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Article

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Treatment of murine lupus with cDNA encoding IFN- γ R/Fc

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IFN- γ , a pleiotropic cytokine, is a key effector molecule in the pathogenesis of several autoimmune diseases, including lupus. Importantly, deletion of IFN- γ or IFN- γ R in several lupus-predisposed mouse strains resulted in significant disease reduction, suggesting the potential for therapeutic intervention. We evaluated whether intramuscular injections of plasmids with cDNA encoding IFN- γ R/Fc can retard lupus development and progression in MRL-Fas^{lpr} mice. Therapy significantly reduced serum levels of IFN- γ , as well as disease manifestations (autoantibodies, lymphoid hyperplasia, glomerulonephritis, mortality), when treatment was initiated at the predisease stage, particularly when IFN- γ R/Fc expression was enhanced by electroporation at the injection site. Remarkably, disease was arrested and even ameliorated when this treatment was initiated at an advanced stage. This therapy represents a rare example of disease reversal and makes application of this nonviral gene therapy in humans with lupus (and perhaps other autoimmune/inflammatory conditions) highly promising.

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Introduction

Lupus, the prototypic systemic autoimmune disease, is characterized by a high female predominance, multiorgan pathology, and a broad spectrum of autoantibodies, of which those against nuclear antigens typically predominate (1). The etiology of this disease is still unknown, but a strong genetic predilection appears to be a dominant factor (reviewed in ref. 2). Despite considerable advances in the management of this disease, morbidity and mortality remain high, and intense efforts are ongoing to develop less toxic and more efficacious treatments. Among the many avenues pursued in experimental models, those related to immunointervention with blocking peptides (3), antibodies, and other agents that inhibit coreceptor or costimulatory molecules (4–6), and those using agonist and antagonists of cytokines (reviewed in ref. 7 and 8), appear to be promising.

Many cytokine abnormalities have been identified in lupus-predisposed mice and humans (reviewed in ref. 7 and 8), the most prominent of which is increased levels of IFN- γ in serum, lymphoid organs, and afflicted tissues. The importance of this Th1 type inflammatory cytokine in lupus pathogenesis was suggested by the initial demonstration of Jacob et al. (9) that (NZBxW)_{F1} lupus mice treated with IFN- γ or its inducers showed accelerated disease, whereas those treated with anti-IFN- γ Ab beginning at the early stage showed significant delay in disease onset. The most compelling evidence for the deleterious effects of IFN- γ in lupus was obtained recently by us (10) and

others (11–14) with several lupus-predisposed mouse strains congenic for deletions of either the *IFN γ* or *IFN- γ R* genes, wherein significant decreases in the humoral and histologic characteristics of the disease were uniformly reported. Of particular interest was our observation that even MRL-Fas^{lpr} mice heterozygous for the IFN- γ deletion (IFN- γ levels half that of the nondeleted mice) were protected from kidney disease and early mortality (10).

Here, we describe the application of intramuscular injections of plasmids (VICAL VR1255) with a cDNA insert encoding an IFN- γ R/IgG1Fc fusion protein as a means to block the disease-promoting effects of IFN- γ in MRL-Fas^{lpr} mice. These mice, due to a defect in the Fas apoptosis-promoting gene, develop an early and severe lupus-like disease, together with massive lymphoaccumulation (reviewed in ref. 2). We applied this regimen at both the predisease and, more relevantly, the advanced disease stages. In addition, we examined the potentiating effects of electroporation at the site of plasmid injection in the systemic expression of the IFN- γ R/Fc fusion protein. We observed significant reduction in all disease manifestations when treatment was initiated early and, notably, such treatment was efficacious even when initiated at the advanced disease stage. This is one of the few examples in which an experimental treatment had a clear-cut effect in halting and, more importantly, ameliorating established systemic autoimmune disease in an appropriate spontaneous mouse model, indicating considerable promise in the treatment of the human disorder.

Methods

Mice. MRL-Fas^{lpr} mice were obtained from the Scripps rodent breeding colony. Mice were given food and water ad libitum and housed in a specific pathogen-free facility. All procedures were approved by the institutional animal research committee.

IFN- γ R/Fc plasmids. The extracellular portion of mouse IFN- γ R alpha-chain and mouse IgG1 constant heavy-chain cDNA were produced by RT-PCR, as we have described previously (15). RNA extraction, reverse-transcription, and PCR amplification were performed using *Pfu* DNA polymerase (Stratagene, La Jolla, California, USA), as described (16). These cDNA fragments were designed for overlap to generate a full-length IFN- γ R/IgG1Fc cDNA segment by PCR. This fragment was then inserted into the *EcoRV* and *EcoRI* restriction sites of the VICAL VR1255 vector (17) from which the original luciferase cDNA sequence had been deleted. The final active plasmid was designated VR1255-IFN- γ R/Fc. VR1255 directs eukaryotic gene expression from a cassette containing the human cytomegalovirus (CMV) immediate-early enhancer/promoter, the CMV intron A sequence, a cloning polylinker for insertion of protein-coding sequences, and a transcriptional terminator region derived from the rabbit β -globin gene. Plasmid DNA was prepared by the alkaline lysis method using an endotoxin-free extraction kit (Qiagen Inc, Santa Clarina, California, USA), diluted to 2 μ g/ μ L in sterile saline and stored at -20°C . The supernatants of COS-7 cells transfected with the VR1255-IFN- γ /Fc vector contained the 130-kD fusion protein, which exhibited more than 90% inhibition of NO release from a macrophage cell line cultured with IFN- γ and lipopolysaccharide (15, and data not shown).

