Injection of IL-12- and IL-18-encoding plasmids ameliorates the autoimmune pathology of MRL/Mp-Tnfrsf6<sup>lpr</sup> mice: synergistic effect on autoimmune symptoms

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Abstract

IL-12 and IL-18 are mediators involved in the onset and progression of the autoimmune disease developing in MRL/Mp-Tnfrsf6<sup>lpr</sup> (lpr) mice, which display symptoms similar to the human systemic lupus erythematosus (SLE). The pathology is characterized by progressive lymphadenopathy and auto-antibody-mediated multiple organ failure, e.g. glomerulonephritis, or pneumonitis and a concomitant increase in serum levels for IFN<sub>c</sub> and tumor necrosis factor-<sub>a</sub> (TNF<sub>a</sub>). In this study, we intramuscularly injected lpr mice with plasmids encoding IL-12 and IL-18, either alone or in combination, in order to affect the development of the autoimmune disease. Five biweekly injections of the combined plasmids starting at 4–5 weeks of age diminished serum levels of TNF<sub>a</sub> and reduced the ability of lymphocytes from treated mice to produce IFN<sub>c</sub> in vitro. Injection of both plasmids synergistically attenuated the development of autoimmune syndromes, lymphoproliferation in secondary lymphoid organs, proteinuria and kidney damage, and pneumonitis. We conclude that IL-12 and IL-18 synergistically affect the pathogenesis of the Th1-dependent autoimmune syndrome of lpr mice and that approaches that target both IL-12 and IL-18 may be a therapeutic option in the treatment of autoimmune SLE.

Introduction

In MRL/Mp-Tnfrsf6<sup>lpr</sup> (lpr) mice, mutation of the <i>fas</i> gene disables expression of the receptor Fas (CD95), which is necessary to induce apoptosis upon Fas-ligand (FasL) binding in the course of negative selection (1). These mice spontaneously develop an autoimmune disease which shares many characteristics with the human systemic lupus erythematosus (SLE) (2, 3) or the human lupus-like pathology autoimmune lymphoproliferative syndrome (4). Hallmarks of the syndrome are spontaneous lymphadenopathy, hypergammaglobulinemia, auto-antibody production and immune complex formation. The lpr mice develop a strong lymphadenopathy in secondary lymphoid organs due to the accumulation of lymphocytes, which are normally eliminated by the Fas pathway (5–7). An unusual, CD3<sup>+</sup> B220<sup>+</sup> CD4<sup>+</sup> CD8<sup>−</sup> (double negative) T cell population persists, which is likely derived from persisting autoreactive T lymphocytes (8, 9). Autoreactive B cells are a major source for hypergammaglobulinemia, finally resulting in auto-antibody-dependent pathological derangements such as vasculitis, arthritis and fatal renal and pulmonary failure (10, 11).

The cytokines tumor necrosis factor-<sub>a</sub> (TNF<sub>a</sub>) and IFN<sub>y</sub> are critically involved in the pathogenesis of mice devoid of the Fas/FasL system. Deletion of the <i>tnfα</i> gene in C57Bl/6-gld mice, which lack expression of Fasl, and develop a disease similar to lpr mice, was shown to ameliorate autoimmune symptoms (12); however, therapeutic inhibition of TNF<sub>a</sub> in...
lpr mice seemed to ameliorate only pulmonary but not renal disease (13). Depletion of the Ifnγ gene in lpr mice lead to reduced lymphadenopathy, diminished endorgan disease and delayed mortality (14, 15). Furthermore, double-negative T cells and auto-antibodies were absent in Ifnγ-deficient lpr mice (15) and renal damage was significantly reduced in lpr mice lacking the gene for the IFNγR (16).

