JCI The Journal of Clinical Investigation

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J Clin Invest. 1997;99(8):1936-1946. https://doi.org/10.1172/JCI119361.

Research Article

The systemic autoimmune syndrome of MRL/Mp-lpr/lpr (MRL/lpr) mice consists of severe pan-isotype hypergammaglobulinemia, autoantibody production, lymphadenopathy, and immune complex-associated end-organ disease. Its pathogenesis has been largely attributed to helper alphabeta T cells that may require critical cytokines to propagate pathogenic autoantibody production. To investigate the roles of prototypical Th1 and Th2 cytokines in the pathogenesis of murine lupus, IFN-gamma -/- and IL-4 -/- lupus-prone mice were generated by backcrossing cytokine knockout animals against MRL/lpr breeders. IFN-gamma -/- animals produced significantly reduced titers of IgG2a and IgG2b serum immunoglobulins as well as autoantibodies, but maintained comparable levels of IgG1 and IgE in comparison to cytokine-intact controls; in contrast, IL-4 -/- animals produced significantly less IgG1 and IgE serum immunoglobulins, but maintained comparable levels of IgG2a and IgG2b as well as autoantibodies in comparison to controls. Both IFN-gamma -/- and IL-4 -/- mice, however, developed significantly reduced lymphadenopathy and end-organ disease. These results suggest that IFN-gamma and IL-4 play opposing but dispensable roles in the development of lupus-associated hypergammaglobulinemia and autoantibody production; however, they both play prominent roles in the pathogenesis of murine lupus-associated tissue injury, as well as in lpr-induced lymphadenopathy.

Roles of Interferon-γ and Interleukin-4 in Murine Lupus

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Abstract

The systemic autoimmune syndrome of MRL/Mp-*lpr*/*lpr* (MRL/*lpr*) mice consists of severe pan-isotype hypergammaglobulinemia, autoantibody production, lymphadenopathy, and immune complex-associated end-organ disease. Its pathogenesis has been largely attributed to helper αβ T cells that may require critical cytokines to propagate pathogenic autoantibody production. To investigate the roles of prototypical Th1 and Th2 cytokines in the pathogenesis of murine lupus, IFN-γ −/− and IL-4 −/− lupus-prone mice were generated by backcrossing cytokine knockout animals against MRL/*lpr* breeders. IFN-γ −/− animals produced significantly reduced titers of IgG2a and IgG2b serum immunoglobulins as well as autoantibodies, but maintained comparable levels of IgG1 and IgE in comparison to cytokine-intact controls; in contrast, IL-4 −/− animals produced significantly less IgG1 and IgE serum immunoglobulins, but maintained comparable levels of IgG2a and IgG2b as well as autoantibodies in comparison to controls. Both IFN-γ −/− and IL-4 −/− mice, however, developed significantly reduced lymphadenopathy and end-organ disease. These results suggest that IFN-γ and IL-4 play opposing but dispensable roles in the development of lupus-associated hypergammaglobulinemia and autoantibody production; however, they both play prominent roles in the pathogenesis of murine lupus-associated tissue injury, as well as in *lpr*-induced lymphadenopathy. (*J. Clin. Invest.* 1997. 99:1936–1946.) Key words: autoimmunity • cytokines • lupus • rodent • autoantibodies

Introduction

MRL/Mp-*lpr*/*lpr* (MRL/*lpr*)[1] mice spontaneously develop a severe autoimmune disease closely resembling human SLE, characterized by hypergammaglobulinemia, autoantibody production of anti-dsDNA, anti-small nuclear ribonucleoprotein (snRNP) and rheumatoid factor specificities, and immune complex–associated end organ disease of the kidney and salivary glands (1–4). The *lpr* mutation, a nearly disabling insertion of an early retrotransposon in the Fas apoptosis gene (5–9), causes defective activation-induced cell death of peripheral αβ T cells (10–13), resulting in the lymphoaccumulation of $CD4^-CD8^-B220^+$ T cells (14).

The pathogenesis of MRL lupus largely requires $CD4^+$ αβ T cells, which provide help to autoreactive B cells (15–18); however, the roles that individual Th1 and Th2 cell subsets play in this process remain unclear. Autoantibody production remains the hallmark of both human and murine lupus, suggesting a requirement for cytokines produced by Th2 cells in autoreactive B cell activation, but detailed studies have revealed conflicting data regarding the relative importance of Th1 versus Th2 cytokines in disease: for example, humoral and end-organ autoimmunity in the New Zealand black/white F1 ([NZB × NZW] F1) murine lupus model could be abrogated by treatment with anti–IFN-γ or anti–IFN-γ receptor antibodies (19, 20), suggesting Th1-dependent mechanisms in pathogenesis; however, treatment of these animals with anti–IL-10 antibodies also significantly diminished autoantibody production and end-organ disease, suggesting the importance of Th2 cytokines in disease pathogenesis (21). Likewise, in MRL mice, disease has been associated with the Th1 cytokines IFN-γ (22–24) and IL-12 (25), as well as the Th2 cytokines IL-4 (26, 27) and IL-10 (23). In fact, treatment with IL-4 antagonists appeared to decrease anti-DNA titers and associated renal disease (26), indicating a substantial requirement for Th2 cytokines, whereas late treatment of MRL/*lpr* mice with antibodies to IFN-γ failed to ameliorate disease (28), suggesting the dispensability of Th1 cytokines. Thus, although some evidence exists for the importance of both types of cytokines in MRL disease pathogenesis, the specific roles that Th1 versus Th2 mechanisms play in various aspects of murine lupus remains incompletely resolved.

To address the roles of the prototypical Th1 cytokine IFN-γ or the Th2 cytokine IL-4 in murine lupus, Fas-deficient and Fas-intact lupus-prone animals genetically deficient in each cytokine were developed, eliminating the technical difficulties of antibody treatment and allowing a more detailed analysis of autoimmune parameters, as well as lymphoaccumulation of T cells. IFN-γ–deficient animals produced lower titers of autoantibodies and Th1-related immunoglobulin isotypes and had reduction in renal and salivary gland disease. Lymphadenopathy was also diminished, with a marked reduction in accumulation of $CD4^-CD8^-B220^+$ T cells. IL-4–deficient animals also had reduced renal and salivary gland disease and decreased lymphadenopathy, with diminution of all T cell subsets; however, these animals maintained titers of autoantibodies similar to their cytokine-intact counterparts, in the setting of lower titers of Th2-related immunoglobulin isotypes. These findings demonstrate that both IFN-γ and IL-4 play prominent roles in the pathogenesis of end-organ disease, even though

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Received for publication 21 October 1996 and accepted in revised form 3 January 1997.

1. *Abbreviations used in this paper:* FANA, fluorescent antinuclear antibody test; Fas+, Fas +/+; IFG+, IFN-γ +/+; IFG−, IFN-γ −/−; IL4+, IL-4 +/+; IL4−, IL-4 −/−; *lpr*, *lpr*/*lpr*; MRL/*lpr*, MRL/Mp-*lpr*/*lpr*; snRNP, small nuclear ribonucleoprotein.

J. Clin. Invest.

0021-9738/97/04/1936/11 $2.00
Volume 99, Number 8, April 1997, 1936–1946

they play opposing roles in the development of elevated levels of class-switched immunoglobulin isotypes and autoantibody production. Hence, murine lupus, at least in animals with MRL background genes, does not fit solely into the paradigm of a Th1- or Th2-cytokine dominated disease.