Treatments. Treatments were initiated at the predisease (1 month of age) or advanced disease stages (4 months of age). Predisease mice received no treatment, intramuscular injections of blank VR1255 plasmid, or VR1255-IFN- γ R/Fc plasmid. In parallel, another set of predisease mice received the above treatments coupled with electroporation at the injection site (electroporation alone; electroporation plus blank VR1255; electroporation plus VR1255-IFN- γ R/Fc). A separate set of mice with advanced disease received either VR1255 or VR1255-IFN- γ R/Fc with electroporation at the injection site. Each of the above groups consisted of 8–14 mice in equal distributions of both sexes. All groups initially received two intramuscular injections at weekly intervals; thereafter, injections were at monthly intervals for up to 6 months, and subsequently bimonthly until termination of the experiment.

Electroporation in vivo. Electroporation was performed essentially as described (18). Briefly, anesthetized mice were injected in the bilateral tibialis anterior muscles with either VR1255 or VR1255-IFN- γ R/Fc plasmids (100 μ g/50 μ L saline) using a 27-gauge needle fitted with a plastic collar limiting muscle penetration to approximately 5 mm. Immediately thereafter, a pair of electrode needles (BTX Corp., San Diego, California,

USA) spaced 5 mm apart were inserted into the muscle bed on either side of the injection sites, and three 100-V square wave pulses (spaced 1 second apart) were applied, followed by three pulses in the opposite polarity for 50 milliseconds in duration using an Electro Square Porator T820M (BTX). No inflammation was observed at the injection sites.

Serologic analysis. IgG subclasses (IgG1, 2a, 2b, 3) were captured on ELISA plates coated with Fc-specific F(ab) $'_2$ fragment of goat anti-mouse IgG (5 μ g/mL) (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA), or with mouse chromatin (3.5 μ g/mL; a gift from R. Rubin). Bound IgG subclasses were measured using alkaline phosphatase-conjugated goat anti-mouse IgG subclass-specific antibodies (Caltag, San Francisco, California, USA) (10). Serum IFN- γ levels were measured with a commercial ELISA kit per the manufacturer's instructions (PharMingen, La Jolla, California, USA). Serum IFN- γ R/Fc fusion protein levels were determined using ELISA plates coated with rat anti-mouse IFN- γ R Ab (clone GR-20; American Type Culture Collection, Rockville, Maryland, USA). After blocking with 5% BSA in saline, sera were added to plates, followed by biotinylated rat anti-mouse IgG1 (PharMingen) and alkaline phosphatase-streptavidin (AP-streptavidin) conjugate (Bio-Rad Laboratories, Hercules, California, USA). Blood urea nitrogen (BUN) levels were ascertained from fresh blood using AZOSTIX strips (Bayer, Elkhart, Indiana, USA). BUN grades were recorded on a scale of 1–4 corresponding to levels from a low of 5–15 mg/dL to a maximum of 50–90 mg/dL.

Flow cytometric analysis and bromodeoxyuridine labeling. Splenocyte comparisons were determined by triple staining using antibodies against CD3, CD4, CD8, CD19, and CD44 (all from PharMingen) and analyses on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, California, USA). For intracellular IFN- γ determination, splenocytes were stimulated with PMA and ionomycin, surface stained for CD4, fixed, permeabilized with saponin, and stained with anti-IFN- γ -phycoerythrin (anti-IFN- γ -PE) (19). For bromodeoxyuridine (BrdU) labeling, mice received drinking water containing 0.8 mg/mL BrdU (Sigma Chemical Co., St. Louis, Missouri, USA) for 9 days, prepared fresh every other day, as described (10). BrdU-labeled cells were stained with one or more of the above antibodies to surface markers, fixed, permeabilized, and stained with FITC-conjugated anti-BrdU Ab (Becton Dickinson) as described (10).

Cytokine levels. Splenic mRNA expression levels for IFN- γ , IL-2, IL-4, IL-10, IL-12, IL-18, and TNF- α were measured using a multiprobe RNase protection assay according to manufacturer's instructions (PharMingen).

Histology and immunohistochemistry. Histologic examination of kidneys was done in a blind manner, and severity of glomerulonephritis (GN) was defined on a scale of 0 to 4+ (10). For immunohistochemistry, sec-

tions of snap-frozen OCT-embedded kidneys were air dried, fixed in acetone, and blocked with an avidin/biotin blocking kit (Vector Labs, Burlingame, California, USA), as well as 10% goat serum or 5% BSA in 0.1 M Tris/0.01% Triton X-100 buffer. Subsequently, sections were incubated with various primary antibodies, including anti-IgG-FITC (Vector Labs), anti-CD3-biotin (PharMingen), anti-F4/80-biotin (Caltag), anti-ICAM-1 (PharMingen), anti-MHC class II (PharMingen), and anti-MCP-1 (Santa Cruz Biotechnology, Santa Cruz, California, USA). When required, sections were sequentially incubated with biotinylated secondary antibodies (Jackson ImmunoResearch). In these cases, sections were incubated with streptavidin horseradish peroxidase (Vector Labs), developed with a peroxidase substrate AEC kit (Vector Labs), and counterstained with Mayer's hematoxylin.