IL-12 and IL-18 synergistically are the most potent physiological inducers of IFNγ known (17, 18). Spleen and peritoneal cells of lpr mice produced higher concentrations of IL-12 after in vivo stimulation than those of control mice (19). In lpr mice, renal IL-12 expression was found to be enhanced (20) and application of recombinant IL-12 worsened the lupus-like autoimmune syndrome (21). Reduction of IL-12 bioactivity by transgenic over-expression of the IL-12p40 subunit, which forms antagonistic homodimers, resulted in the suppressed production of auto-antibodies and of IFNγ with limited improvement of the clinical manifestations (22). On the other hand, deficiency of IL-12, achieved by deletion of the ifnγp40 gene, led to a reduction of the systemic pathology, which was less pronounced in the kidneys (23).

In a previous study, we observed that cells from lpr mice produced substantial amounts of IL-18 spontaneously and that lymphocytes derived from their lymph nodes or spleens in comparison with those from wild-type mice displayed an increased reactivity to stimulation with IL-18 (24). Enhanced expression of IL-18 was found in several T1,-dependent autoimmune diseases, e.g. in Crohn's disease or rheumatoid arthritis (18), and neutralizing anti-IL-18 antibodies prevented the development of experimental autoimmune encephalomyelitis (25). Finally, Kinoshita et al. (26) reported that blocking IL-18 signaling by targeted deletion of the gene encoding the IL-18R α-chain in lpr mice reduced autoimmune symptoms to a certain extent and prolongs survival. In summary, high levels of both IL-12 and IL-18 induce or support T1,-dependent autoimmune diseases, whereas neutralization of either IL-12 or IL-18 seems to be of some, but limited, benefit. Thus, it seems that the synergistic function of IL-12 and IL-18 is centrally involved in the pathogenesis of SLE, leaving open the question whether perturbing both IL-12 and IL-18 together leads to a substantial improvement of the lpr disease.

The aim of our study was to therapeutically interfere with the development of the autoimmune disease of lpr mice based on the key T1,-promoting cytokines IL-12 and IL-18 in combination. A cDNA vaccination strategy was employed, shown to be able to modulate cytokine activity in vivo (27, 28). Here we report that repeated intramuscular (i.m.) injection of a combination of cDNAs encoding IL-12 and IL-18 reduced TNFα serum levels, decreased the ability of lymphocytes to secrete IFNγ in response to mitogens or to IL-12 plus IL-18 in vitro and markedly ameliorated characteristic pathological signs of the autoimmune disease.

Methods

Plasmid construction and animal treatment

The construct pEF2-IGIF was kindly provided by W. M. F. Lee and the constructs pcDNA3-IL-12p35 and pcDNA3-IL12p40 by D. B. Weiner (both from the University of Pennsylvania, Philadelphia). The insert coding for IL-18 (IGIF) was excised from pEF2-IGIF and inserted into the pcDNA3 vector (Invitrogen). pcDNA3-based constructs were amplified in Escherichia coli XL1-Blue and purified using anion-exchange columns according to the manufacturer's instructions (Qiagen, Hilden, Germany). For injection, plasmid DNA was precipitated and redissolved in sterile salt solution (0.9% NaCl) to a final concentration of 2 µg µl−1. The lpr mice were obtained from Harlan/ Winkelmann (Borchen, Germany), housed and bred under special pathogen-free conditions in the animal facility of the Hannover Medical School. All animal procedures were approved and authorized by the government of Hannover (Bezirksregierung Hannover). Female mice were treated starting at 4–5 weeks of age by injecting 2 × 100 µg of plasmid DNA into the thigh muscles of each hind leg. This procedure was repeated four times in 2-week intervals. If not stated otherwise, mice were killed 1 week after the last injection, and serum, organs and cells were prepared for analysis.

Quantification of serum cytokine and anti-dsDNA antibody levels

Whole blood of treated mice was collected by cardiac puncture of the anesthetized animal and sera were prepared after 1 h of incubation on ice by 10 min centrifugation at 10 000 × g. The sera were either processed immediately or stored until analysis at −80°C. The concentrations of TNFα, IFNγ, IL-2, IL-4 and IL-5 were determined using the CBA system and flow cytometry (BD/PharMingen, Heidelberg, Germany), accordingly to the instructions supplied by the manufacturer. Concentrations of IL-12 and IL-18 were evaluated by ELISA (IL-12: R&D Systems, Minneapolis, MN, USA; IL-18: MBL, Nagoya, Japan).

