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IFN-γ Receptor Deletion Prevents Autoantibody Production and Glomerulonephritis in Lupus-Prone (NZB × NZW)F_1 Mice[1]

Cordula Haas,* Bernhard Ryffel,† and Michel Le Hir[2]*

(NZB × NZW)F_1 female (BW) mice spontaneously develop an autoimmune disease, characterized by the production of autoantibodies (autoAbs) and glomerulonephritis, which can be delayed by neutralizing IFN-γ Abs and accelerated by IFN-γ injections. To define the role of IFN-γ in the pathogenesis of glomerulonephritis, we established a population of BW mice deficient in IFN-γR (BWγR−/−) by repeated crossing; these mice were compared with BWγR+/+ and +/− littermates. Of the BWγR+/+ and +/− mice, 50% showed immune complex glomerulonephritis with heavy proteinuria at 8 mo of age, while only 10% of the BWγR−/− mice were affected at 14 mo. The serum concentration of anti-dsDNA and anti-histone Abs was dramatically reduced in BWγR−/− mice. The role of IFN-γ in promoting class switch to IgG2a and IgG3 could not fully account for the impaired production of anti-dsDNA in BWγR−/− animals since, IgM and IgG1 levels were also reduced. There was a high incidence of B cell lymphoma in the BWγR−/− mice, which might be related to the suppression of autoAb production. Thus, the absence of glomerulonephritis in BWγR−/− mice is likely due to a dramatic yet unexplained effect of the inactivation of IFN-γ signaling on autoAb production. *The Journal of Immunology,* **1998, 160: 3713–3718.**

Female BW mice constitute one of the best-studied animal models of spontaneous systemic autoimmunity. Their characteristic features include polyclonal B cell activation, production of autoantibodies (autoAbs),[3] and development of glomerulonephritis (1–3). The major cause of death is renal failure due to glomerulonephritis. BW females are considered a useful animal model for human systemic lupus erythematosus (1).

Th2-type cytokines have been implicated in murine lupus. IL-4 mRNA expression increased with aging in BW females, while IL-2 mRNA expression decreased (4). Cytokine profiles of mitogen-stimulated T cells in BW mice showed high Th2 cell-related cytokine production (IL-4, IL-5, IL-10), while Th1 cell products (IL-2, IFN-γ) were low (5). The administration of anti-IL-10 or anti-IL-6 Abs in BW mice delayed the onset of disease, while injection of IL-6 accelerated onset (6–8). Surprisingly, BW mice were protected from autoimmune disease by inhibition of Th1-type responses as well. This protection was achieved by either transgenic expression of IL-4 (9) or inhibition of IFN-γ signaling. Neutralization of IFN-γ by Abs or soluble IFN-γR prevented glomerulonephritis (10, 11), while administration of exogenous IFN-γ accelerated autoAb production and renal disease (10–12).

How IFN-γ contributes to autoimmune disease in BW mice remains a matter of speculation. In an attempt to elucidate that point, we established a BW population that was deficient in IFN-γR. Such an approach has advantages over neutralization protocols. The signaling pathway is completely disrupted, no prolonged treatment is required, and reproducibility is presumably better. BW mice deficient in IFN-γR (BWγR−/−) were obtained by a crossing protocol. Essentially, a comparison of the BWγR−/− mice with BWγR+/+ and BWγR+/− littermates confirmed previous data obtained by neutralization of IFN-γ (10, 11). However, the protection from glomerulonephritis and the inhibition of autoAb production was more striking in the present study. Unexpectedly, the effect of the IFN-γR−/− mutation on autoAb production was not limited to class switch, since it inhibited not only the IFN-γ-dependent isotypes IgG2a and IgG3 but also IgM and IgG1. The dramatic inhibition of autoAb production may be sufficient to explain the absence of glomerulonephritis in BWγR−/− mice.