Statistics. For group mean comparisons, statistical significance was determined by the Student's *t* test, whereas significance for survival curves was determined by the Kaplan-Meier method. *P* values of less than 0.05 were considered significant.

Results

Serum IFN- γ R/Fc levels and IFN- γ -neutralizing activity in vivo. An ELISA detecting IFN- γ R/Fc fusion protein, but not native IFN- γ R, was performed to determine serum levels of this protein in MRL-Fas^{lpr} mice injected with the cDNA plasmid with or without local electroporation. Mice (*n* = 16) injected 1.5 months earlier with two intramuscular doses of this plasmid (200 μ g each, 2 weeks apart) had IFN- γ R/Fc levels below the sensitivity level of our assay (< 10 ng/mL). In contrast, mice (*n* = 16) in which plasmid injections were coupled with local electroporation had dramatically increased values, ranging from 10 to 360 ng/mL (mean \pm SEM = 144.4 \pm 18.3). Among individual mice, however, there was considerable variability: four mice had values between 10–30 ng/mL, three between 40–55 ng/mL, and nine between 100–360 ng/mL. Reassessment one month later showed fusion protein levels had declined in half these animals, whereas the remainder had steady or even moderately increased levels. Based on these results, the treatment schedule was initially set at monthly intervals (up to 6 months of age) and bimonthly thereafter until sacrifice.

The *in vivo* blocking activity of the expressed receptor was ascertained by measuring serum IFN- γ levels at the time of sacrifice. Surprisingly, MRL-Fas^{lpr} mice injected with the VR1255-IFN- γ R/Fc plasmid (active plasmid) without electroporation, whose serum receptor levels were undetectable in our assay, had half the IFN- γ levels of either nontreated mice or mice injected with control VR1255 plasmid (blank plasmid) (Figure 1). Declines in IFN- γ values were more pronounced in both young and older mice in which injections were coupled with electroporation (approximately 10–25% of those receiving the blank plasmid), in accordance with the high levels of expressed IFN- γ R/Fc.

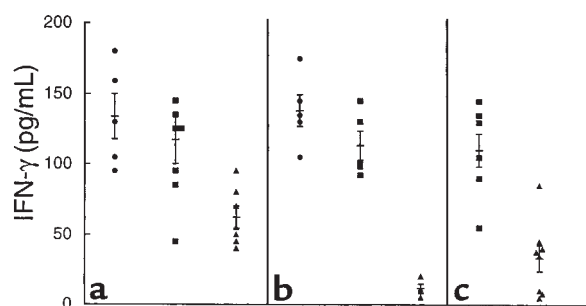


Figure 1

Serum IFN- γ levels for treatment and control groups. Levels were determined by solid-phase ELISA. (a) Treatments initiated at 1 month of age without local electroporation. (b) Treatments initiated at 1 month of age with electroporation. (c) Treatments initiated at 4 months of age with electroporation. Circles, no treatment; squares, blank plasmid (VR1255); triangles, active plasmid (VR1255-IFN- γ R/Fc). The mean and SEM are shown. In a, *P* < 0.001, and in b and c, *P* < 10⁻⁶ between active plasmid- and blank plasmid-treated groups (*n* = 3–8).

Survival rates. Treatment of MRL-Fas^{lpr} mice initiated at the predisease stage (1 month old) with VR1255-IFN- γ R/Fc injections without electroporation was associated with increased survival compared with controls (Figure 2a). At 8 months of age, the point at which the study of these groups was terminated, 62% of the mice injected with the active plasmid were alive, whereas unmanipulated mice or those injected with the blank plasmid had all died. Survival increased to 90% at 11 months of age when injections with the VR1255-IFN- γ R/Fc plasmid initiated at an early age were coupled with local electroporation, whereas control mice (local electroporation only or together with the blank plasmid) died by 7.5 months (Figure 2b).

To determine whether the VR1255-IFN- γ R/Fc plasmid, in addition to prevention, could control established systemic autoimmune responses, we tested its efficacy in a cohort of mice with advanced disease (4 months old). Remarkably, mice treated with the active plasmid and local electroporation were all alive at 14 months of age, the latest point of observation, where-

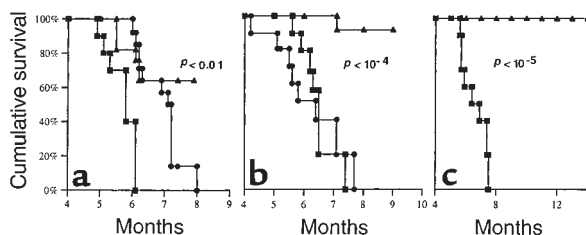


Figure 2

Cumulative survival rates for treatment and control groups. (a) Treatments initiated at 1 month of age without local electroporation. (b) Treatments initiated at 1 month of age with electroporation. (c) Treatments initiated at 4 months of age with electroporation. Circles, no treatment; squares, blank plasmid (VR1255); triangles, active plasmid (VR1255-IFN- γ R/Fc). Indicated *P* values are derived from comparisons of IFN- γ R/Fc cDNA plasmid-treated vs. blank plasmid-treated mice (*n* = 8–14 mice per group).