Anti-dsDNA antibodies were detected using a standard ELISA protocol with slight modifications (29). In brief, salmon sperm DNA (Fluka, Buchs, Switzerland) was coated onto 96-well immunoplates (Nunc, Roskilde, Denmark) overnight at 4°C. Plates were blocked with PBS/3% FCS and mice sera, diluted 1:500 in blocking buffer, and were incubated for 2 h at 37°C. The dsDNA-bound antibodies were detected with biotinylated sheep anti-mouse IgG (Dianova, Hamburg, Germany) and peroxidase (POD)-coupled streptavidin. Relative serum anti-dsDNA levels were calculated based on a standard curve obtained from serial diluted serum of a 5-month-old lpr mouse.

Analysis of lymph node weight

In order to compare the size of lymph nodes from cDNA-treated animals with those of control animals, axillary and inguinal lymph nodes were dissected and weighted. For standardization, individual ratios of lymph node weight to total mouse weight were calculated. Control mice and treated animals were compared by plotting the means ± SD of the obtained ratios.

Lymphocyte phenotyping and determination of IFNγ production

Single-cell suspensions of lymph node cells were prepared by teasing the dissected organ in complete medium [RPMI 1640 supplemented with 5% (v/v) FCS, 1 mM pyruvate, non-essential amino acids, 2 mM L-glutamine, 50 µM 2-mercaptoethanol 100 IU ml−1 penicillin and 100 IU ml−1 streptomycin; all from GIBCO/Invitrogen, Karlsruhe, Germany].
Resulting cells were analyzed by cytometry (FACScan, Becton-Dickinson, Heidelberg, Germany) using directly labeled mAbs specific for CD4, CD8, CD3 and B220 (BD/PharMingen).

In order to measure IFNγ secretion, cells were seeded in 96-well flat-bottomed plates (2.5 × 10^5 cells per well) and were either left unstimulated or stimulated with 2.5 μg ml⁻¹ Con A (Sigma, Taufkirchen, Germany), 100 ng ml⁻¹ LPS (E. coli 055: B5; Sigma), 10 ng ml⁻¹ IL-12 plus 100 ng ml⁻¹ IL-18 (both from PeproTech, London, UK) or 50 nM 12-ß-tetradecanoylphorbol-13-acetate (TPA) plus 0.5 μg ml⁻¹ ionomycin (both from Sigma). After 24, 48 and 72 h of incubation, IFNγ concentrations in the supernates were determined using a commercially available ELISA kit (Endogen, Woburn, MA, USA).

Analysis of urine protein concentration
Protein concentrations of spontaneously dropped out urine were assessed using test sticks (Combur®Test; Roche Diagnostics, Mannheim, Germany) in weekly intervals, at the same day and hour by the same person. Protein concentrations were assorted in four categories: 0–30, 30–100, 100–500 or >500 mg dl⁻¹. Means were calculated using the lowest value of each category.

In situ analysis of infiltrating lymphocytes
One hour before organ preparation, mice were injected intraperitoneally with 0.05 mg bromodeoxyuridine (BrdU) per gram body weight. Kidneys, lymph nodes and spleens were removed and shock frozen in liquid nitrogen. Lungs were filled with 1 ml of a mixture of Tissue-Tek (O.C.T. compound; Sakura Europe, Zoeterwoude, The Netherlands) embedding medium and PBS (1:4) and shock frozen. Serial cryostat sections of the kidneys were obtained along a longitudinal axis near the hilus, whereas the lung sections were done in a horizontal axis near the hilus. At least five sections of each organ within a distance of 50 μm were stained for nuclear BrdU and a surface marker as follows: (i) BrdU staining of denaturated DNA using anti-BrdU/POD (Fab fragments; Roche) developed with diaminobenzidine (brown color); (ii) Surface staining with anti-murine CD3 using the alkaline phosphatase–anti-alkaline phosphatase antibody technique and Fast Blue (Sigma) as substrate (blue color); followed by counterstaining with hematoxylin and mounting in glycergel. At least five non-serial sections of both kidneys and both lungs were analyzed. The tissue slices were evaluated by light microscopy (Axiophot; Zeiss, Jena, Germany) by two independent investigators.