Methods

Mice. IFN-γ −/− DBA (29) or IL-4 −/− C57BL/6 (30) mice were crossed against MRL/*lpr* animals (all from Jackson Laboratories, Bar Harbor, ME) to generate IFN-γ +/− Fas +/*lpr* or IL-4 +/− Fas +/*lpr* double-heterozygotic offspring, which were intercrossed to generate IFN-γ–intact (IFG+) and IFN-γ–deficient (IFG−) Fas-intact (Fas+) or *lpr*/*lpr* (*lpr*) F2 animals, as well as IL-4–intact (IL4+) and IL-4–deficient (IL4−) Fas+ or *lpr* animals. Genotypes were screened via PCR of tail DNA, as described elsewhere for IFN-γ (Jackson Laboratory), IL-4 (Jackson Laboratories), and Fas (10). All animals were maintained under specific pathogen-free conditions at the Yale University School of Medicine (New Haven, CT).

Cytokine assessment. To assay for cytokine production, 5×10^5 erythrocyte-cleared splenocytes were incubated for 18-20 h in 0.5 ml of DMEM containing 10% fetal bovine serum with or without 5 μg/ml concanavalin A (Sigma Chemical Co., St. Louis, MO). 50 μl of culture supernatant was assayed for IFN-γ and IL-4 activity via specific cytokine ELISA (Endogen Inc., Cambridge, MA).

Antibody analyses. Serum immunoglobulin and autoantibody studies were performed as previously described (18). Briefly, antinuclear antibodies were assayed by indirect immunofluorescence (FANA) on sera diluted at 1:50 using HEp-2 substrate cells (Quidel, San Diego, CA). Fluorescence was visualized with a microscope at a magnification of 1000 (Axioskop; Carl Zeiss, Inc., Thornwood, NJ), with intensity rated 0–4$^+$, as determined by the lightmeter's estimated required exposure time for 10–12 cells/hpf on ASA 400 film (> 120 s, 0; < 120 s, 1$^+$; < 60 s, 2$^+$; < 30 s, 3$^+$; < 15 s, 4$^+$). IgG isotype-specific FANAs were performed using FITC-conjugated goat F(ab′)$_2$ anti–mouse IgG1, IgG2a, IgG2b, and IgG3 (Southern Biotechnology Associates, Inc., Birmingham, AL). Specific autoantibody titers (IgG anti-dsDNA, IgG anti-snRNP, and kappa rheumatoid factor) were determined by ELISA using specific substrates on sera at 1:100 dilution. Some anti-dsDNA antibodies were further assayed by indirect immunofluorescence on *Crithidia luciliae* substrates (1:10 serum dilution; Antibodies Inc., Davis, CA). IgG isotype-specific autoantibody ELISAs utilized alkaline phosphatase-conjugated rat anti–mouse IgG1, IgG2a, IgG2b, and IgG3 (PharMingen, San Diego, CA). Serum immunoglobulin titers were determined by ELISA (Pierce, Rockford, IL, or PharMingen). Positive titers were determined as OD values which exceeded three standard deviations above those of mean normal controls, which included B10.BR and/or RAG-1 −/− mice (Jackson Laboratories). Statistical significance was evaluated by paired student's *t* test on mean immunoglobulin titers or autoantibody OD values.

Cell analyses. Spleens and six peripheral nonmesenteric lymph nodes were weighed, homogenized, and cleared of erythrocytes by osmotic lysis. Live cell count was determined by trypan blue exclusion. For phenotypic analyses, cells were analyzed by FACSort® flow cytometry and CellQuest 1.1 software (Becton Dickinson, Bedford, MA): antibodies included H129.19-FITC or PE (anti-CD4), GL3-PE (anti-TCR γδ), RA3-6B2-biotin (anti-CD45R/B220), H57-597-FITC, or PE (anti-TCR β; all from PharMingen), and/or 53-6.7-Quantum Red™ (anti-CD8α; Sigma Chemical Co.), followed by Streptavidin-Texas Red® (Gibco Life Technologies, Gaithersburg, MD).

Histopathology and renal function tests. Tissue specimens were fixed in 10% buffered formalin, and sections were stained with Hematoxylin and Eosin by standard procedures at the Department of Pathology, Yale University School of Medicine (18, 31, 32). Creatinine was measured by standardized Jaffé reaction (Sigma Chemical Co.).

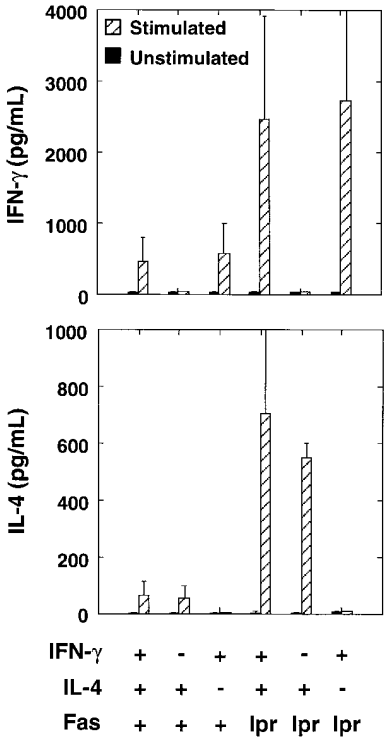

Figure 1. Cytokine production by IFN-γ −/− and IL-4 −/− mice. Supernatants of unstimulated or ConA-stimulated splenocytes from 7–8-mo-old control, IFN-γ −/− or IL-4 −/− animals were assayed for IL-4 or IFN-γ by ELISA. Shown are standard deviations for two to six animals tested in each group. The limit of assay sensitivity was 47 pg/ml for IFN-γ, and 3 pg/ml for IL-4.

Results

Generation of cytokine-deficient lupus-prone mice. IFG-*lpr* and IL4-*lpr* mice were generated by backcrossing IFN-γ −/− or IL-4 −/− breeders against the MRL/*lpr* background. To confirm the phenotype of the animals, their splenocytes were assayed for IL-4 or IFN-γ production by ELISA (Fig. 1). As expected, IL4- animals produced undetectable levels of IL-4, while IFG- animals produced undetectable levels of IFN-γ, regardless of Fas genotype.

Serum immunoglobulin production in cytokine-deficient mice. Sera from mice at ages 3 and 7–8 mo were analyzed for immunoglobulin isotype levels (Fig. 2). At 3 mo of age, IFG-*lpr* animals had significantly lower levels of IgG2a, IgG2b, IgG3, IgA, and IgM compared with IFG+*lpr* counterparts ($P < 0.001$), but maintained comparable or perhaps increased levels of IgG1 and/or IgE (Fig. 2 *A*). Similarly, IFG-Fas+ animals had lower levels of IgG2a, IgG2b, IgG3, IgA, and IgM ($P < 0.05$) compared with IFG+Fas+ mice. Older animals generally had preservation of such differences: IFG-*lpr* animals produced significantly lower levels of IgG2a and IgG2b ($P < 0.001$) as well as IgM and IgA ($P < 0.01$) compared with IFG+*lpr* counterparts, but had comparable levels of IgG1, IgG3, and IgE. IFG-Fas+ animals maintained lower levels of IgG2a and IgG2b compared with IFG+Fas+ animals at this age ($P < 0.001$), but had comparable or higher levels of IgG1, IgG3, IgM, IgA, and IgE. IFG- animals thus developed diminished titers of Th1-associated isotypes, particularly IgG2a and Ig2b, compared with their IFG+ counterparts, but produced comparable or excess titers of Th2-associated isotypes, including IgG1 and IgE.