Table 1Reduction of lymphoid hyperplasia and T-cell phenotype changes of IFN- γ /Fc-treated mice

Disease stage and treatment	Lymphoid Hyperplasia ^B			DN subset ^C		CD8 ⁺ Subset ^C		CD4 ⁺ subset ^C		
	LN	Spleen	Total	DN CD44 ^{hi}	DN BrdU ^{hi}	CD8 ⁺ CD44 ^{hi}	CD8 ⁺ BrdU ^{hi}	CD4 ⁺ CD44 ^{hi}	CD4 ⁺ BrdU ^{hi}	CD4 ⁺ IFN- γ
Prediseased ^A										
No Rx	3.9 ± 0.4	0.74 ± 0.1	38.6 ± 2.2							13.8 ± 1.5
Blank plasmid	3.6 ± 0.3	0.71 ± 0.1	42.2 ± 2.1							16.3 ± 5.8
IFN- γ /Fc plasmid	2.3 ± 0.3 ^D	0.54 ± 0.1	21.2 ± 1.8 ^D							35.2 ± 3
Diseased ^A										
Electroporation alone	3.2 ± 0.2	0.63 ± 0.2	42.5 ± 3.5	43.3 ± 0.5	46.9 ± 3.2	20.4 ± 0.5	15.6 ± 1.2	36.1 ± 0.9	20.4 ± 0.9	17.1 ± 2.9
Blank plasmid + electroporation	3.7 ± 0.6	0.88 ± 0.1	42.1 ± 2.1	48.6 ± 3.5	52.2 ± 4.3	19.9 ± 2.1	16.1 ± 1.1	39.5 ± 1.6	20.7 ± 2.1	14.8 ± 0.8
IFN- γ /Fc plasmid + electroporation	0.7 ± 0.4 ^D	0.41 ± 0.1 ^D	15.4 ± 2.2 ^D	21.1 ± 1.7 ^D	17.4 ± 0.7 ^D	19.3 ± 1.2	23.3 ± 1.1	59.0 ± 2.3 ^D	31.3 ± 2.1 ^D	42.1 ± 6.2 ^D
Diseased ^A										
Blank plasmid + electroporation	3.1 ± 0.5	0.66 ± 0.1	41.2 ± 5.1	40.9 ± 4.1	42.2 ± 2	15.9 ± 2.0	17.6 ± 1	42.7 ± 2.3	24.1 ± 3.1	19.3 ± 2.2
IFN- γ /Fc plasmid + electroporation	1.6 ± 0.3 ^D	0.32 ± 0.1 ^D	16 ± 2.1 ^D	5.9 ± 1.5 ^D	8.2 ± 0.7 ^D	17.7 ± 1.6	13.1 ± 0.6	75.7 ± 1.0 ^D	46.7 ± 2.4 ^D	31.8 ± 2.4 ^D

^APrediseased, treatment started at 1 month of age; diseased, treatment started at 4 months of age. ^BLymph node (axillary, inguinal, cervical, and mesenteric) and spleen weights in grams. ^CTotal DN cells are given as percentages of CD3⁺ splenic cells, whereas all others denote percentages of the indicated subsets (mean ± SEM). ^D $P < 0.05$ between active plasmid- and blank plasmid-treated groups ($n = 8-14$ mice per group, except for the BrdU^{hi} and CD4⁺IFN- γ groups where $n = 4$).

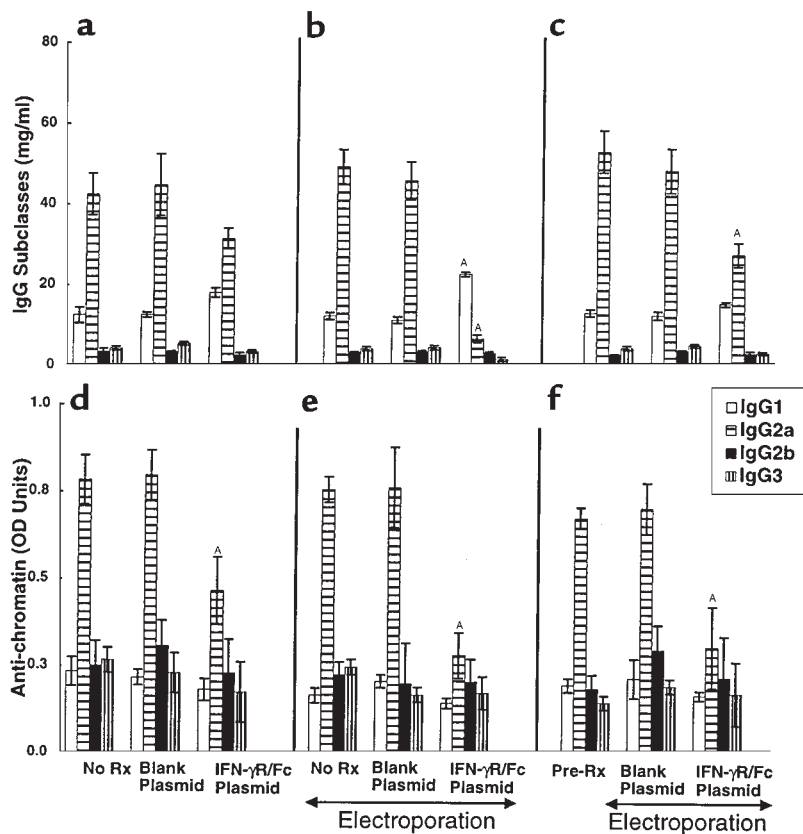
as control mice receiving blank plasmid injections with electroporation died by 7.5 months (Figure 2c).

