IFN-gamma is essential for the development of autoimmune glomerulonephritis in MRL/Ipr mice.

C Haas, B Ryffel and M Le Hir

*J Immunol* 1997; 158:5484-5491;

http://www.jimmunol.org/content/158/11/5484
IFN-γ Is Essential for the Development of Autoimmune Glomerulonephritis in MRL/lpr Mice

Cordula Haas, Bernhard Ryffel, and Michel Le Hir

MRL/lpr mice develop lymphoproliferation and accelerated autoimmune glomerulonephritis from which they ultimately die. To investigate the role of IFN-γ in the manifestation of the disease, we generated MRL/lpr mice lacking the IFN-γ receptor (MRL/lpsyR−/−). The absence of IFN-γ signaling had no effect on generalized lymphoproliferation, expansion of CD4+CD8− double-negative T cells, or hypergammaglobulinemia. By contrast, glomerulonephritis as detected by proteinuria and histology was absent in MRL/lpsyR−/− mice. While serum IgG1 anti-dsDNA Abs were increased in all three strains of MRL/lpr mice (yR+/+, +/-, −/−), those of the IgG2a and IgG3 isotypes were low in MRL/lpsyR−/− mice. Immune complexes and C3 deposition were dramatically reduced in the glomerular capillaries of MRL/lpsyR−/− mice compared with MRL/lpsyR+/+ and +/- mice. Therefore, IFN-γ plays a key regulatory role in the development of nephritis in MRL/lpr mice. Low levels of IFN-γ-dependent IgG2a and IgG3 autoantibodies in MRL/lpsyR−/− mice might protect them from the pathogenic features of IgG3 cryoglobulins and complement-activating IgG2a and IgG3.


Materials and Methods

Animals and experimental protocol

MRL/lpr/lpr (MRL/lpr) and MRL+/+ mice were provided by Bomholtgaard Breeding and Research Centre (Ry, Denmark). IFN-γ-deficient MRL/lpr mice were created by backcrossing. The carrier of the mutation (34) had the background 129Sv. Mice were screened for the disrupted gene by PCR analysis of tail DNA lysate according to standard protocols using the following primers: 5'-CCCATTTAGATCCTACATACGAAAC (antisense). The IFN-γR defect was backcrossed into MRL/lpr mice by using the following three genotypes: mice homozygous for the disrupted IFN-γR gene (MRL/lpsyR−/−), homozygous wild-type mice (MRL/lpsyR+/+), and heterozygous mice (MRL/lpsyR+/−). MRL+/+ mice were used as controls. The experimental groups were constituted as follows: 31 (15 f, 16 m) MRL/lpsyR+/+, among which 12 F6, 6 F7, and 7 F8; 31 (15 f, 16 m) MRL/lpsyR−/−, of which 16 were F6, 6 F7, 3 F8, and 6 F9; 23 (9 f, 14 m) MRL/lpsyR−/−, of which 11 were F6, 4 F7, 5 F8, and 3 F9; and 26 (14 f, 12 m) MRL/lpsyR+/+. They were kept under standard conditions. Mice where killed when urine protein levels reached 3 mg/ml or at the age of 9 mo if proteinuria did not occur. One kidney was fixed in 4% buffered paraformaldehyde; the other kidney was shock frozen.

Definition of abbreviations in this paper:

