed, acceleration of glomerulonephritis was reported in MRL/lpr mice after estrogen administration.[52] Another study showed an opposite effect, in MRL/lpr mice.[53] Elevation of serum estriol is often found in female patients with SLE, and sometimes is linked to active disease.[54] Progesterone favors a shift to Th2 type immune response,[55] thus it may worsen lupus nephritis. Thus, the role of sex-hormones in pregnancy associated changes of lupus disease activity is controversial. A possible background of this controversy could be the organ-dichotomy described in our present report.

The design of the present study deserves some comment. Allogeneic matings (with NMRI males) were performed primarily for their relevance to humans. This raises the question of how fetal chimerism ('allograft' fetus) influences autoimmunity. As congenic MRL/lpr males have low testosterone level[56] and low fertility, the rate of matings can not be calculated exactly, which does not allow the exact timing of parturition required in our study. Therefore, we could not include a syngenic control group. Thus, the present study is not conclusive regarding the influence of fetal microchimerism on the progression of autoimmune disease. However, we have observed similar amelioration of skin disease in our MRL/lpr breeder colony, but a shortened life span compared to non-breeder females. This suggests that the observed alterations in disease activity are rather a consequence of pregnancy *per se* and not of the repeated allogenic exposure. In conclusion, although the present study cannot dissect the effects of repeated allo-exposures from the effects of repeated pregnancies, the two cannot be separated in the clinical situation either, as human pregnancies are always allogenic.

In summary, skin disease of MRL/lpr mice is regulated differently from lupus nephritis during pregnancy. In the present study, we first demonstrated a dramatic improvement of skin pathology by repeated pregnancies. However, mice undergoing multiple pregnancies showed a shortened lifespan. Furthermore, our data suggest that skin and kidney diseases may have different pathomechanisms during pregnancy in MRL/lpr mice with a divergent cytokine regulation. Revealing the local pathomechanisms contributing to organ-specific lesions of SLE during or after pregnancy may lead to a better understanding of the role of pregnancy in SLE.

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