Polyclonal and anti-chromatin IgG subclass levels. At the time of sacrifice, or the latest observation point, mice in which treatment with VR1255-IFN- γ /Fc plus electroporation was initiated at an early age had significantly reduced levels of polyclonal IgG2a and, conversely, significant increases in polyclonal IgG1 compared with controls receiving electroporation alone or with blank plasmid (Figure 3, upper panel). A significant reduction in polyclonal IgG2a and a minimal increase in IgG1 were detected when this treatment was initiated at the late disease stage. Mice injected with the active plasmid at an early age without electroporation also showed a decline in polyclonal IgG2a and an increase in IgG1, but these changes did not reach statistical significance.

The anti-chromatin autoantibody response in all control groups was dominated by the IgG2a subclass (Figure 3, lower panel), as reported previously (10, 20). Mice receiving the active plasmid either early or late in the disease process showed significant reductions in this autoantibody subclass, particularly when injections were coupled with electroporation. Significantly, this reduction was detectable not only when comparing the active plasmid-treated and control groups, but also between pre-

and posttreatment levels in late-disease mice. It is also noteworthy that the drop in IgG2a anti-chromatin levels caused by IFN- γ blockade was not associated with a compensatory increase in anti-chromatin autoantibody of the Th2-dependent IgG1 subclass.

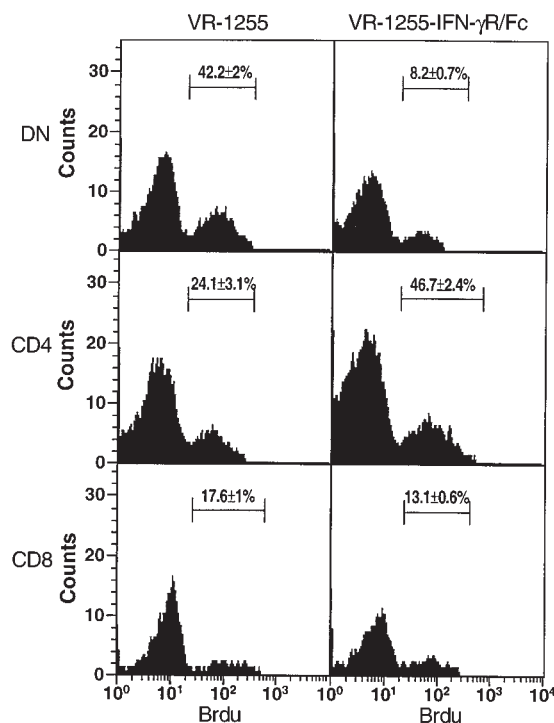
Lymphoid hyperplasia and cellular composition. The Fas^{lpr} mutation is associated with massive lymphoid hyperplasia, primarily due to the expansion of the apoptosis-defective double-negative (DN; CD4⁻8⁻) cells (reviewed in ref. 2). Thus, we assessed the effects of the various treatments on lymphoid hyperplasia as well as phenotypic and cycling characteristics of splenic T cells (Table 1). VR1255-IFN- γ /Fc treatment initiated at predisease

**Figure 3**

Serum polyclonal IgG subclasses (upper panels) and anti-chromatin IgG subclasses (lower panels). (a and d) Treatments initiated at 1 month of age without electroporation. (b and e) Treatments initiated at 1 month of age with electroporation. (c and f) Treatments initiated at 4 months of age with electroporation. ^A $P < 0.001$, $n = 8-14$ mice per group.

Figure 4

Long-term in vivo BrdU incorporation (9 days in drinking water) in T-cell subsets of mice treated late in the disease with either the blank plasmid (left panels) or the IFN- γ /Fc cDNA plasmid (right panels) coupled with electroporation. Assessments were made 7 months after initiation of treatment. Cells were first stained with the indicated surface markers, fixed, stained with anti-BrdU, and analyzed by FACS. Profiles are representative of one mouse, and mean \pm SEM of BrdU^{hi} (cycling) cells are derived from four mice per group.



or advanced disease stages resulted in drastic reductions of both lymph node and spleen weights, as well as the DN cell population. These reductions were even more pronounced when treatment was coupled with local electroporation. Furthermore, the frequency of activated DN CD44^{hi} cells was reduced from 48.6% in the blank plasmid-treated mice to 21.1% in the group receiving the active plasmid plus electroporation beginning at an early age, and an even more impressive reduction in the frequency of these cells was observed in mice when this treatment was initiated at the advanced disease stage (40.9% in the blank plasmid-treated vs. 5.9% in the active plasmid-treated mice). The proportions of both CD19⁺ and CD8⁺ cells (not shown), including those with the activated phenotype (CD44^{hi}) (Table 1), was unaltered in the various treatment groups. Although the proportion of CD4⁺ cells was similar in all groups (not shown), there was a significant increase in CD4⁺ cells expressing the activation CD44^{hi} marker, unlike DN cells, as well as intracellular IFN- γ staining in mice treated with the active plasmid (Table 1). The phenotypic results on activation markers coincided well with the frequency of cycling cells, as assessed by in vivo BrdU uptake, i.e., reduction in DN BrdU^{hi} cells, unaltered frequency in CD8⁺ BrdU^{hi} cells, and increased frequency in CD4⁺ BrdU^{hi} cells (Table 1 and Figure 4).

As defined by multiprobe RNase protection assay, with the exception of a twofold increase in IL-18, there were no changes in expression levels for IFN- γ , IL-2, IL-4, IL-10, IL-12, and TNF- α in splenic RNA derived from the various groups of mice at sacrifice.