IL4-*lpr* animals had significantly lower levels of IgG1 and IgE compared with IL4+*lpr* counterparts ($P < 0.001$) at 3 mo of age, but maintained comparable levels of IgG2a, IgG2b,

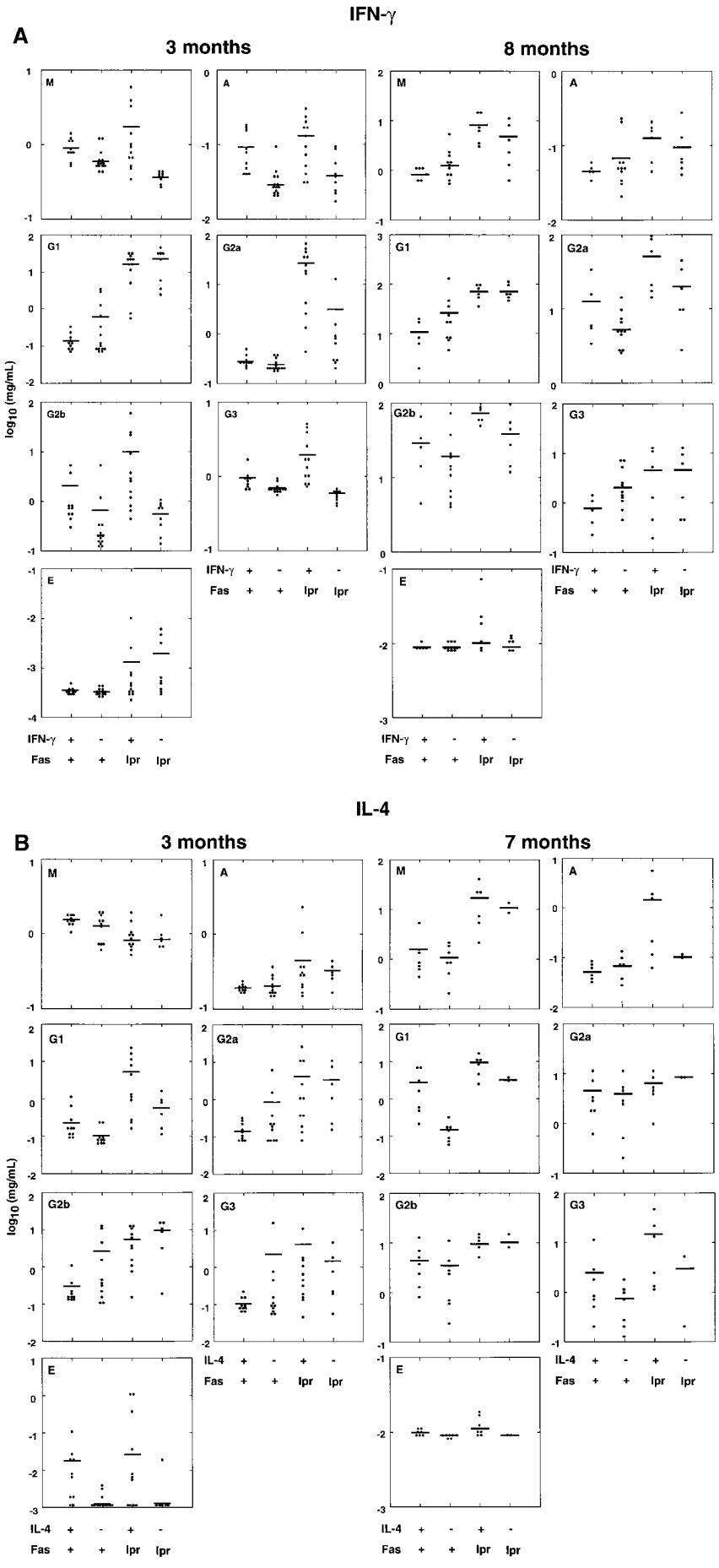

Figure 2. Serum immunoglobulin isotype titers in cytokine mutant mice. Sera from 12-wk-old or 7–8-mo-old animals were assayed for immunoglobulin titers by ELISA. (*A*) For IFG+Fas+, IFG-Fas+, IFG+*lpr*, and IFG-*lpr* animals, respectively, $n = 10$, 14, 12, and 9 at 12 wk of age, and $n = 5$, 11, 6, and 6 at 8 mo of age. (*B*) For IL4+Fas+, IL4-Fas+, IL4+*lpr*, and IL4-*lpr* animals, respectively, $n = 10$, 11, 11, and 6 at 12 wk of age, and $n = 7$, 7, 7, and 2 at 7 mo of age. Horizontal bars indicate mean titers; note the use of a logarithmic scale.