Materials and Methods

Animals and experimental protocol

NZB and NZW mice were provided by Bomholtguard Breeding and Research Center (Ry, Denmark). The IFN-γR null mutation (13) was backcrossed separately in the NZB and NZW background. The carrier of the mutation had the background 129/Sv. Mice were screened for the disrupted gene by PCR analysis of tail DNA lysate according to standard protocols using the following primers: 5′-CCCATTTAGATCCTACATACGAAA CATACGG-3′ (sense) and 5′-TTTCTGTCATCATGGAAAGGAGG GATACAG-3′ (antisense). The F_1 backcross generation was produced by mating a 129/Sv IFN-γR−/− female with a NZB or a NZW male. For further generations, IFN-γR+/− offspring were mated with their fathers. Heterozygous NZB as well as NZW mice of the F_5 backcross generation were intercrossed to achieve the following three genotypes of littermates: mice homozygous for the disrupted IFN-γR gene (BWγR−/−), homozygous wild-type mice (BWγR+/+), and heterozygous mice (BWγR+/−). NZW mice that were 4 mo old were used as controls. The experimental groups were constituted as follows: 25 BWγR+/+ mice, 26 BWγR+/− mice, 20 BWγR−/− mice, and 11 NZW mice. They were housed under standard conditions. Mice were killed when urinary protein levels reached 3 mg/ml or if they showed signs of severe disease, such as ≥15% weight loss or prostration. The remaining mice were killed at the age of 14 mo. One kidney was fixed in 4% buffered paraformaldehyde, and the other kidney was shock-frozen. Additional organs were fixed in paraformaldehyde if they showed macroscopic signs of disease.

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[3] Abbreviations used in this paper: autoAb, autoantibody; sIg, serum Ig.

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Clinical scoring

Body weights and urinary protein levels were measured weekly. Proteinuria was assessed semiquantitatively using dip sticks (Albustix, Bayer Diagnostics, Basingstoke, U.K.). Starting at 2 mo of age, blood was taken every month from the retroorbital plexus in 12 BWγR+/+, 14 BWγR+/−, 13 BWγR−/−, and 10 NZW mice.

Serum IgG (sIg)G levels

sIgG levels were quantified by ELISA. All reagents were obtained from Sigma (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 second Ab, and the disodium salt of *p*-nitrophenyl phosphate was used 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 IgG anti-dsDNA autoAbs

sIgG anti-dsDNA levels were quantified by ELISA essentially as described above for sIgG alone. Microtiter plates were coated with dsDNA from salmon testes at 10 μg/ml in PBS. The mouse sera to be tested were incubated overnight at a dilution of 500×. A standard curve was constructed using serial dilutions of a pool of sera from MRL/*lpr* mice showing glomerulonephritis. The titer of a given sample was the reciprocal value of the standard dilution yielding the same OD as the tested serum multiplied by 1×10^7.

Serum titers of IgM, IgG1, IgG2a, IgG3 anti-dsDNA, and IgG anti-histone

Serum levels of IgG anti-dsDNA subclasses and IgG anti-histone were determined by ELISA when the mice were 7 mo old. Serum levels of IgM anti-dsDNA were measured in 3- and 7-mo-old mice. The alkaline phosphatase-labeled mouse IgG subclass-specific Abs were obtained from Southern Biotechnology Associates (Birmingham, AL), and the alkaline phosphatase-labeled mouse IgG- and IgM-specific Abs were supplied by Sigma. The assays were performed basically as described above for total IgG. The microtiter plates were coated with dsDNA from salmon testes at 10 μg/ml in PBS or with total histone from calf thymus at 2.5 μg/ml in PBS (both from Sigma). Mouse sera were diluted from 30 to 10^5. The titer was calculated as the dilution giving an OD of 0.2 over background as estimated by a linear regression analysis of plots of the OD against the reciprocal value of the dilution.

Cytokine production by splenocytes in vivo

Spleens from three BW+/+ and three BW−/− 5-mo-old female mice were squeezed through an 80-mesh sieve (Bellco, Vineland, NJ) and cleared of erythrocytes by osmotic lysis. Splenocytes were resuspended at a concentration of 5×10^6 cells/ml in RPMI 1640 including 25 mM HEPES (supplemented with 10% FCS, 1% penicillin/streptomycin mix, 1% L-glutamine (200 mM), and 0.1% 2-ME (50 mM)). They were cultured in 1 ml aliquots in 24-well tissue culture plates either in medium alone or with 5 mg/ml Con A (Sigma). The supernatants were collected after 24 h and stored at −20°C. Two ELISA kits were used for the detection of IFN-γ (Genzyme, Cambridge, MA) and IL-4 (ImmunoKontact, Bioggio, Switzerland). Cytokine concentrations were calculated with reference to standard curves using recombinant cytokines.