Statistics
Student’s t-test or one-way analysis of variance with Dunnett’s post-test was performed using GraphPad Prism version 3.03 for Windows, GraphPad Software (San Diego, CA, USA). A P-value <0.05 was considered statistically significant.

Results
IL-12/IL-18 cDNA treatment leads to reduced lymph node mass in lpr mice
One of the most prominent symptoms of the autoimmune pathology in lpr mice is the accumulation of atypical lymphocytes in secondary lymphoid organs, leading to their enhanced size and weight. Mice treated with the empty vector (ctr.), IL-12 cDNA or IL-18 cDNA alone develop huge lymph nodes and a pronounced splenomegaly. However, the combined injection of both IL-12 and IL-18 cDNA together significantly reduces lymph node size (Fig. 1A) and cellularity (ctr.; 2.4 ± 0.3 × 10^6; IL-12 cDNA: 1.9 ± 0.3 × 10^6; IL-12/IL-18 cDNA: 2.1 ± 0.5 × 10^6; IL-12/IL-18 cDNA: 1.0 ± 0.7 × 10^6; numbers are from the pooled four lymph nodes obtained from each individual mice) in comparison with the injections of the empty vector or of a single cytokine cDNA. The cellular composition of
IL-12/IL-18 cDNA treatment reduces serum TNFα concentrations

One week after the last cDNA injection, serum concentrations of IL-12 and IL-18 are measured by ELISA. The IL-12p70 and IL-18 concentrations are always below the detection limit of 50 pg ml⁻¹. However, the serum concentration of IL-12p40 monomers is about 1000 pg ml⁻¹ in empty vector-treated control mice and does not change significantly due to the cDNA treatment (Fig. 2A).

Both TNFα and IFNγ serum concentrations increase age dependently in lpr mice (data not shown). A slight decrease in the IFNγ concentration is detectable upon injection of either the single plasmids or the combined IL-12/IL-18 cDNAs; however, none of the differences is statistically significant (Fig. 2A). In contrast, the TNFα concentration is found to be reduced from about 30 pg ml⁻¹ after empty vector injection to about 15 pg ml⁻¹ after injection of either the IL-12 or the IL-18 cDNA. Moreover, injection of both cytokine cDNAs significantly enhances the reducing effect of the single cDNA injections, resulting in <10 pg ml⁻¹ TNFα in the sera of the treated mice (Fig. 2A). These data indicate that the constitutively enhanced serum TNFα level in lpr mice decreases more efficiently by the i.m. injection of both cDNAs encoding IL-12 and IL-18 when compared with the single injections.

IL-12/IL-18 cDNA treatment does not affect anti-dsDNA IgG levels

High anti-DNA auto-antibody titers are found in lpr mice, however, with considerable variation. In order to determine whether the cDNA vaccination strategy affects this feature, we determine the relative anti-dsDNA IgG serum concentrations. None of the cDNA injections, IL-12 or IL-18 individually or both combined, decreases the mean anti-dsDNA IgG serum level in lpr mice at 13–14 weeks of age (Fig. 2B).

Lymph node cells from IL-12/IL-18-treated mice produce less IFNγ after in vitro stimulation