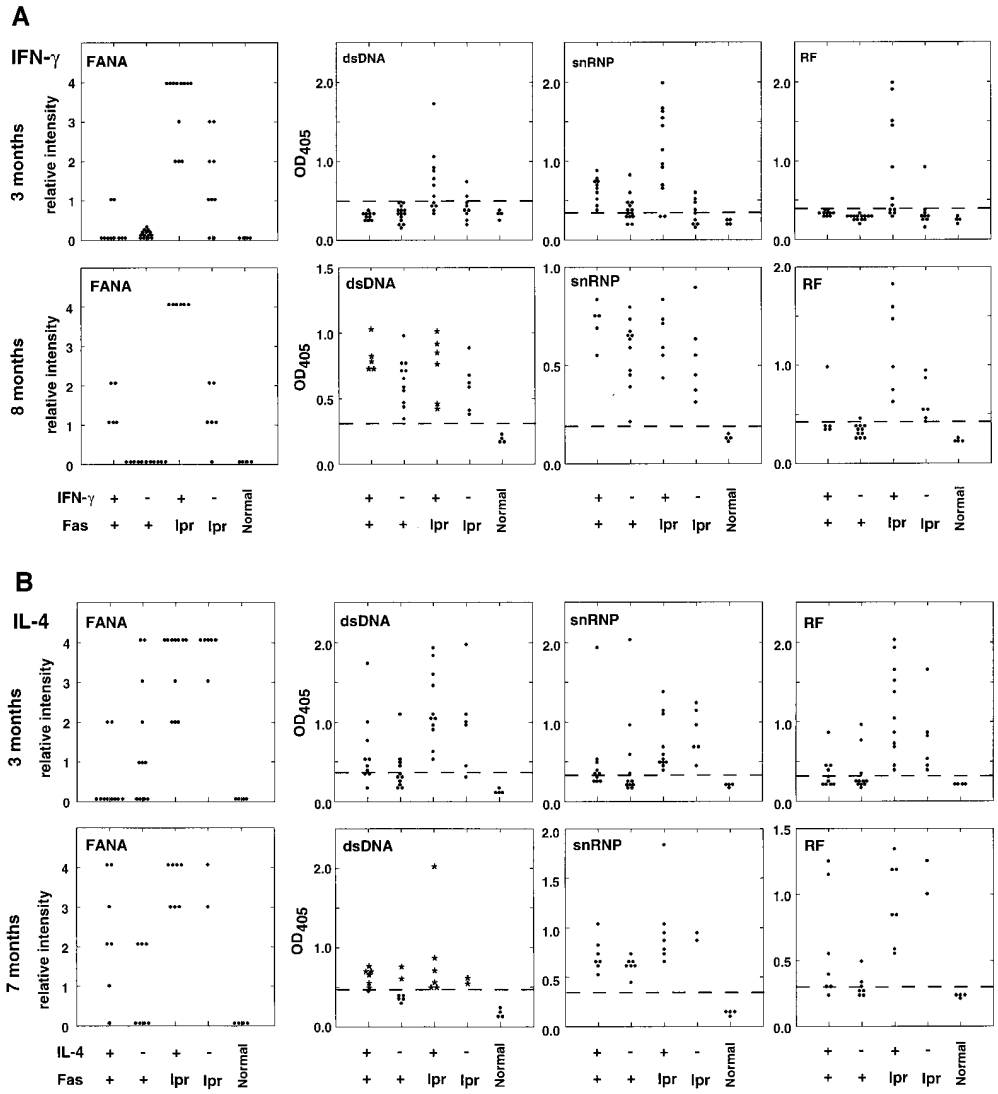

Figure 3. Serum autoantibodies in cytokine mutant mice. Sera from 12-wk-old or 7–8-mo-old IFG+ and IFG− (*A*), and IL4+ and IL4− (*B*), animals were assayed for autoantibodies via the fluorescent antinuclear antibody test (*FANA*), or via specific autoantibody ELISAs for anti-dsDNA, anti-snRNP, and rheumatoid factor antibodies. Sera from 7–8-mo-old animals were also assayed for anti-dsDNA activity by immunofluorescence on *Crithidia* substrates; positive results are indicated by asterisks. ELISA assays for differently aged mice were performed at separate times, such that differences in absolute OD values do not necessarily indicate differences in autoantibody activity between mice of different ages. Dashed lines indicate thresholds for positivity, as determined by three standard deviations greater than the OD_{mean} of age-matched normal animals (B10.BR or RAG-1 −/−). Numbers of animals in each group are identical to those in Fig. 2.

IgG3, IgA, and IgM (Fig. 2 *B*). IL4-Fas+ animals had lower levels of IgG1 and IgE compared with IL4+Fas+ animals ($P < 0.05$), but contained higher levels of IgG2a, IgG2b, and IgG3. Older IL4-*lpr* animals produced significantly less IgG1, but similar levels of IgG2a and IgG2b, in comparison to their IL4+*lpr* counterparts ($P < 0.001$). These IL4-*lpr* animals also appeared to produce less IgE, as well as IgG3 and IgA ($P < 0.05$), but the significance of these findings remains unclear given the small number of IL4-*lpr* animals available for analysis at this age ($n = 2$). Still, IL4-Fas+ animals at this age produced significantly less IgG1, but comparable titers of IgG2a and IgG2b, compared to IL4+Fas+ counterparts ($P < 0.001$). Thus, IL4- animals developed diminished titers of Th2-associated isotypes, especially IgG1, compared with their IL4+ counterparts, but maintained comparable titers of Th1-associated isotypes, especially IgG2a and IgG2b.

Autoantibody production in cytokine-deficient mice. 3-mo-old IFG- animals had lower titers of autoantibodies compared with IFG+ animals (Fig. 3 *A* and data not shown). This comparison was most apparent between groups of *lpr* animals, where IFG-*lpr* sera contained lower-titer antinuclear antibodies, as judged by FANA ($P < 0.01$; Fig. 3 *A* and data not shown), and contained lower titers of anti-dsDNA, anti-snRNP and rheumatoid factor antibodies ($P < 0.001$; Fig. 3 *A*), compared with IFG+*lpr* sera. This difference was less penetrant between the Fas+ groups, where 2/10 IFG+Fas+ animals versus 0/14 IFG-Fas+ animals tested positive by FANA ($P < 0.01$), and 100% of IFG+Fas+ animals versus 50% of IFG-Fas+ mice tested weakly to moderately positive for anti-snRNP antibodies by ELISA ($P < 0.01$). Older animals demonstrated a similar effect of IFN-γ deficiency, where IFG-*lpr* animals had lower FANA and RF titers compared with IFG+*lpr* counterparts ($P < 0.001$), and IFG-Fas+ animals had lower FANA titers compared with IFG+Fas+ counterparts ($P < 0.001$). Interestingly, these older IFG- animals appeared to produce similar levels of anti-dsDNA and anti-snRNP antibodies compared with their IFG+ counterparts. However, such autoantibodies likely were of low affinity, since in contrast to anti-dsDNA antibodies from IFG+ animals, those from the IFG- cohort failed to bind the dsDNA-containing kinetoplast of *Crithidia luciliae* substrates. This failure to bind the latter substrate correlates with the inability of anti-snRNP antibodies to immunoprecipitate radiolabeled snRNPs, and suggests the presence of polyreactive, rather than antigen-specific

and affinity-matured, autoantibodies (Fig. 3 *A* and data not shown) (18).

In contrast, IL-4 deficiency had little effect upon autoantibody production (Fig. 3 *B*). A higher percentage of IL4-Fas+ sera did test positive by FANA compared with IL4+Fas+ counterparts (64 versus 20%; $P < 0.10$) at 3 mo of age, and a higher percentage of IL4-*lpr* sera contained high-titer antinuclear antibodies compared with IL4+*lpr* counterparts at this same time point (100 versus 73% testing 3+ or 4+; P = NS); however, differences in anti-dsDNA, anti-snRNP, and rheumatoid factor titers were not statistically significant among IL4+Fas+ versus IL4-Fas+, and IL4+*lpr* versus IL4-*lpr* mice, even though some IL4-*lpr* animals appeared to have lower titers of anti-dsDNA and rheumatoid factor antibodies. Such findings appeared to persist among older IL4- animals, both Fas+ and *lpr.* Notably, IL4- animals appeared capable of generating antibodies with affinities and/or specificities comparable to IL4+ counterparts, since IL4- anti-dsDNA antibodies tested positive by *Crithidia* immunofluorescence (18).

Significant autoantibody activity, in terms of FANA, anti-dsDNA, and anti-snRNP, was found among all IgG isotypes in both IFG+ and IL4+ animals (Fig. 4, *closed circles*), analogous to previous studies in MRL mice that have shown pan-isotype elevation in autoantibody levels, albeit with a predominance of IgG2a, IgG2b, and IgG3 (22, 33–37). In contrast, autoantibodies in IFG- animals were predominantly of the IgG1 subclass, with relative paucities of IgG2a, IgG2b, and/or IgG3 ($P < 0.001$; Fig. 3, *three left panels*, *open circles*), whereas autoantibodies in the IL4- cohorts were predominantly of the IgG2a, IgG2b, and/or IgG3 subclasses, with a relative paucity of IgG1 ($P < 0.001$; Fig. 3, *three right panels*, *open circles*). This isotype-specific penetrance of autoantibodies may account in part for the relative maintenance of total autoantibody levels in the IL4- cohorts versus the IFG- groups (see Figs. 2 and 3).