Histology

The kidneys of all animals along with six livers showing macroscopic abnormalities were analyzed histologically. Organ slices were fixed in 4% buffered paraformaldehyde and processed for paraffin sectioning. Sections of 3-μm thickness were stained with the periodic acid Schiff reagent followed by hematoxylin.

Immunofluorescence

The left kidneys of four BWγR+/+ and four BWγR+/− mice that were killed after showing proteinuria and the left kidneys of six BWγR−/− mice that reached 14 mo of age, as well as the livers of four BWγR−/− mice in which lymphomas were suspected, were shock-frozen. Sections of 6-μm thickness 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 immunolabeling. After a rinse in PBS, the sections were incubated for 16 h at 4°C with the primary Abs diluted in PBS. The sections were then washed in PBS and incubated for 1 h at room temperature with cyanine (Cy3)-labeled goat anti-rat or rabbit anti-goat Ig Ab diluted 1:200. After rinsing in PBS, the sections were mounted in Immu-mount (Shandon, Pittsburgh, PA). Goat anti-mouse C3 was purchased from Bethyl laboratories (Montgomery, TX), and both goat anti-mouse Ig(G+M) (Cy3-conjugated $F(ab)_2$ fragment) as well as Cy3-conjugated rabbit anti-goat IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). The following clones of rat anti-mouse Abs were used: OKT3 (anti-CD3), RA3–6B2 (anti-B220), 114.15.2 (anti-MHC class II), and F4/80 (anti-macrophage).

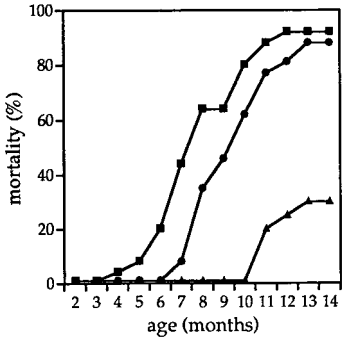

FIGURE 1. Mortality of BWγR+/+ (■), BWγR+/− (●), and BWγR−/− (▲) mice.

Statistics

A variance analysis was performed using ANOVA software, and the significance was tested with the Bonferroni/Dunn test.

Results

Mortality

Most BWγR+/+ (23 of 25) and BWγR+/− (23 of 26) mice were killed because they showed protein uria. Of the 20 BWγR−/− mice, 2 were killed at the ages of 10 and 12 mo because of proteinuria, while 4 others became moribund at the ages of 11 and 13 mo and were killed while showing normal urinary protein levels (≤0.3 mg/ml). Of the latter, three had lymphomas, which were readily visible macroscopically, appearing as pale spots on the liver surface. The remaining mice were killed at the age of 14 mo.

Since we killed the mice as soon as they showed heavy proteinuria or other symptoms of severe pathology, the time-course of mortality (Fig. 1) does not reveal the actual longevity of the experimental groups. However, since proteinuria (3 mg/ml) in BWγR+/+ and +/− mice (Fig. 2) developed over a time-course similar to that seen with BW mice in other studies (1, 6, 10), it is likely that in those two experimental groups, the longevity is close to that seen in the wild-type BW, in which a 50% mortality rate

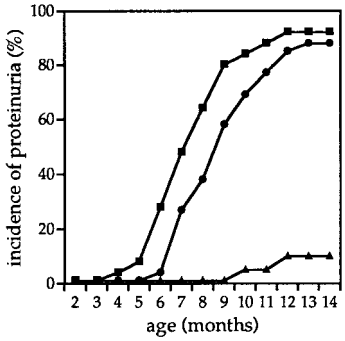

FIGURE 2. Time-course of the incidence of proteinuria in BWγR+/+ (■), BWγR+/− (●), and BWγR−/− (▲) mice. The starting numbers of animals were 25, 26, and 20, respectively. The numbers on the vertical axis represent the percentage of animals that have reached a value of 3 mg/ml.