SLA, systemic lupus erythematosus; DN, double-negative; lpr, lymphoproliferation mutation; MRL/lpr, MRL/lpr/lpr mice; MRL/lpsyR−/−, MRL/lpr mice lacking the IFN-γ receptor; f, female; m, male; PAS, periodic acid Schiff reagent; TBS, Tris-buffered saline; G3, glomerulonephritis; IFN, interferon; SLE, systemic lupus erythematosus; C3, complement-activating IgG2a and C3; period acid Schiff reagent; TBS, Tris-buffered saline; normal thymus (14). Several studies clearly dissociate DN T cell apoptosis and defective IFN-γ regulation has been suggested in humans and mice. IFN-γ levels in the sera of patients with autoimmune disorders are significantly elevated (22, 23). Overexpression of IFN-γ was also shown in lymph node cells of MRL/lpr mice (24–29). Comparison of three colonies derived from MRL/lpr mice revealed a positive correlation between the rapidity of disease onset and IFN-γ production (28). Treatment with exogenous IFN-γ accelerated autoimmune and renal disease in (NZB×NZW)F1 mice (30, 31). Blocking the effect of IFN-γ with anti-IFN-γ or soluble IFN-γ receptor inhibited the onset of glomerulonephritis in the latter strain (31, 32). In contrast, treatment of MRL/lpr mice with anti-IFN-γ affected neither the survival nor the incidence of glomerulonephritis in MRL/lpr mice (33).

In this study, we examined MRL/lpr/lpr mice lacking the IFN-γ receptor. The IFN-γR−/− genotype (34) was backcrossed into the MRL/lpr strain. MRL/lpr IFN-γR−/− (MRL/lpsyR−/−) mice were protected from renal injury despite development of lymphadenopathy and DN T cell expansion.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
just before the immunolabeling. After a rinse in Tris-buffered saline (TBS), no  
Research Laboratories (West Grove, PA). Blinded scoring of intraglom-
mice: 

The Journal  
Bethyl Laboratories (Montgomery, TX). and goat anti-mouse Ig(G+M)  
membrane: 2  
scale:  
room temperature with  

in TBS. The sections were then washed in TBS and incubated for  

levels were measured weekly. Protein- 

uria was assessed semiquantitatively using dip sticks (Albustix, Bayer Di-
agnosis, Basingstoke, U.K.). Mice were screened monthly for enlarged  

ery month starting at the age of 2 mo. For all serologic examinations, mice  

and/or cellular crescent. A score was attributed to each of at least 40 glo-

in PAS-stained sections. The following scale was applied:  

Serum IgG levels  
Serum IgG levels were quantified by ELISA. All reagents were from Sigma  
Chemical Co. (St. Louis, Mo). Microtiter plates were coated with goat anti-mouse IgG at 5 mg/ml in PBS. Plates were then blocked with 2% BSA-PBS. The mouse sera to be tested were incubated overnight at a 10^6  
dilution. A goat anti-mouse IgG coupled to alkaline phosphatase was used as secondary Ab and the disodium salt of p-nitrophenyl phosphate as the substrate. The absorbance at 405 nm was measured. The total IgG concentration was calculated with reference to an internal standard of pure mouse IgG.  
Serum titers of anti-dsDNA autoantibody subclasses  
Serum levels of IgG subclasses were determined by ELISA at the age of 6 mo. The alkaline phosphatase-labeled IgG subclass-specific Abs were ob-
tained from Southern Biotechnology Associates (Birmingham, AL). The assays were performed basically as described above for total IgG. The microtiter plates were coated with dsDNA from salmon testes (Sigma Chemical Co.) at 10 mg/ml in PBS. Mouse sera were diluted from 10^-2 to 2 x 10^-6. Results are expressed as the dilution at which the OD is 0.2 over background.  
Histology  
All animals were analyzed histologically. Kidneys were fixed in 4% buffer-
eparated paraformaldehyde and processed for paraffin sectioning. Then, 3-μm-thick sections were stained with the periodic acid Schiff reagent (PAS) followed by hematoxylin. Blinded scoring of glomerular alterations was conducted in PAS-stained sections. The following scale was applied: 0 = morphology as in the 3-month-old MRL+/- mice; 1 = moderate expansion of the glomerular matrix, but no glomerulonephritis; 2 = mild glomerulonephritis with mesangial hypercellularity and/or segmental necrosis; 3 = severe glomerulonephritis with extended sclerosis and/or loop necrosis and/or cellular crescent. A score was attributed to each of at least 40 glomeruli per mouse, and a mean score was calculated for each mouse.  
Immunohistochemistry  
The left kidney of 5 MRL/lpr/lpr +/- (1 F, 2 F2, 2 F3), 3 MRL/lpr/lpr +/-  
(t 1 F, 2 F2), 10 MRL/lpr/lpr +/-(4 F, 5 F4, 1 F5), and 6 MRL/lpr/lpr mice  
was shock frozen. Sections 6 μm thick were cut on a cryostat and air dried  
for storage at 80°C. Fixation in acetone (10 min at 0°C) was performed  
just before the immunolabeling. After a rinse in Tris-buffered saline (TBS),  
the sections were incubated for 16 h at 4°C with the Primary Abs diluted in  
TBS. The sections were then washed in TBS and incubated for 1 h at  
temperature with indocarbocyanine-labeled goat anti-rat Ig Ab diluted  
1:200. After rinsing in TBS, the sections were mounted in Immu-
mount (Shandon, Pittsburgh, PA). Rat anti-mouse MHC class II, clone  
7/7, was obtained from Serotec (Oxford, U.K.), goat anti-mouse C3 from  
Bethyl Laboratories (Montgomery, TX), and goat anti-mouse IgG+M  
(indocarbocyanine-conjugated Fab2 fragment) from Jackson Immuno-
Research Laboratories (West Grove, PA). Blinded scoring of intraglo-
merular IgG+M and of C3 was performed according to the following  
hallocked phenotypes were displayed by only a minority of spleen cells in MRL +/- mice (Fig. 4, Table I). MRL/lpr/lpr +/-, +/-, and -/- mice already had elevated serum IgG levels at the age of 2 mo (Fig. 5). From
FIGURE 2. Time course of the incidence of skin lesions in MRL/lprR +/+ (■), MRL/lprR +/− (○), and MRL/lprR −/− (▲) mice.