Lymphadenopathy in cytokine-deficient mice. In both IFG-*lpr* and IL4-*lpr* animals, *lpr*-induced lymphadenopathy as determined by organ weights and cellularity was significantly reduced compared with their respective cytokine-intact counterparts ($P < 0.001$; Fig. 5, *A* and *B*). In IFG-*lpr* animals, lymphoid organ weights and cell counts were reduced to values comparable to their IFG+Fas+ counterparts at 3 mo of age, although some older IFG-*lpr* animals contained modestly increased spleen and lymph node size compared to IFG+Fas+ counterparts ($P < 0.01$). The weight and cell counts of both younger and older IL4-*lpr* spleens and lymph nodes still remained significantly elevated in comparison with IL4+Fas+ animals ($P < 0.001$), despite the gross reduction in comparison with IL4+*lpr* mice.

IFG-*lpr* animals contained severely diminished numbers of $CD4^-CD8^-B220^+$ αβ T cells that characteristically predominate in the T cell population of cytokine-intact *lpr* animals (Fig. 5, *A* and *C*), while IL4-*lpr* animals maintained similar percentages of $CD4^-CD8^-B220^+$ as well as conventional $CD4^+B220^-$ and $CD8^+B220^-$ αβ T cells (Fig. 5, *B* and *C*). Notably, the $CD4^+B220^+$ αβ T cell population, which contributes 5–20% of the $B220^+$ αβ T cell population (38), was proportionately diminished in IL4-*lpr* animals but absent in IFG-*lpr* animals (Fig. 5, *A–C*). These differences were apparent in both younger and older mice. Thus, IFN-γ was required for the *lpr*-induced lymphoaccumulation of T cells in *lpr* mice, affecting the $CD4^-CD8^-B220^+$, $CD4^+B220^+$, and $CD4^-CD8^-B220^-$ subsets. In comparison, IL-4 was required for the development and propagation of full-blown lymphadenopathy, but was not associated with any significant changes in the percentages of individual T cell populations.

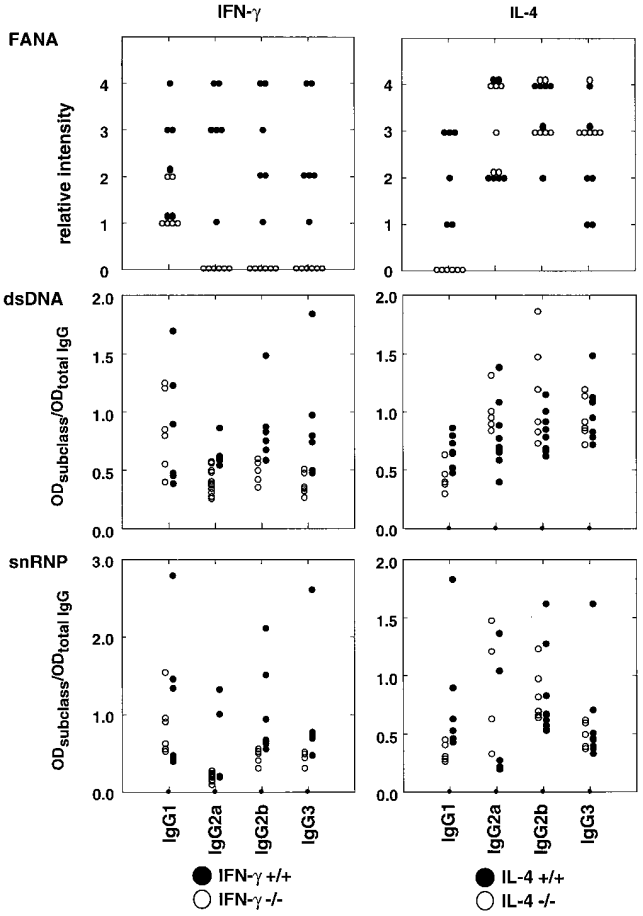

Figure 4. Relative IgG isotype distributions of autoantibodies in cytokine mutant mice. FANA-, anti-dsDNA-, or anti-snRNP-positive sera from 7–8-mo-old animals were assayed for specific IgG isotype autoantibody activities. IFG+ (*IFN-γ +/+*, *closed circles*), IFG- (*IFN-γ −/−*, *open circles*), IL4+ (*IL-4 +/+*, *closed circles*), and IL4- (*IL-4 −/−*, *open circles*) groups include both Fas+ and *lpr* sera.

End-organ disease in cytokine-deficient mice. Both IFG+*lpr* and IL4+*lpr* animals developed typical autoimmune lesions in the kidney, salivary gland, and, at older ages, liver (Fig. 6 and Tables I and II). Here, renal disease was characterized by diffuse glomerulonephritis along with severe interstitial infiltrates, as well as perivasculitis (not shown). Sialoadenitis was characterized by periductal infiltration with hyperplasia of the ductal epithelia and acinar atrophy. Hepatic disease included periportal and perivascular infiltrates.

At 3 mo of age, both IFG-*lpr* and IL4-*lpr* animals failed to develop such end-organ lesions, although mild glomerular hypercellularity and perivascular disease was present in some animals. Older IFG-*lpr* mice likewise demonstrated substantially decreased end-organ disease compared to their IFG+ counterparts. Older IFG-Fas+ animals also appeared to be protected from end-organ disease compared with IFG+Fas+ animals, although the severity of disease in IFG+Fas+ animals at this age remained relatively low. In contrast, older IL4-*lpr* ani-