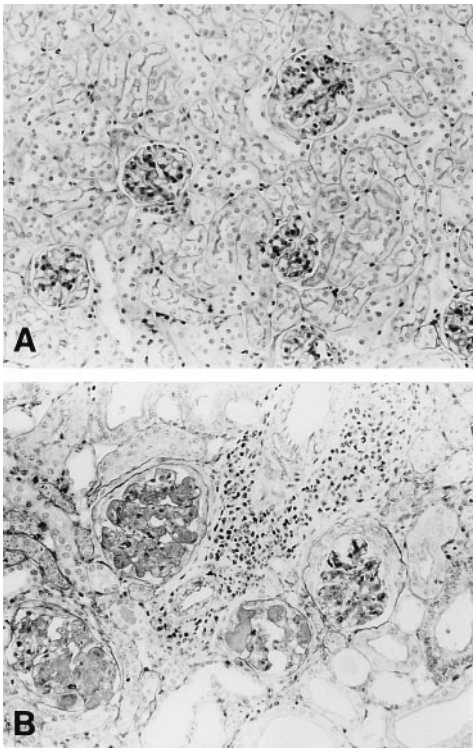

FIGURE 3. Histology of the renal cortex. Periodic acid Schiff reagent-stained sections of paraffin-embedded kidneys from BWγR−/− (*A*) and BWγR+/+ (*B*) mice at the age of 14 and 8 mo, respectively, are shown. Magnification: ×250.

occurs over a range of ~7 mo to 9 mo in various studies (1, 6, 10–12, 14). Disruption of IFN-γ signaling had a profound effect on mortality, since 70% of the BWγR−/− mice reached 14 mo of age.

Glomerulonephritis

About 90% of the BWγR+/+ and +/− but only 10% of the BWγR−/− mice developed proteinuria during the experimental period (Fig. 2). Histology revealed glomerulonephritis in all mice that showed proteinuria, and exclusively in those mice (Fig. 3).

Deposits of Ig and C3 were abundant in the mesangium and along the capillary walls of mice that showed proteinuria, whereas in the BWγR−/− mice that survived 14 mo, they were as low as in the non-lupus-prone NZW mice (Fig. 4). Thus, 90% of the BWγR−/− mice did not show any functional or histologic signs of glomerulonephritis.

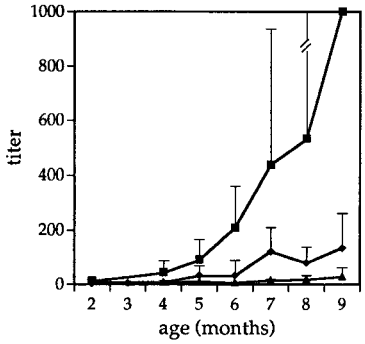

FIGURE 5. Time-course of sIgG anti-dsDNA titers in BWγR+ (equals +/+ and +/−) (■), BWγR−/− (▲), and NZW (♦) mice.

AutoAbs and total sIgG

Evaluation of IgG anti-dsDNA Abs by ELISA showed a continuous and massive increase in BWγR+/+ and +/− mice with age, whereas the titers remained low in the BWγR−/− mice for up to 9 mo (Fig. 5). At 7 mo, IgG1, IgG2a, and IgG3 anti-dsDNA Abs were elevated in the BWγR+/+ and +/− mice, while BWγR−/− mice displayed levels similar to those seen in NZW mice (Fig. 6). The effect of IFN-γR deficiency on autoAb production at 7 mo was not restricted to anti-dsDNA, since IgG anti-histone Abs were also low in BWγR−/− mice (Fig. 7). At 3 mo, IgM anti-dsDNA Abs were elevated in BWγR+/+, +/−, and −/− mice but at 7 mo, the levels of BWγR−/− mice were as low as the levels in NZW mice (Fig. 8). Thus anti-dsDNA Ab production was initiated normally in BWγR−/− mice but was then repressed. In contrast to IgG autoAbs, there was no statistically significant difference in total sIgG