Kidney disease and immunocytochemistry. The grade of GN in control mice (nontreated, blank plasmid-treated, with or without electroporation at predisease or advanced disease stages) was 3.62 ± 0.05 (mean \pm SEM), whereas that of the prediseased or diseased mice treated with VR1255-IFN- γ /Fc plus electroporation was 2.36 ± 0.03 ($P < 10^{-5}$). BUN grades were similarly reduced in active plasmid-treated mice compared with controls (3.95 ± 0.03 vs. 1.22 ± 0.11 , $P < 10^{-7}$) (Table 2).

Kidney-infiltrating T cells (CD3⁺) and macrophages (F4/80⁺) were diminished in mice injected with VR1255-IFN- γ /Fc at both the predisease and advanced disease stages regardless of electroporation (Figure 5). Decreased kidney IgG deposits as well as protein expression of MHC class II, ICAM-1, and MCP-1 were also observed in the active plasmid-treated mice (Figure 6).

Discussion

We demonstrated that: (a) intramuscular injections of plasmids with cDNA encoding IFN- γ /Fc protected MRL-Fas^{lpr} mice from development of lymphoid hyperplasia and lupus-like disease, (b) electroporation at the injection site significantly enhanced the expression of the fusion protein and its protective effects, and (c) this mode of treatment was highly effective in prolonging survival and ameliorating the serologic and histologic parameters of the disease, even when initiated at the advanced stage, thereby making it a good candidate for human application.

Previous studies have attempted to neutralize IFN- γ in mouse lupus models using polyclonal Ab's (9) or mAb's (21), as well as soluble recombinant IFN- γ (22). These approaches, however, have limitations: for example, with regard to Ab's, large quantities may be required, the Ab may not attain sufficient concentrations in secondary lymphoid organs or sites of inflammation, and it may be neutralized by host immune

Table 2

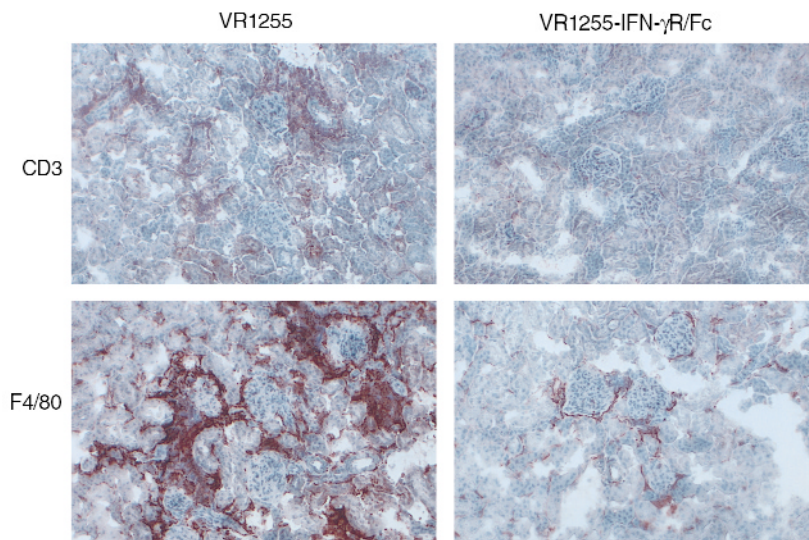
Reduction of kidney disease in IFN- γ /Fc plasmid-treated mice

Disease stage	Treatment	Kidney disease	
		GN ^A	BUN ^A
Prediseased	No Rx	3.5 \pm 0.1	3.9 \pm 0.1
	Blank plasmid	3.5 \pm 0.1	4.0 \pm 0.0
	IFN- γ /Fc plasmid	2.8 \pm 0.1	2.1 \pm 0.3 ^B
Prediseased	Electroporation alone	3.7 \pm 0.2	4.0 \pm 0.0
	Blank plasmid + electroporation	3.7 \pm 0.2	4.0 \pm 0.0
	IFN- γ /Fc plasmid + electroporation	2.4 \pm 0.1 ^B	1.3 \pm 0.3 ^B
Diseased	Blank plasmid + electroporation	3.8 \pm 0.1	3.9 \pm 0.1
	IFN- γ /Fc plasmid + electroporation	2.3 \pm 0.2 ^B	1.1 \pm 0.4 ^B

^AGN and BUN grades (mean \pm SEM) scored from 0-4. ^B $P < 0.001$ between active plasmid- and blank plasmid-treated groups ($n = 8-14$ mice per group).

Figure 5

CD3⁺ and F4/80⁺ cells in kidney tissue from MRL-Fas^{lpr} mice treated with blank (VR1255) or active (VR1255-IFN- γ R/Fc) plasmid with electroporation from 1 month of age. The numbers of T cells (CD3⁺), and especially macrophages (F4/80⁺), are markedly reduced in the active plasmid-treated groups. Similar reductions were observed in mice treated from 4 months of age with active plasmid (data not shown). Tissue sections were stained with either biotinylated anti-CD3 or anti-F4/80, incubated with streptavidin-horseradish peroxidase, and developed with AEC (see Methods). Photomicrographs are representative sections from four mice in each group. $\times 25$.



responses. With regard to soluble recombinant receptors, rapid turnover may affect efficacy and necessitate frequent administration. These constraints might explain the negative result with anti-IFN γ mAb treatment of MRL-Fas^{lpr} mice reported previously (21) and the finding that treatment with recombinant soluble IFN- γ R in (NZB \times W)F₁ lupus mice was effective only when initiated early, but not late, when IFN- γ levels are significantly higher (22).