FIGURE 3. Mortality of MRL/lprR +/+ (■), MRL/lprR +/− (○), and MRL/lprR −/− (▲) mice. Most mice were killed when proteinuria reached 3 mg/ml. However, one MRL/lprR +/+ mouse, one MRL/lprR +/−, and five MRL/lprR −/− became moribund because of reasons other than glomerulonephritis (see Results).

FIGURE 4. Flow cytometric analysis of spleen cells in 4- to 6-mo-old MRL/lprR +/+ , MRL/lprR −/−, and MRL +/+ mice. CD3+CD4+ T cells (left lane) decreased from 47% in MRL+/+ to 15.6 and 17.1% in MRL/lprR +/+ and MRL/lprR −/− mice, respectively. CD3+CD8+ T cells (right lane) decreased from 48.2% in MRL +/+ to 10.5 and 19.6% in MRL/lprR +/+ and MRL/lprR −/− mice, respectively. These results are representative of several experiments and are summarized in Table I.
The Journal of Immunology 5487

Table I. Flow cytometric analysis of spleen cells

<table>
<thead>
<tr>
<th></th>
<th>CD4⁺ T cells (%)</th>
<th>CD8⁺ T cells (%)</th>
<th>DN T cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRL +/+</td>
<td>45.5 ± 3.6</td>
<td>50.0 ± 4.6</td>
<td>4.5 ± 1.0</td>
</tr>
<tr>
<td>MRL/lprR +/+</td>
<td>21.0 ± 8.4</td>
<td>12.8 ± 6.8</td>
<td>66.2 ± 14.8</td>
</tr>
<tr>
<td>MRL/lprR +/-</td>
<td>22.7 ± 4.7</td>
<td>12.5 ± 3.2</td>
<td>64.8 ± 7.7</td>
</tr>
<tr>
<td>MRL/lprR -/-</td>
<td>31.4 ± 9.8</td>
<td>23.2 ± 4.3</td>
<td>45.4 ± 12.8</td>
</tr>
</tbody>
</table>

*These analyses are the means of several experiments (see Fig. 4). The percentages of DN T cells were calculated as 100% minus the sum of CD4⁺ and CD8⁺ T cells.