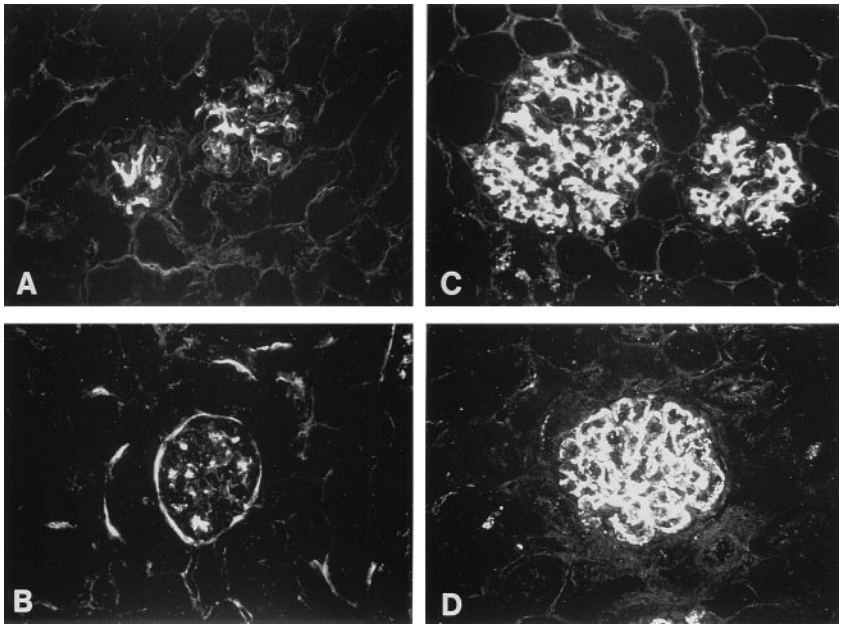

FIGURE 4. Ig(G+M) immunodeposits (*A* and *C*) and complement factor C3 (*B* and *D*) in the renal cortex of BWγR−/− (*A* and *B*) and BWγR+/+ (*C* and *D*) mice, aged of 14 and 8 mo, respectively, are shown. Magnification: ×360.

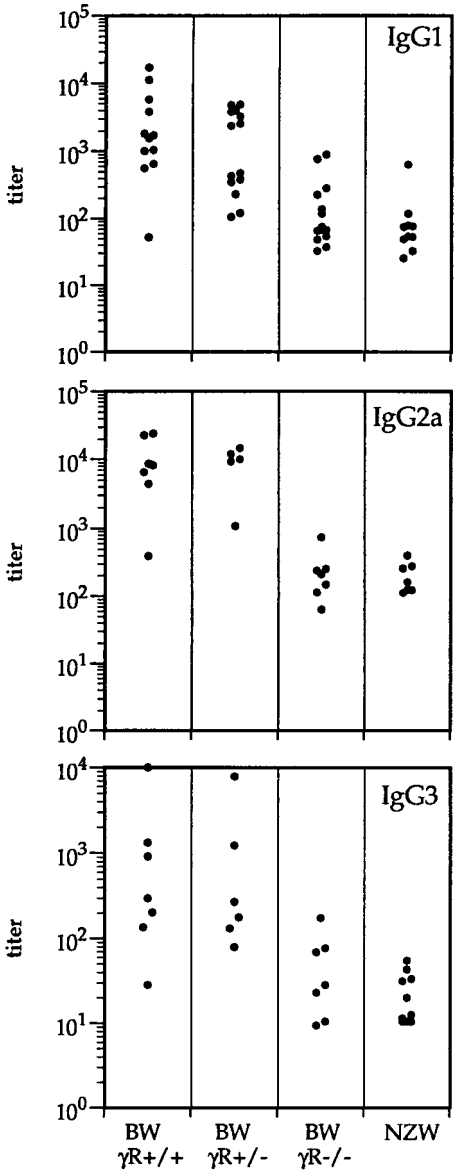

FIGURE 6. Serum titers of IgG1, IgG2a, and IgG3 anti-dsDNA autoAbs from 7-mo-old mice. Results are expressed as the dilution in which the OD is 0.2 over background. The log values of the titers were analyzed statistically. For all tested IgG subclasses, the levels for BWγR+/+ and +/− mice were significantly increased compared with the levels for BWγR−/− and NZW mice.

levels between the populations of BW mice at 7 mo (5.5 ± 1 mg/ml in γR+/+, 5.8 ± 2.7 mg/ml in γR+/−, and 4.2 ± 0.8 mg/ml in γR−/−; mean ± SD, $n = 6$).