Several of these problems may be overcome by intramuscular injection of nonviral vectors, which can induce sustained production of cytokine agonists or antagonists. This approach was originally implemented in MRL-Fas^{lpr} mice by Raz et al. (23, 24), who showed that injections of a cDNA vector encoding TGF- β commencing early in life significantly reduced disease parameters. We have now used a nonviral vector encoding a secreted IFN- γ R/Fc (IFN- γ inhibitory fusion protein) as a means to assess its prophylactic and therapeutic effects in this lupus model. We used IFN- γ R fused to IgG1 Fc as the inhibitor instead of the truncated receptor alone, since fusion molecules secreted as homodimers have been reported to have much longer half-lives than truncated IFN- γ R (40 vs. 1–3 hours, respectively) (25, 26), and dimeric IFN- γ R/Fc fusion proteins exhibit higher ligand avidity than single-chain receptors (27). Finally, IgG1 Fc, commonly used in these cases, was chosen as the partner to create the enhanced half-life–displaying biomolecule because this IgG subclass does not activate complement. In addition, to enhance systemic fusion protein expression, we coupled the intramuscular injections with local electroporation. Among the many methods used to promote naked plasmid DNA cellular transfer and gene expression, application of low field strength, square-wave electric pulses through external or invasive electrodes appears to produce markedly higher transfer efficiency (18, 28–31). This has been attributed to increased numbers of muscle fibers that take up plasmid DNA and probably increased copy number of plas-

mid introduced into each muscle cell. Indeed, when we applied this procedure, levels of IFN- γ R/Fc in the majority of treated animals exceeded 100 ng/mL, and the ligand levels were consequently reduced to approximately 10–25% of those in controls.

Prophylactic treatment initiated at the predisease stage with injections of the VR-1255-IFN- γ R/Fc plasmid without local electroporation effectively protected mice from early lethality and reduced serologic and histologic disease markers. These benefits were observed despite low serum levels of the fusion protein, which may, to some extent, be due to the sequestration of the receptor in IFN- γ -producing lymphoid organs and inflammatory sites. This possibility is supported by the fact that decreases in IFN- γ serum levels were still substantial. The degree of prophylaxis afforded by this regimen is not unlike the protection we reported previously in MRL-Fas^{lpr} mice heterozygous for the *IFN- γ* gene deletion (10) and that reported by others (32) in a subline of long-lived MRL-Fas^{lpr} mice in which IFN- γ levels were one-third that of conventional MRL-Fas^{lpr} mice. In the heterozygous *IFN- γ* gene–deleted mouse, however, despite reduced GN and prolonged survival, autoantibody levels and IgG kidney deposits were unaffected (10). It appears, therefore, that the active plasmid treatment is more potent than the drop in serum IFN- γ levels suggests, presumably due to the postulated effects of the sequestered receptor in inflammatory sites. Overall, the data indicate that even a fractional inhibition of IFN- γ is sufficient to reduce disease progression and severity. Nevertheless, injections of the active plasmid combined with electroporation significantly improved protection commensurate with several-fold increases in serum levels of the IFN- γ inhibitory fusion protein.

The positive results with early-life treatment provided the impetus to apply this mode of therapy to mice with established disease. Despite the severity of the underlying illness, survival was extended beyond

expectations, with 100% of the mice alive at 14 months of age, the latest point of observation. The effectiveness of this treatment in late disease is, to our knowledge, unprecedented, and it is remarkable that inhibition of a single molecule has such a profound effect in the course of this multifactorial disease. Only a few other attempts have been made to modify late-stage lupus disease in animal models, and only those using reagents that block costimulation (CTLA-4Ig, anti-CD40L, or combination) have shown any success (6, 33, 34). These treatments, however, may severely compromise immune responsiveness in general, and early trials in humans with anti-CD40L had to be discontinued due to complications from thromboembolic episodes (35).

Regardless of whether the VR-1255 IFN- γ /Fc plasmid treatment was initiated early or late, there was, as expected, a drop in serum levels of polyclonal IgG2a (Th1 dependent) and a concomitant increase in the levels of polyclonal IgG1 (Th2 dependent). Similarly, there was a significant decline in the dominant IgG2a anti-chromatin subclass, even when treatment was initiated late. Unlike the situation with polyclonal IgG subclasses, however, there was no compensatory switch of anti-chromatin from IgG2a to IgG1. These results are, again, compatible with our previous observations with the IFN- γ gene-deleted MRL-Fas^{lpr} mice (10) and strongly indicate that an autoimmune response against chromatin is highly dependent on the presence of IFN- γ .