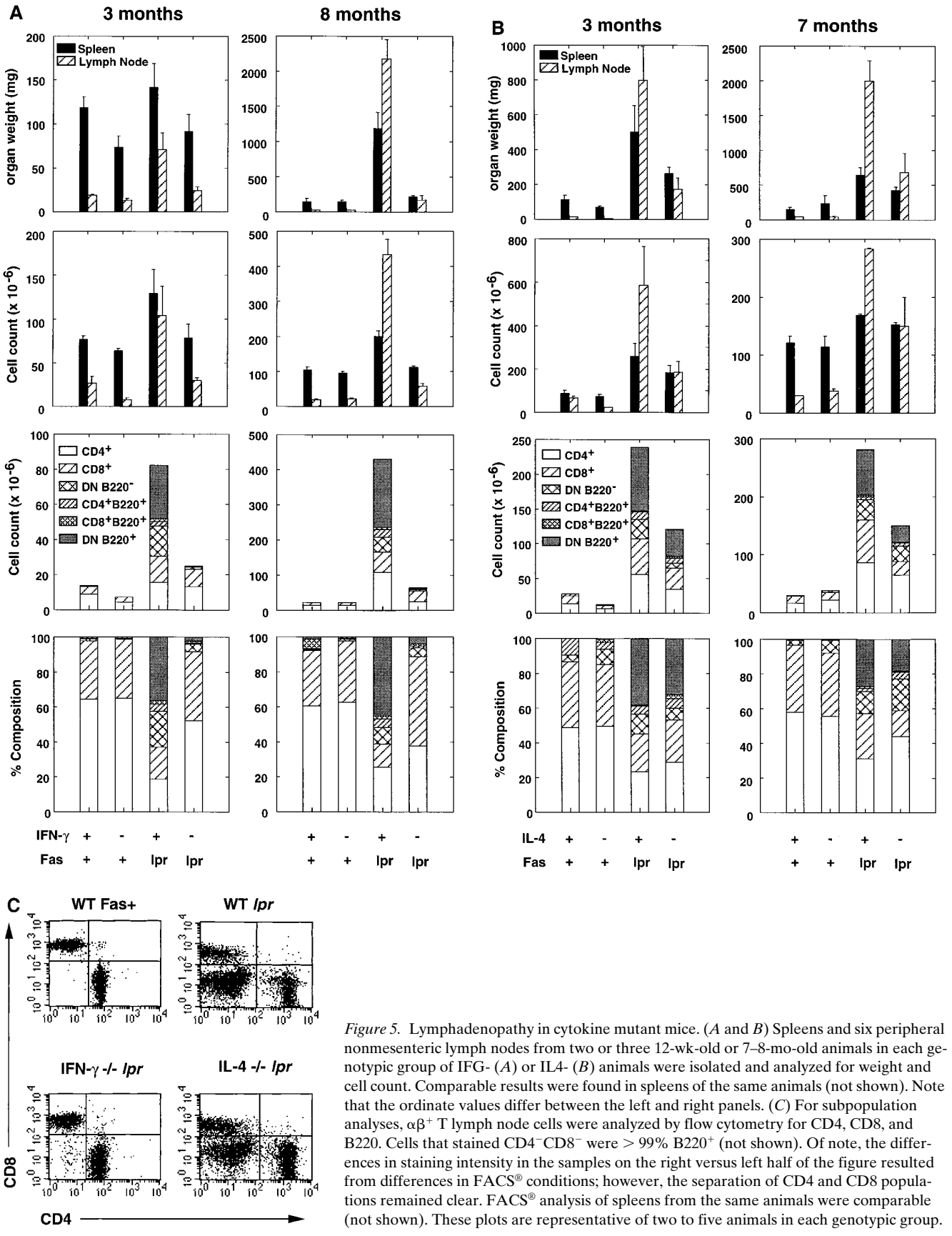

Figure 5. Lymphadenopathy in cytokine mutant mice. (*A* and *B*) Spleens and six peripheral nonmesenteric lymph nodes from two or three 12-wk-old or 7–8-mo-old animals in each genotypic group of IFG- (*A*) or IL4- (*B*) animals were isolated and analyzed for weight and cell count. Comparable results were found in spleens of the same animals (not shown). Note that the ordinate values differ between the left and right panels. (*C*) For subpopulation analyses, $\alpha\beta^+$ T lymph node cells were analyzed by flow cytometry for CD4, CD8, and B220. Cells that stained $CD4^-CD8^-$ were > 99% $B220^+$ (not shown). Of note, the differences in staining intensity in the samples on the right versus left half of the figure resulted from differences in FACS® conditions; however, the separation of CD4 and CD8 populations remained clear. FACS® analysis of spleens from the same animals were comparable (not shown). These plots are representative of two to five animals in each genotypic group.

Table I. Pathology of IFN-γ Mutant Lupus-prone Mice

			Renal disease			Salivary gland disease		
IFN-γ	Fas	Age	Glomerular	Tubular	Perivascular	Acinar	Perivascular	Hepatic
		mo						
+	+	3	−	−	−	−	−	−
+	+	3	−	−	−	−	−	−
+	+	3	−	−	−	−	−	−
−	+	3	−	−	−	−	−	−
−	+	3	−	−	−	−	−	−
−	+	3	−	−	−	−	−	−
+	lpr	3	+++	++	++	+	++	−
+	lpr	3	++++	+++	+++	++	++	−
+	lpr	3	+++	++	+++	++	+	−
−	lpr	3	+/−	−	−	−	−	−
−	lpr	3	+/−	−	−	−	−	−
−	lpr	3	−	−	−	−	−	−
+	+	8	+	+/−	+	−	−	−
+	+	8	+	−	+	−	−	−
+	+	8	+/−	−	−	−	−	−
−	+	8	−	−	−	−	−	−
−	+	8	−	−	−	−	−	−
−	+	8	−	−	−	−	−	−
+	lpr	8	++++	++	+++	++	++	+++
+	lpr	8	+++	++	+++	++	+	+
+	lpr	8	++++	+	++	++	++	+
−	lpr	8	+/−	−	+/−	+	−	+
−	lpr	8	+/−	−	−	−	−	−
−	lpr	8	+/−	−	−	−	−	−

Table II. Pathology of IL-4 Mutant Lupus-prone Mice

			Renal disease			Salivary gland disease		
IL-4	Fas	Age	Glomerular	Tubular	Perivascular	Acinar	Perivascular	Hepatic
		mo						
+	+	3	−	−	−	−	−	−
+	+	3	−	−	−	−	−	−
+	+	3	−	−	−	−	−	−
−	+	3	−	−	−	−	−	−
−	+	3	−	−	−	−	−	−
−	+	3	−	−	−	−	−	−
+	lpr	3	++	++	++	++	+	−
+	lpr	3	++++	++	+++	+	+	−
+	lpr	3	++++	+++	+++	++	+	−
−	lpr	3	−	−	+/−	+	−	−
−	lpr	3	+/−	−	−	+/−	−	−
−	lpr	3	−	−	−	−	−	−
+	+	7	+	−	+	++	−	−
+	+	7	+	−	−	−	−	−
+	+	7	−	−	−	−	−	−
−	+	7	+	−	+	−	−	+
−	+	7	+	−	+	−	−	−
−	+	7	+	−	−	+	−	−
+	lpr	7	+++	+	+++	++	+	+
+	lpr	7	++++	++	+++	++	+	+
+	lpr	7	+++	++	+++	++	+	+
−	lpr	7	++	−	++	−	−	+
−	lpr	7	++	−	++	++	+	+++