Production of IL-4 and IFN-γ by Con A-stimulated splenocytes in vitro

Splenic T cells from BWγR−/− mice produced significantly more IL-4 (477 ± 39 pg/5 × 10^6 cells) than those from BWγR+/+ mice (302 ± 66 pg/5 × 10^6 cells). There was no difference in the production of IFN-γ between BWγR−/− (5838 ± 118 pg/5 × 10^6 cells) and BWγR+/+ mice (5599 ± 553 pg/5 × 10^6 cells). These data indicate that the production of Th1 and Th2 cytokines is not impaired in BWγR−/− mice.

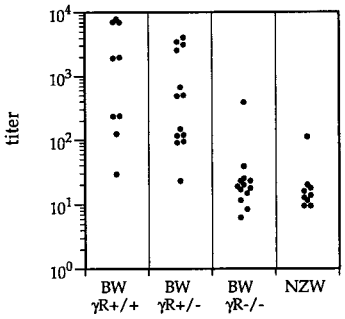

FIGURE 7. Serum titers of IgG anti-histone autoAbs from 7-mo-old mice. Results are expressed as the dilution in which the OD is 0.2 over background. The log values of the titers were analyzed statistically. The levels for BWγR+/+ and +/− mice were significantly increased compared with the levels for BWγR−/− and NZW mice.

Lymphomas

Of the six BWγR−/− mice that showed signs of disease and were killed before the end of the experimental period, three showed pale foci at the liver surface. Histology of those livers revealed accumulations of large lymphocytes with cleaved nuclei and prominent nucleoli (Fig. 9). Similar foci were found in the kidneys of the same mice but, with the exception of one mouse, they were much less conspicuous than those in the liver and were restricted to the periarterial connective tissue (data not shown). Of the remaining 14 BWγR−/− mice that were killed at the end of the experimental period (after 14 mo), 3 more showed macroscopically abnormal livers and accumulations of large lymphocytes in the liver and kidney. The spleens of the latter three mice were also fixed and processed for histology. Periarterial lymphatic sheaths (PALS), germinal centers, and the marginal zone were not visible. The cellular composition of the white pulp appeared monotonous, consisting mainly of large lymphocytes with the same morphology as those infiltrating the liver and kidney (Fig. 9). In the four livers that were processed for immunofluorescence, the lymphocytic foci expressed MHC class II, B220, and IgM and could thus be identified as B cells (Fig. 10). $CD3^+$ T cells were intermingled among B cells in all foci, but they clearly represented a minority of the lymphocyte population (data not shown). Macrophages, identified by the F4/80 Ab, were rare (data not shown).

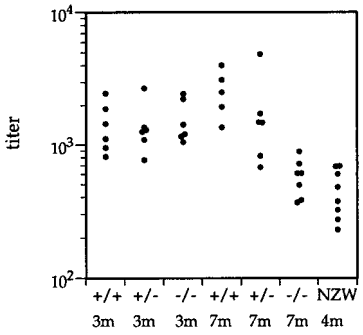

FIGURE 8. Serum titers of IgM anti-dsDNA autoAbs from 3- and 7-mo-old mice. Results are expressed as the dilution in which the OD is 0.2 over background. The log values of the titers were analyzed statistically. The levels for BWγR+/+, +/−, and −/− mice at 3 mo of age were significantly increased compared with the levels for NZW mice. At 7 mo of age, IgM anti-dsDNA titers were still significantly increased in BWγR+/+ and +/− mice, but BWγR−/− titers were decreased to values similar to those seen in 4-mo-old NZW mice.