IFNγ is expressed by T lymphocytes and NK cells upon induction by e.g. IL-12 plus IL-18. In order to test their ability to produce IFNγ, lymph node cells from mice treated with the empty vector (ctr.) or with cDNAs coding for IL-12 and/or IL-18 are prepared and stimulated in vitro. The accumulated IFNγ in the supernates is assessed after 24, 48 and 72 h of incubation. All of the stimuli used (Con A, LPS, IL-12/IL-18, TPA/ionomycin) induce the accumulation of substantial amounts of IFNγ in the supernates of cells from mice treated with the empty vector or with one of the cDNAs encoding IL-12 or IL-18 alone. In contrast, in the supernates of cells from IL-12/IL-18 cDNA-injected lpr mice, considerably less IFNγ accumulates after induction by either of the stimuli within 48 h (Fig. 3). The kinetics of the accumulation of IFNγ are basically similar in all groups, increasing until 48 h and remaining constant until 72 h, with the exception of those cells treated with TPA/ionomycin, which showed a maximal IFNγ accumulation already after 24 h and reduced values after 72 h. However, in comparison with the supernates of cells from empty vector- or single cDNA-injected mice, in the supernates of cells obtained from the IL-12/IL-18-injected mice, significantly less IFNγ is accumulated at all time points analyzed (data not shown). Thus, the capability of freshly prepared lymphocytes from lpr mice to produce (or consume) IFNγ is significantly affected by the in vivo treatment with IL-12/IL-18 cDNAs.

IL-12/IL-18 cDNA treatment reduces the lupus-typical disruption of immune architecture in secondary lymphoid organs

Pronounced differences between empty vector-, IL-12 cDNA- or IL-18 cDNA-injected mice and those treated with the combined IL-12 and IL-18 cDNAs are found by histological comparison of their lymph nodes and spleens. The lymph nodes and spleens of empty vector (ctr.)-treated as well as of IL-12 or IL-18 cDNA-treated animals are largely disorganized. In contrast, sections of lymph nodes and of spleens from IL-12/IL-18 cDNA-injected animals display an organized structure (Fig. 4). In addition, in the organs of control and IL-12 and IL-18 cDNA-treated animals, a large number of proliferating cells, i.e. BrdU-incorporating cells, are detectable, whereas significantly fewer proliferating cells are found in the organs of lpr mice injected with IL-12/IL-18 cDNA (Fig. 5).

| Table 1. Lymphocyte distribution in lymph nodes obtained from cDNA-treated lpr mice |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| CD3* B220⁺ | 16.75 ± 4.02 | 16.00 ± 2.12 | 16.75 ± 4.60 | 15.00 ± 3.61 |
| CD3* B220⁺ | 30.75 ± 1.79 | 34.50 ± 2.87 | 41.25 ± 4.87 | 35.50 ± 6.95 |
| CD3* B220⁺ | 51.00 ± 3.08 | 47.25 ± 4.32 | 38.75 ± 5.17 | 46.00 ± 7.71 |
| CD4⁺ CD8⁺ | 15.75 ± 1.92 | 17.50 ± 1.66 | 16.25 ± 1.09 | 15.50 ± 1.12 |
| CD4⁺ CD8⁺ | 11.25 ± 2.59 | 9.25 ± 1.79 | 12.00 ± 4.00 | 10.25 ± 2.86 |
| CD4⁺ CD8⁺ | 71.25 ± 4.32 | 72.00 ± 2.00 | 68.25 ± 6.30 | 73.00 ± 3.94 |

Single-cell suspensions were prepared from axillary and inguinal lymph nodes obtained from lpr mice after treatment with the cDNAs indicated in the upper lane. Lymphocyte populations were analyzed and quantified by FACS. Reported are the relative numbers of cells (mean % ± SD) within the dot plot quadrants as shown in Fig. 1(B). Data are from nine mice of each group.
...and IL-12p40, quantified by ELISA, at a concentration of 1057 pg ml\(^{-1}\). Data are from 5–15 mice of each group (*P < 0.05, **P < 0.005). (B) Anti-dsDNA IgG levels in sera were determined by ELISA and expressed in arbitrary units relative to a standard lpr serum. The mean anti-dsDNA IgG level found in empty vector-treated lpr mice was set as 100% and the values of the other treatment groups (IL-12 cDNA: light gray bars, IL-18 cDNA: dark gray bars, IL-12/IL-18 cDNA: black bars) were calculated accordingly. Data are from 5–10 animals of each group (*P < 0.05, **P < 0.005).