Then on, the IgG serum concentrations steadily increased, reaching a maximum at the age of 6 mo. At this time, the IgG levels of all three groups were increased 5- to 10-fold compared with MRL +/+ mice (Fig. 5).

Autoantibody production

Anti-dsDNA IgG subclass levels were assessed at the age of 6 mo. IFN-γ is not essential for the production of autoantibodies, since MRL/lprR -/- mice produced anti-dsDNA Abs of the IgG1 subclass. However, in these mice, in contrast to MRL/lprR +/+ and +/- mice, anti-dsDNA Abs of the IgG2a and IgG3 subclass were as low as in control MRL +/+ mice (Fig. 6).

Histology

In the kidneys of MRL/lprR +/+ , +/-, and -/- mice, mononuclear cell infiltrates, often of huge dimensions, occurred around large vessels (Figs. 7 and 9). Glomeruli of MRL/lprR +/+ and +/- mice showed proliferative glomerulonephritis with segmental sclerosis, protein thrombi, and frequent cellular crescents. MRL/lprR -/- mice had no signs of glomerulonephritis (Fig. 7, Table II). Vasculitis was detected in the renal arteries of most MRL/lprR +/+ and +/- mice, but not in -/- mice. In contrast, histologic examination of ears displaying necrotizing lesions revealed vasculitis in the three populations (not shown).

Igs and C3 in the glomeruli

Granular deposits of mouse Ig(G+M) were distributed along the capillary walls and in the mesangium in MRL/lprR +/+ , +/-, and -/- mice (Fig. 8). In the latter group, the abundance of the deposits was distinctly lower than in the two others (Table II). In control MRL +/+ mice, Ig(G+M) deposits were located exclusively in the mesangium.

FIGURE 5. Serum IgG levels in MRL/lprR +/+ (■), MRL/lprR +/- (□), MRL/lprR -/- (▲), and MRL +/+ (●) mice.

FIGURE 6. Serum titers of IgG1, IgG2a, and IgG3 anti-dsDNA autoantibodies at the age of 6 mo. Results are expressed as the dilution at which the OD is 0.2 over background. Circles represent single mice, and the line shows the mean. The log values of the reciprocal titers were analyzed statistically (variance analysis using ANOVA and Scheffe's test for significance). For IgG1, MRL/lprR +/+ , +/-, and -/- were significantly increased compared with MRL +/+ mice, and there was no significant difference between MRL/lprR +/- and -/- mice. For IgG2a and IgG3, there was no significant difference between MRL/lprR +/+ and +/- or between MRL/lprR -/- and MRL +/+ mice, but the values for MRL/lprR +/- and -/- were significantly higher than for MRL/lprR -/- and MRL +/+.
with Scheffe's test.

The recessive mutation lpr has been mapped to mouse chromosome 19 (35). Analysis of backcross mice revealed renal deposits, the difference between MRULpryR and MRUlpryR -/- on the one hand and MRULpryR +/- and +/- on the other hand was statistically significant. Variance analysis was performed using ANOVA software and the significance was tested with Scheffe's test.