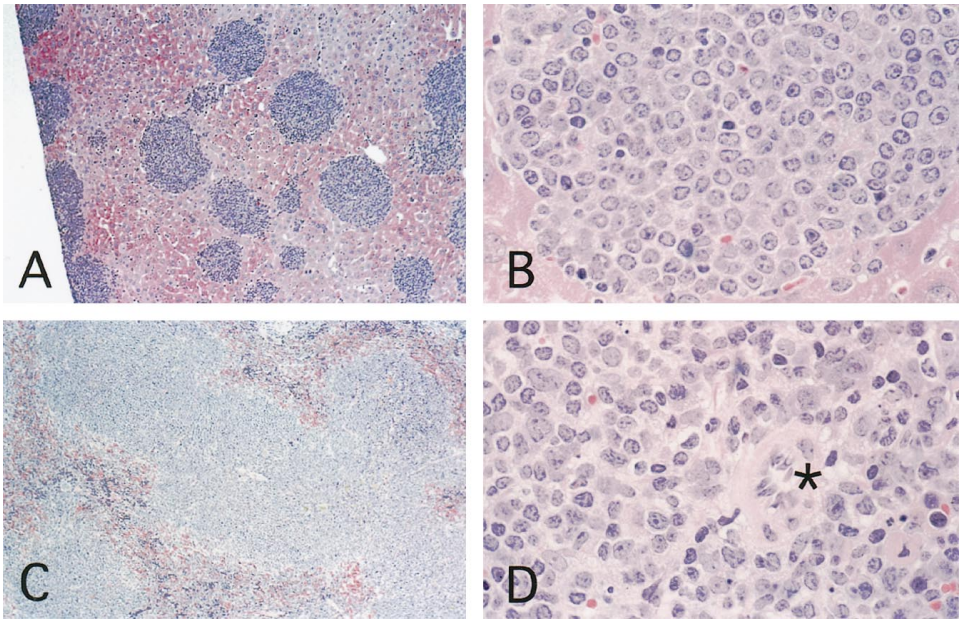

FIGURE 9. Histology of lymphoma in the liver (*A* and *B*) and spleen (*C* and *D*) of a BWγR−/− mouse. The lymphocytic foci in the liver appear cytologically homogeneous (*A* and *B*). In the spleen, the cytology of the white pulp is monotonous, and it is not possible to identify PALS, germinal centers, or a marginal zone (*C* and *D*). In the periphery of a central arteriole (asterisk in *D*), the small lymphocytes, characteristic of the PALS, represent only a minor population among large, likely neoplastic, lymphocytes. The latter show the same morphology in the liver (*B*) as in the spleen (*D*). Stain: periodic acid Schiff (*A*), hematoxylin-eosin (*B–D*). Magnification: ×55 (*A*), ×490 (*B* and *D*), and ×150 (*C*).

Discussion

In an attempt to find out how IFN-γ influences glomerulonephritis in a murine model of systemic lupus erythematosus (10, 11), we generated BW mice that were deficient in IFN-γR by backcrossing. The development of glomerulonephritis and the production of autoAbs were assessed in BWγR−/−, +/+, and +/− littermates. The data confirm previous studies showing the importance of IFN-γ in the pathogenesis of glomerulonephritis in this animal model (10–12). The protection observed in BWγR−/− mice was actually better than that obtained by neutralization of IFN-γ in previous studies. Indeed, at 11 mo, ~40% of mice treated with IFN-γ-neutralizing Abs showed proteinuria (10) compared with 5% in BWγR−/− mice. Furthermore, although neutralizing treatments did delay the production of IgG anti-DNA, the levels were clearly increased at 6 mo (10, 11). Incomplete neutralization of IFN-γ in previous studies might explain the lesser degree of protection from autoimmunity compared with the present study in which IFN-γ signaling was completely abolished. Also, in earlier studies, neutralizing treatment was conducted for a limited period of time, namely from 4 to 7 or 8 mo of age (10, 11).

The very low levels of IgG anti-dsDNA and IgG anti-histone in BWγR−/− mice might reflect a defective Th cell function. In this respect, it is interesting that the impairment of the Th1 response to parasites provoked by deletion of the IFN-γR in mice did not result in the expected shift to a Th2 response (15, 16). However, since splenocytes of BWγR−/− mice and BWγR+/+ mice produced similar amounts of IL-4 and IFN-γ in vitro, Th2 cell help for Ig isotype switch is presumably available in the mutants. The similarity of total sIgG levels among the BWγR−/− mice and their γR+/− and γR+/+ counterparts supports this possibility.