Because of the highly pleiotropic properties of IFN- γ (36), it is very difficult to define the exact mechanism(s) by which this cytokine promotes autoimmunity. Nevertheless, based on the elevated MHC class I and II expression on splenic and peritoneal monocytes of IFN- γ -hyperproducing MRL-Fas^{lpr} mice (10, 37, 38), we postulate that a major effect of IFN- γ blockade would be downregulation of MHC expression, as we documented previously in IFN- γ gene-deleted MRL-Fas^{lpr} mice (10), and consequently reduced autoreactivity. This reduction in MHC expression may encompass not only professional antigen-presenting cells, but also nonprofessional antigen-presenting cells in the afflicted tissues, such as tubular epithelial cells in the kidney (ref. 10, and the present study). These cells have been shown to hyperexpress class II MHC in these mice and to function as antigen-presenting cells (39, 40). The postulated inhibition of autoreactivity was, in fact, reflected in the reduced lymphoid hyperplasia and the lower frequency of both cycling (BrdU^{hi}) and activated (CD44^{hi}) DN cells, presumably because these cells, lacking coreceptors, would require upregulation of MHC and increased Ag presentation to be engaged. In contrast, the frequency of BrdU^{hi} CD8⁺ cells was unaltered by IFN- γ inhibition. Curiously, however, the frequency of BrdU^{hi} CD4⁺ cells was increased, an unexplained finding that may be attributed to a regulatory or compensatory mechanism, since a high proportion of these cells was also positive for intracellular IFN- γ .

Additional mechanisms by which kidney disease may be reduced in mice treated with the VR-1255-IFN- γ /Fc plasmid are decreased expression of inflammatory response-promoting molecules, such as ICAM-1 and MCP-1, as documented herein. ICAM-1, a cell-surface protein that regulates immune cell interactions, and MCP-1, a macrophage-attracting chemokine, have been shown previously to be highly expressed in diseased kidneys of MRL-Fas^{lpr} mice (41, 42). In addition, congenic MRL-Fas^{lpr} mice with deleted *MCP-1* (42) or *ICAM-1* (43) genes showed reduced GN.

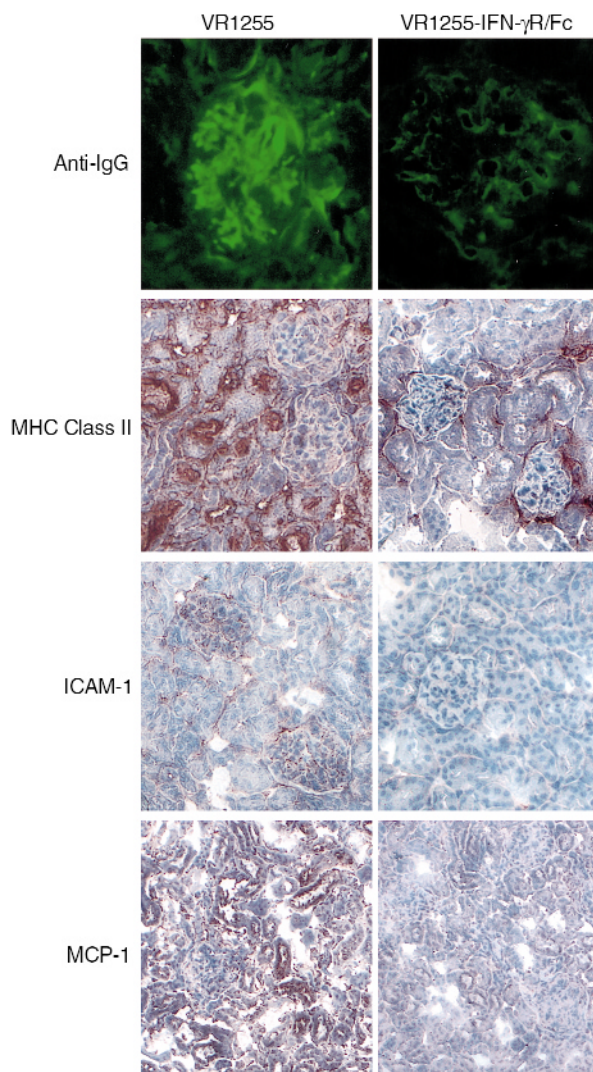


Figure 6 IgG deposits and expression of MHC class II, ICAM-1, and MCP-1 proteins in kidney tissues from MRL-Fas^{lpr} mice treated from 1 month of age with blank (VR1255) or active (VR1255-IFN γ /Fc) plasmid with electroporation. Active plasmid-treated mice had substantially reduced glomerular IgG deposits, as well as decreased levels of MHC class II, ICAM-1, and MCP-1. Similar reductions were observed in mice treated from 4 months of age with active plasmid (data not shown). IgG deposits were detected with FITC-anti-IgG antibody ($\times 45$), and the immunoperoxidase procedure for detection of MHC class II, ICAM-1, and MCP-1 was performed as described in Figure 5 ($\times 25$). Photomicrographs are representative samples from four mice per group.

Gene therapy for autoimmune diseases continues to receive considerable attention (44, 45). The delivery of IFN- γ inhibitory molecules by intramuscular injection of plasmid vectors is simple and appears to be nontoxic and safe. The use of this approach for gene therapy of autoimmune diseases circumvents several problems encountered with viral vectors (46) because the plasmid will not reactivate to a pathogenic state, is unlikely to be incorporated in genomic DNA or to be neutralized by the host's immune response, and does not stimulate a local inflammatory response. This approach may also be superior to recombinant soluble molecules in that it provides a depot of genetic material for long-term expression of the active biomolecule. In addition, desirable effects on affected microenvironments may be more potent, since recent studies (47) have shown that naked DNA is transported from the injection site to not only the regional lymph nodes, but also to distant organs such as the spleen and very likely to the afflicted tissues. Finally, since inhibition of IFN- γ has been shown to be of benefit in other autoimmune diseases, such as myasthenia gravis (48, 49) and insulin-dependent diabetes mellitus (50), the mode of therapy outlined here may have broader application.

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