The complement factor C3 was found abundantly in MRL/lpryR +/- and +/- mice along the glomerular capillary walls in the proximal tubules and in the perivascular infiltrates (Fig. 9). In contrast, MRL/lpryR -/- mice showed a much lower level of glomerular deposits of Igs and C3 than their +/- and +/+ littermates. This might account for the protection from glomerulonephritis in the MRL/lpryR +/- mice. Indeed, a requirement for B cells and/or autoantibodies in initiating autoimmune-mediated tissue destruction in SLE has been demonstrated repeatedly (38-40). MRL/lpryR +/- mice that were made B cell deficient by introducing the Jh mutation failed to develop nephritis or vasculitis (38). Constitutive expression of Bcl-2 in B cells promotes the development of a lupus-like autoimmune syndrome, probably by providing survival signals for autoreactive B cells (39, 41). In the present study, serum IgG concentrations were abnormally high in MRL/lpryR +/-, ++, -/-, and --/ mice. Likewise, anti-dsDNA Abs of the IgG1 subclass reached similar titers in the three strains. In contrast, IgG2a and IgG3 anti-dsDNA titers were very low in MRL/lpryR +/- compared with MRL/lpryR ++ and +/+ mice. This undoubtedly reflects the role of IFN-γ in promoting the switch to the IgG2a and IgG3 subclasses (34). There is evidence suggesting a particularly high nephritogenic capacity for these two isotypes. IgG3 cryoglobulins are important in causing glomerular lesions (42-46). Deposition of IgG3 cryoglobulins itself, without involvement of immune complexes, results in the generation of "wire loop" deposits, scores of 0 to 3 were given (see Materials and Methods). For all three evaluated parameters, the difference between MRL/lpryR -/- on the one hand and MRL/lpryR +/- and +/- on the other hand was statistically significant. Variance analysis was performed using ANOVA software and the significance was tested with Scheffe's test.

The complete protection of MRL/lpryR +/- mice from glomerulonephritis during the experimental period of 9 mo seems to be in contradiction with the data of a previous study using anti-IFN-γ Abs in MRL/lpr mouse (33). However, the two experimental protocols were basically different. In the MRL/lpryR +/- mice in our study, IFN-γ signaling was constitutively deficient, whereas Nicoletti and colleagues started the treatment when the mice were 12 wk of age. Moreover, neutralization of IFN-γ by Abs might not have been complete.

MRL/lpryR +/- mice showed a much lower level of glomerular deposits of Igs and C3 than their +/- and +/+ littermates. This might account for the protection from glomerulonephritis in the MRL/lpryR +/- mice. Indeed, a requirement for B cells and/or autoantibodies in initiating autoimmune-mediated tissue destruction in SLE has been demonstrated repeatedly (38-40). MRL/lpryR +/- mice that were made B cell deficient by introducing the Jh mutation failed to develop nephritis or vasculitis (38). Constitutive expression of Bcl-2 in B cells promotes the development of a lupus-like autoimmune syndrome, probably by providing survival signals for autoreactive B cells (39, 41). In the present study, serum IgG concentrations were abnormally high in MRL/lpryR +/-, ++, -/-, and --/ mice. Likewise, anti-dsDNA Abs of the IgG1 subclass reached similar titers in the three strains. In contrast, IgG2a and IgG3 anti-dsDNA titers were very low in MRL/lpryR +/- compared with MRL/lpryR ++ and +/+ mice. This undoubtedly reflects the role of IFN-γ in promoting the switch to the isotypes IgG2a and IgG3 (34). There is evidence suggesting a particularly high nephritogenic capacity for these two isotypes. IgG3 cryoglobulins are important in causing glomerular lesions (42-46). Deposition of IgG3 cryoglobulins itself, without involvement of immune complexes, results in the generation of "wire loop" deposits, scores of 0 to 3 were given (see Materials and Methods). For all three evaluated parameters, the difference between MRL/lpryR -/- on the one hand and MRL/lpryR +/- and +/- on the other hand was statistically significant. Variance analysis was performed using ANOVA software and the significance was tested with Scheffe's test.

Table II. Semiquantitative analysis of histologic and immunohistologic parameters in glomeruli

<table>
<thead>
<tr>
<th>Histology</th>
<th>IgG+M</th>
<th>C3</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRL/lpryR +/-</td>
<td>2.56 ± 0.25 (13)</td>
<td>2.60 ± 0.55 (5)</td>
</tr>
<tr>
<td>MRL/lpryR +/-</td>
<td>2.15 ± 0.52 (14)</td>
<td>2.33 ± 0.58 (3)</td>
</tr>
<tr>
<td>MRL/lpryR -/-</td>
<td>0.84 ± 0.24 (10)</td>
<td>0.89 ± 0.60 (9)</td>
</tr>
</tbody>
</table>