**Fig. 2.** IL-12/IL-18 cDNA treatment reduces TNF\(\alpha\) serum levels in lpr mice. One week after the last injection, lpr mice were bled and sera were prepared and analyzed. (A) The sera of empty vector (ctr.; open bars)-treated mice contained IFN\(\gamma\) and TNF\(\alpha\), tested by CBA, at concentrations of 4.9 ± 1.8 and 28.7 ± 12.5 pg ml\(^{-1}\), respectively, and IL-12p40, quantified by ELISA, at a concentration of 1057 ± 477 pg ml\(^{-1}\). The mean values of empty vector-treated mice were set as 100% and those of the other treatment groups (IL-12 cDNA: light gray bars, IL-18 cDNA: dark gray bars, IL-12/IL-18 cDNA: black bars) were calculated accordingly. Data are from 5–10 animals of each group (*P < 0.05, **P < 0.005). (B) Anti-dsDNA IgG levels in sera were determined by ELISA and expressed in arbitrary units relative to a standard lpr serum. The mean anti-dsDNA IgG level found in empty vector-treated lpr mice was set as 100% and the values of the other treatment groups were calculated accordingly. Data are from 5–10 animals of each group.

Derangements of lung and kidney are reduced in IL-12/IL-18 cDNA-treated mice

Lungs and kidneys are among the affected organs in the lpr autoimmune disorder, characterized by lymphoid infiltrates (30, 31) finally leading to fatal glomerulonephritis and pneumonia, probably causing the death of the animal. Accordingly, we find very strong perivascular lymphoid infiltrations in these organs from 13- to 14-week-old lpr mice treated with the empty vector and also in those from IL-12 or IL-18 cDNA-injected animals. In contrast, virtually no infiltrates are detectable in kidneys and lungs from lpr mice injected with IL-12/IL-18 cDNAs (Table 2).

During progression of the disease, the barrier function of the glomerular basement membrane is gradually lost and an increase in proteinuria can be measured. During the period of CDNA treatment, proteinuria increases time dependently in mice injected with the empty vector. In contrast, in mice being treated with the IL-12/IL-18 cDNA combination, proteinuria does not increase much above starting levels. Injection of either one of the cytokine cDNAs alone also reduces proteinuria, but the pronounced protective effect observed with the combination of both IL-12 plus IL-18 cDNA is not achieved (Fig. 6).

**Fig. 3.** IL-12/IL-18 cDNA treatment reduces in vitro IFN\(\gamma\) production of lymph node cells. One week after the last cDNA injection, single-cell suspensions were prepared from lymph nodes of empty vector (ctr.; open bars)-, IL-12 cDNA (light gray bars)-, IL-18 cDNA (dark gray bars)- and IL-12/IL-18 cDNA (black bars)-treated lpr mice. Resulting cells were cultured and stimulated in vitro (Con A: 2.5 \(\mu\)g ml\(^{-1}\), LPS: 1 \(\mu\)g ml\(^{-1}\), IL-12/IL-18: 10 ng ml\(^{-1}\) IL-12 + 100 ng ml\(^{-1}\) IL-18, TPA/ionomycin: 50 nM TPA + 0.5 \(\mu\)g ml\(^{-1}\) ionomycin) as indicated on the abscissa. After 48 h of incubation, IFN\(\gamma\) concentrations were measured in the supernates by ELISA. IFN\(\gamma\) concentrations in supernates from unstimulated cells were always <80 pg ml\(^{-1}\) and are not reported. Data are from 5–15 mice of each group (*P < 0.05, **P < 0.005).

**Discussion**

The aim of our study was to interfere in a therapeutic fashion with the development of the spontaneous autoimmune disease of lpr mice at an early time point. In several disease models, the i.m. injection of cDNA encoding (auto-)antigens has been shown to induce protective immunity and to ameliorate pathologies, thus being a real cDNA vaccination (32–38). We administered cDNAs encoding the two IFN\(\gamma\)-inducing cytokines IL-12 and IL-18 (23, 26) to young lpr mice and monitored key characteristics of the developing autoimmune disease. The injection of cDNA encoding either IL-12 or IL-18 alone has been shown to provoke some but limited amelioration in the pathogenesis of the SLE model lpr mice (32, 36). Here we could demonstrate that their combined application leads to a far stronger reduction of the autoimmune syndrome.