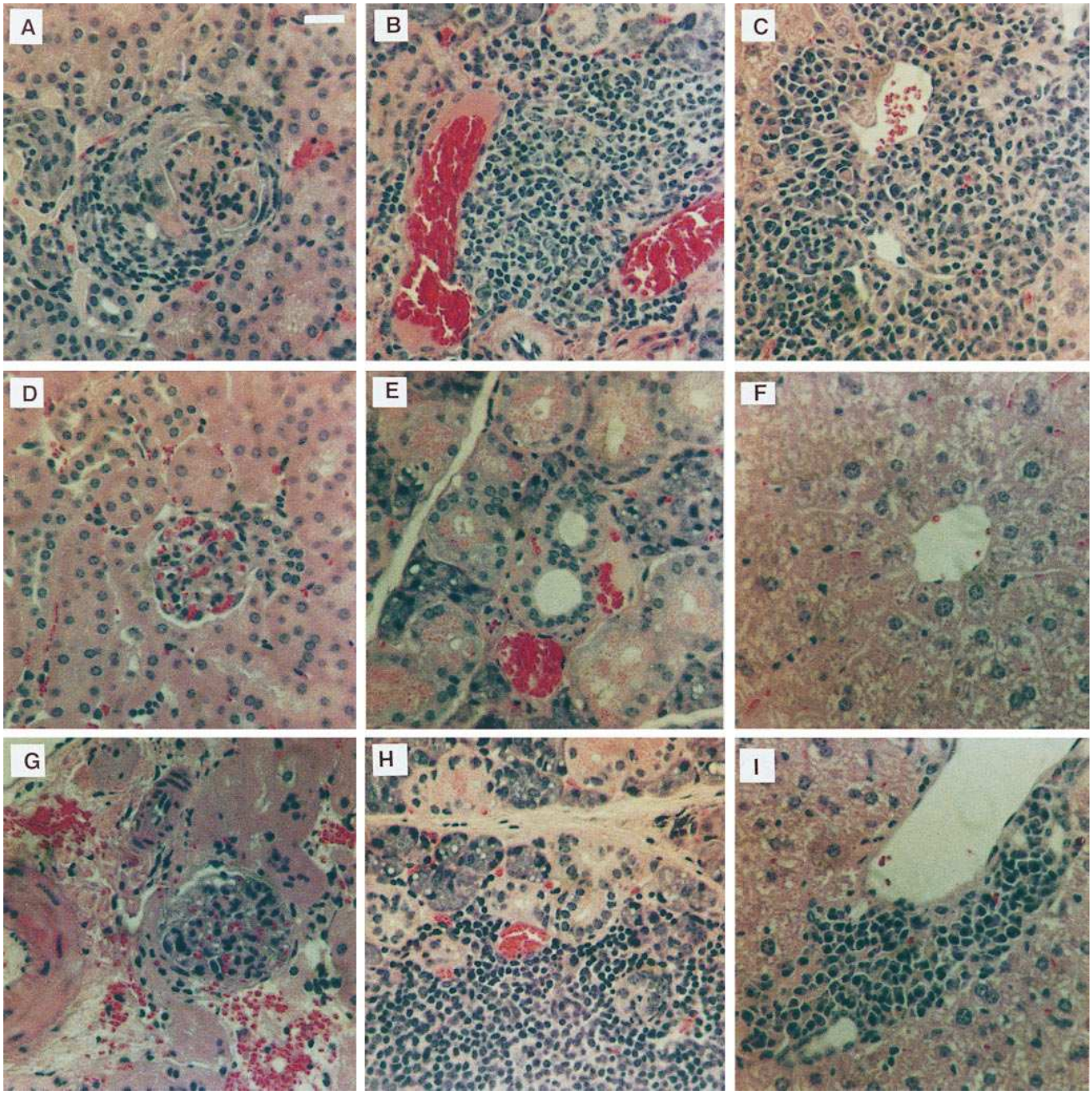

Figure 6. Representative histopathology of cytokine mutant mice. (*A–C*) Cytokine-intact *lpr* animals, as demonstrated in this IL4+*lpr* animal, characteristically developed severe renal disease (*A*), consisting of diffuse glomerulonephritis and severe interstitial and perivascular infiltrates (not shown), (*B*) severe sialoadenitis, consisting of periductal infiltration with hyperplasia of the ductal epithelia and acinar atrophy, and (*C*) periportal hepatic infiltrates. Such pathology was evident by 3 mo of age. In contrast, 3-mo-old Fas+ animals (*IFG+*, *IFG-*, *IL4+*, and *IL4-*) as well as IL4-*lpr* and IFG-*lpr* animals had grossly normal kidneys, salivary glands, and livers (not shown). (*D–F*) Older IFG-*lpr* animals, as demonstrated in this 8-mo-old animal, maintained grossly normal (*D*) kidneys, although mild glomerular hypercellularity may have been present; (*E*) salivary glands; and (*F*) livers. (*G–I*) However, older IL4-*lpr* animals, as demonstrated in this 7-mo-old animal, developed mild disease, characterized by (*G*) some glomerular hypercellularity with proliferation and occlusion of capillary loops, as well as mild perivasculitis (not shown), (*H*) moderate sialoadenitis, consisting of periductal inflammation; and (*I*) mild periportal hepatic infiltrates. These specimens are representative of two to six such animals examined in each genotypic group. Scale bar, 25 μm.

mals developed mild renal, salivary gland, and even hepatic disease; while significantly diminished in comparison with the IL4+*lpr* counterparts, these animals were more diseased in comparison to age-matched IL4+Fas+ animals. Differences between IL4-Fas+ and IL4+Fas+ animals could not be ascertained given the mild penetrance of end-organ abnormalities at this age.

Pathologic findings correlated with serum creatinine levels (Fig. 7), where older cytokine-intact *lpr* animals had significantly higher creatinine levels than either their IFG- or IL4-

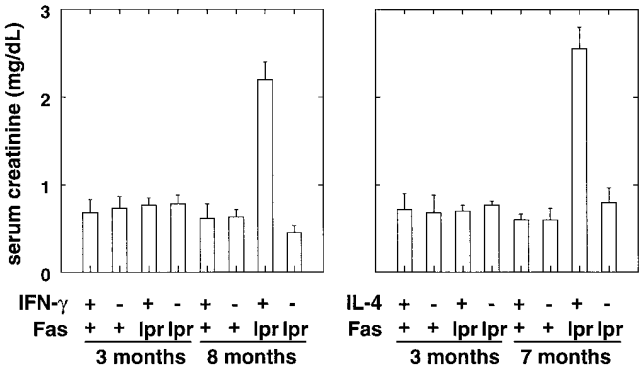

Figure 7. Serum creatinine levels in cytokine mutant mice. Sera from 3- or 7–8-mo-old animals were tested for creatinine concentration by a standardized Jaffé reaction. Standard error bars are shown for two to six animals tested in each group.

counterparts ($P < 0.001$), demonstrating the presence of compromised renal function in cytokine-intact *lpr* animals, but conserved renal function in IFG-*lpr* and IL4-*lpr* animals. Thus, given the time course and nature of disease in the IFG and the IL4 groups, both IFN-γ and IL-4 were required for the typical, severe progression of end-organ lupus disease in *lpr* mice; however, IFN-γ deficiency provided more lasting protection than IL-4 deficiency, with the latter animals having some disease, albeit much less than the IL-4 intact group.

Discussion

To elucidate more clearly the roles of prototypical Th1 and Th2 cytokines in the pathogenesis of murine lupus, this study generated lupus-prone mice genetically deficient in either IFN-γ or IL-4. Cytokine deletion altered the penetrance of autoantibodies and total serum immunoglobulin isotypes, diminishing total autoantibody production as well as Th1-dependent isotypes in IFN-γ deficiency, but maintaining autoantibody production while diminishing Th2-dependent isotypes in IL-4 deficiency. However, both cytokines were found necessary for the full penetrance of end-organ disease and lymphadenopathy, dissociating autoantibody production from histological disease parameters. These results thus emphasize the importance of both IFN-γ and IL-4 in disease pathogenesis.

Despite the theoretical difficulty of genetically identical F2 intercross animals, as analyzed here, the dominance of MRL disease-inducing genes in crosses with nonautoimmune strains (38, 39) results in a penetrance of hypergammaglobulinemia, autoantibodies (including the characteristic anti-snRNPs of the MRL background [2–4]), and end-organ disease at a rate comparable to wild-type MRL animals as evidenced in several previous studies (18, 40, 41, and this study). Indeed, it is notable that F2 animals develop renal and salivary gland lesions, as well as anti-snRNP antibodies, since MRL disease itself is stochastic, with incomplete penetrance of autoantibody production and end-organ disease (24, 36, 42). Thus, while our mice may not technically reflect wild-type ("100%") MRL disease, their lupus syndrome closely resembles the severe MRL phenotype. Nevertheless, because of such issues, the various genetic groups in this study have been analyzed as populations, rather than individual mice: each genetic group as a whole being 50% MRL overall, and each population of animals at similar risk for disease. Moreover, since IL4–deficient animals were originally derived from C57BL/6 breeders while IFN-γ–deficient animals were from DBA breeders, the two groups were not directly compared. Rather, the roles of the respective cytokines were examined within each respective set of four animal groups, using internal controls: IFG+Fas+ and IFG+*lpr* for IFG- animals, and IL4+Fas+ and IL4+*lpr* for IL4- animals.