There is no previous report of defective autoAb production in mice with deficient IFN-γ signaling. Production of IgG anti-dsDNA Abs after LPS-treatment was not inhibited in IFN-γR−/− mice with the 129/Sv background (17). In the lupus-prone strain MRL/*lpr*, IFN-γ−/− mutants (18) and IFN-γR−/− mutants (19) produced high amounts of IgG autoAbs, even though the IFN-γ-promoted IgG2a and IgG3 isotypes remained at low levels as expected. The defective IgG autoAb production in γR−/− mice in the present study might therefore reflect some peculiarity of the BW background. In that context, one relevant characteristic of NZB-derived strains is the high incidence of $CD5^+$ B cell malignancy (20–24). In the present study, foci of neoplastic B cells were observed in the livers and kidneys of 6 of 20 BWγR−/− mice. Since we focused on glomerulonephritis and observed lymphoma by chance at the end of the experimental period, the true incidence of B cell neoplasy may have been underestimated. During passaging in vivo, some $CD5^+$ B cell clones derived from

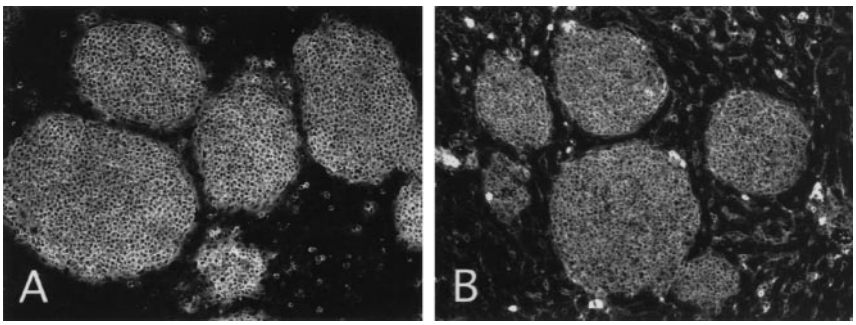

FIGURE 10. Immunofluorescent detection of MHC class II (*A*) and IgM (*B*) in the liver of a BWγR−/− mouse. Both Ags are expressed in a majority of cells in the lymphocytic foci. Magnification: ×110.

NZB mice with chronic lymphatic leukemia acquired the capacity to infiltrate various organs and produce large-cell lymphomas that are strikingly similar to those observed in the present study in BWγR−/− mice. Indeed, the B cell clones colonize preferentially the liver and consist of large lymphocytes with cleaved nuclei as well as prominent nucleoli (21, 25). Importantly, spontaneous development of that type of lymphoma has not been described before in either NZB or BW mice. Likewise, lymphomas were not observed in BWγR+/+ mice in the present study. Thus, it appears that the IFN-γR−/− mutation might accelerate or modify a pathway of neoplastic development of B cells that is typical for NZB-derived strains. An accelerated development of malignant B cells in BWγR−/− mice is plausible, since proliferation of those cells might be under the control of specific $NK1^+$ T cells via IFN-γ (26).

There is convincing evidence in the literature for a link between B cell neoplasy and the inhibition of Ab production. Malignant $CD5^+$ B cells seem to be responsible for the inhibition of B cell function in multiple myeloma patients (27). The decrease of IgM anti-dsDNA between the ages of 3 mo and 7 mo in BWγR−/− mice is reminiscent of the decrease of total sIgM and IgM autoAb in recipients of NZB malignant B cells (28, 29). Clearly, a detailed comparison of the development of B cells between BWγR−/− mice and BWγR+/+ mice will be necessary to substantiate the hypothesis that neoplastic B cells suppress autoAb production in BWγR−/− mice.

IFN-γ may contribute to glomerulonephritis by up-regulating the expression of MHC gene products and adhesion molecules as well as by activating macrophages. However, the IFN-γR−/− mutation did not protect mice from mild glomerular inflammation induced by LPS (17) or from anti-glomerular basement membrane glomerulonephritis (30). In BW mice, it is not necessary to invoke the proinflammatory properties of IFN-γ to explain the profound protective effect of blocking IFN-γ signaling. The low titers of circulating autoAbs and intraglomerular immune complexes constitute the most likely explanation.

The crucial importance of IFN-γ for the production of autoAbs in BW mice was unexpected, and it remains to be explained. The BWγR−/− mice might represent a useful tool in the study of the regulation of autoAb production in murine lupus.

Acknowledgments

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