The number of mice used for the evaluations is shown in parentheses.
lesions characteristic of lupus nephritis (45). There is a good correlation of IgG3 production with the development of lupus nephritis, as shown in MRL/lpr X (MRL/lpr X C3Hlpr) backcross mice (43) and in a new substrain of MRL/lpr, MRL/lpr II (long lived) (47). The transfer of the xid (X chromosome-linked immune deficiency) gene, which causes a defect of IgM and IgG3 production (48), delays the development of lupus nephritis (49). Murine IgG2a and IgG3 activate the complement system (28, 50) and, additionally, IFN-γ up-regulates the expression of complement factors C3 and C4 (51). Therefore, MRL/lprR −/− mice should be largely protected from the pathogenic features of IgG3 cryoglobulins and of complement-activating IgG2a and IgG3.

The majority of autoimmune diseases are mediated by Th1 CD4+ T cells; for example, experimental autoimmune encephalomyelitis (EAE), the nonobese diabetic mouse (NOD), and collagen-induced arthritis (52). On the contrary, most cases of SLE appear to be mediated by Th2 cytokines. Indeed, in patients with SLE and in (NZB×NZW)F1 mice, there is an imbalance toward Th2 cytokine production as a result of either an increase in Th2 cytokine production or a decrease in Th1 cytokine production or both (27, 53). MRL/lpr and (SWR×NZB)F1 mice might represent an exception in promoting the Th1 pathway (27, 28, 54). The protective effect of the IFN-γR −/− mutation in MRL/lpr mice in the present study is compatible with that proposal. However, the initiation of autoimmunity may not require IFN-γ, since anti-dsDNA of the IgG1 isotype reached similar levels in the MRL/lprR −/− mice as in the MRL/lprR +/+ and +/− mice.

Also, the development of skin vasculitis shows that MRL/lprR −/− mice are not protected from autoimmunity. In MRL/lpr mice, rheumatoid factors of the IgG3 isotype seem to play an essential role in the pathogenesis of vasculitis, whereby the cryoglobulin activity of IgG3 appears important (43, 44, 46). MRL/lprR +/+ , +/−, and −/− mice developed necrotizing vascular lesions of the skin of the ears and neck, but only MRL/lprR +/+ and +/− mice had vasculitis in renal arteries. Cryoglobulins precipitate at low temperature (45); thus, the skin of the ears is especially sensitive to damage by cryoglobulinemia. MRL/lprR −/− mice might have sufficiently high levels of IgG3 rheumatoid factors to induce skin lesions but too low a level for induction of vasculitis in the kidney.

MHC class II in proximal tubules was widespread in MRL/lprR +/+ and +/− mice, but only was found occasionally in MRL/lprR −/− mice. MRL/lpr mice have an enhanced MHC class II expression on their proximal tubular cells preceding deterioration of renal function, suggesting that tubular MHC class II expression may play a role in the initiation and progression of nephritis (55). Since IFN-γ promotes up-regulation of MHC Ags (56, 57), the interruption of IFN-γ signaling might explain the lack
of MHC expression in proximal tubular cells of MRL/lpr/lpr. However, an IFN-γ-independent pathway for MHC class II regulation in renal tubules exists in the mouse (58, 59).

Lymphopenopathy and DTN cell expansion occurred in MRL/lpr/lpr → +/+, +/+ mice, whereas the latter strain was not affected by glomerulonephritis. This constitutes a further evidence for the dissociation between the DTN cell expansion and autoimmune disease, which has already been suggested by others (15–18).

In summary, the lack of a functional IFN-γ signaling pathway protects MRL/lpr mice from glomerulonephritis and early death. IFN-γ plays a role in Ig class switching to the isotopes IgG2a and IgG3, which might be particularly potent in inducing glomerulonephritis in this model. IFN-γ does not play a role in aberrant lymphocyte development in MRL/lpr mice nor is it required for initiation of autoimmune disease.

Acknowledgments

The authors thank T. Karich for technical assistance in animal care and S. Lugi and M. Müller for support in FACS analysis and PCR techniques.

References