This study helps resolve the otherwise conflicting data of previous studies on Th1 cytokines in MRL lupus (22–24, 28) by providing genetic evidence for a relative requirement for IFN-γ. In conventional immune responses, regulatory T cells may produce IFN-γ to downregulate autoreactive B cells (43), or to modulate pathogenic Th2 cells (41). However, in MRL/*lpr* mice, IFN-γ–related regulation may be ineffective in the face of intrinsic B cell (42, 44, 45) or T cell (46, 47) abnormalities, paradoxically propagating pathogenic autoantibody isotypes (e.g., IgG2a) (24); enhanced vascular leakage of organ-specific pathogenic T cells (48, 49); enhancing autoantigen presentation by macrophages, endothelial or epithelial cells (29, 50); and/or enhancing release of related inflammatory cytokines, such as IL-1, TNF, IL-12, or nitric oxide (25). Thus, the failure of anti–*IFN*-γ to ameliorate disease in 12-wk-old animals (28) likely reflects the importance of IFN-γ in the initiation of autoimmunity, suggesting that blocking Th1 cytokines may modulate lupus only when affected in the setting of early, perhaps preclinical disease. Th2-dependent isotype-specific hypergammaglobulinemia and autoantibody production persists, but remains of limited pathological consequence perhaps due to noncomplement fixing isotypes (IgG1) (51). In addition, IFN-γ may be directly required for the development of pathological autoantibodies by promoting autoantigen specificity and/or affinity (24), as suggested by analyses of anti-dsDNA antibodies in IFG- animals (Fig. 3 *A*).

These findings also reconcile the seemingly conflicting results of previous studies on Th2 cytokines in MRL autoimmunity (22, 23, 27), indicating a requirement for IL-4 in disease pathogenesis. Conventionally, IL-4 may also be evoked to downregulate ongoing autoimmune responses (18). However, in MRL/*lpr* animals, intrinsic B and T cell defects may cause IL-4–related mediators to promote autoantigen presentation, activate pathogenic myeloid cells, and/or diminish other modulatory cytokines like IL-1, IL-8, or TNFα (21, 52). Additionally, like IFN-γ, IL-4 may be directly required to promote pathogenic autoantibody specificity, affinity, or isotype via a costimulatory function in T–B cell collaboration; this view is supported by the diminished absolute numbers of conventional CD4+B220- T cells in IL4-*lpr* animals, suggesting the presence of fewer IL-4–dependent T cells capable of providing B cell help for pathogenic autoantibody production. In contrast to IFN-γ, however, IL-4–deficient animals appear capable of developing pathogenic autoantibodies, given their development of *Crithidia*-positive anti-dsDNA antibodies (Fig. 3 *B*) and the penetrance of renal disease in older *lpr* animals, albeit markedly reduced in comparison to IL-4–intact mice. Nevertheless, IL-4 is required for the maintenance of murine lupus: despite the titers of autoantibodies, non–Th2-type hypergammaglobulinemia, and relative cellular phenotypes in the IL4-*lpr* group which were comparable with their IL4+*lpr* counterparts, the former animals had significantly reduced end-organ disease, as well as diminished absolute numbers of

conventional T cells and lymphadenopathy. This view is supported by the results of one previous study, which found that administration of IL-4 antagonists could diminish disease parameters even if first given during established autoimmune disease (26). Thus, these results suggest that future cytokine-based therapeutic investigations will require further evaluation of individual cytokines in the pathogenesis of pathogenic autoantibodies versus the mediation of end-organ disease.

Additional findings of interest in the present study include the alterations in lymphadenopathy in both IFG-*lpr* and IL4-*lpr* animals, especially considering the paucity of knowledge on cytokines in lymphadenopathy of *lpr* mice. The absence of $B220^+$ T cells in IFG-*lpr* mice clearly demonstrate a requirement for IFN-γ in the development of the $B220^+$ T cell subsets characteristic of *lpr* disease, whereas the overall diminution in cell counts with essentially an unaltered cell subpopulation distribution in IL4-*lpr* animals suggests a role for IL-4 in the propagation of *lpr* lymphadenopathy. These findings may relate to the roles of IFN-γ and Fas in the downregulation of immune responses: the $B220^+$ T cell subset likely arises from the defective activation-induced cell death of peripheral *lpr* T cells, and such T cells may be told to die via IFN-γ as provided by regulatory $CD4^+$ or $CD8^+$ T cells, which themselves may be dependent upon IFN-γ (10–13, 37). On the other hand, the role of IL-4 in lymphadenopathy may primarily relate to its role in T cell activation, in which deficiency leads to fewer activated T cells, resulting in fewer total conventional T cells as well as fewer postactivation $B220^+$ T cells (52). This dissociation of Th1 versus Th2 cytokines in *lpr* disease further stresses the importance of regulatory dysfunction in the pathogenesis of autoimmune syndromes.

The 40–50% $CD4^-CD8^-$ T cell content of lymph nodes found in animals in this study is notable, and is in contrast to prior analyses of MRL/*lpr* mice which report percentages of such cells as high as 90% (53). This difference may reflect background genes in our F2 intercross mice, since *lpr*-induced lymphadenopathy strongly depends upon genetic background (38, 54). However, we should note that our MRL/*lpr* colony, even wild-type animals, typically has a 50–80% $CD4^-CD8^-$ T cell content of lymph nodes, regardless of age (data not shown), more in concert with findings reported herein. This may reflect local housing conditions, in which a variety of infectious organisms may affect the development of such cells, which likely represent postactivation T cells unable to complete activation-induced cell death (10–13); in this context, we note that our animals are all housed in filter top cages or in barrier facilities. Nevertheless, given these differences, our analyses here have focused not upon the extent of lymphadenopathy, but whether or not it develops, with comparisons made within the respective cytokine-deficient groups as was done for hypergammaglobulinemia, autoantibody production, and end-organ disease.

A final note is warranted regarding the use of congenitally cytokine-deficient animals, which may confound analyses of the functions of IFN-γ versus IL-4 in disease pathogenesis because the prolonged in utero and neonatal absence of either cytokine may alter lymphocyte development and hence effector functions (55). The present study has instead assumed that such effects of cytokine deficiency reflect the ongoing autoimmune processes in these animals, and has drawn conclusions from its results and the additional support of past studies in lupus animals and humans. Nevertheless, such concerns stress the need for constant referral to both the human and animal disease, where physiological analyses may confirm hypotheses generated by studies in knockout systems.

Acknowledgments

We thank Tom Taylor for assistance with flow cytometry.

This work was supported in part by grants from the National Institutes of Health (AR40072 and AR44076) and from the Arthritis and Lupus Foundations, their Connecticut chapters, and donations to Yale Rheumatology in the memories of Irene Feltman, Albert L. Harlow, and Chantal Marquis (to J. Craft). S.L. Peng was supported by the Medical Scientist Training Program, Yale University School of Medicine.

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