Reduced lymphadenopathy and serum TNF\(\alpha\) levels

A main characteristic of the autoimmune pathology of lpr mice is the marked lymphadenopathy dominated by...
CD4⁺CD8⁻B220⁺T cells. The enhanced lymph node cellularity in these mice presumably results from the persistence of cells otherwise eliminated by the Fas pathway. We could provide evidence that CD3⁺ cells accumulating in lymph nodes or spleens are still proliferating. Thus, lymphadenopathy in lpr mice seems to be a matter of both lymphoaccumulation and lymphoproliferation. TNFα plays a role in the generation of lymphadenopathy since TNFα-deficient B6-gld mice (with a defect in FasL expression and displaying symptoms similar to lpr mice) show clear reduced lymphadenopathy as compared with their respective cytokine-expressing controls (12). Accordingly, the reduced lymphocyte number within the lymph nodes due to the cDNA treatment performed in this study could be attributed to the concomitant reduction of the serum level of TNFα.

Reduced IFNγ concentration in lymph node cell culture supernates after in vitro stimulation

In addition, we observed a striking functional difference between lymph node cells from empty vector-, IL-12 cDNA- or
IL-12/IL-18 cDNA-treated mice and those from IL-12/IL-18 cDNA-injected mice. The latter ones accumulated less IFNγ upon in vitro stimulation in their supernates, which could be due to reduced production and/or enhanced consumption. The IL-12/IL-18 cDNA injection affected the IFNγ accumulation, but not its kinetic, of the lymph node cells in response to specifically IFNγ-inducing factors (IL-12 and IL-18) as well as to a general T cell activating (Con A) or other stimuli (LPS, a microbial substance acting on B cells, macrophages and dendritic cells; TPA plus ionomycine, non-physiological stimulation to reach maximum cellular activation). Thus, the IL-12/IL-18 cDNA treatment resulted in the reduced ability of the lymph node cell preparation to either generate or consume/degrade IFNγ, at least after in vitro stimulation. Regarding the possibly reduced IFNγ generation, the alterations might be either due to the specific elimination of IFNγ-producing T lymphocytes or NK cells from the whole-cell population or, if they persist, due to their reduced capability to produce IFNγ. The latter of these two possibilities seems to be more likely, since comparable proportions of CD3+/IFNγ+ cells were found in lymph node cells prepared from mice treated by any of the four in vivo regimen after their identical in vitro stimulation (data not shown). The mechanism responsible for the reduced accumulation is currently under investigation.

**Reduced proteinuria and kidney damage**

Proteinuria, a symptom indicative of renal damage and characteristic of the autoimmune disease of lpr mice, is significantly reduced in mice injected with cDNAs encoding both IL-12 and IL-18. Injection of cDNAs coding for either IL-12 or IL-18 alone reduced proteinuria as well, however, mainly in the initial phase of the observation phase and is overall less pronounced than the combined application. Histologically, a severe renal peri-vascular infiltration of lymphocytes has been found in empty vector-treated mice, which was reduced in mice treated with plasmids coding for either IL-12 or IL-18 alone, while it was completely absent in mice injected with the IL-12/IL-18-coding cDNAs. Accordingly, in preliminary analysis (data not shown), we found less mRNA for chemokines and inflammatory cytokines and less ultrastructural derangements in kidneys from IL-12/IL-18 cDNA-treated mice in comparison with those from empty vector-injected mice. These differences could account for the reduction of proteinuria in IL-12/IL-18-injected mice. Thus, the treatment with both cDNAs indeed is synergistically effective in reducing autoimmune kidney derangements as compared with the single injection.
Reduced pulmonary disease

The massive lymphocyte infiltration seen in lungs of the empty vector-treated mice is diminished in the organs of lpr mice injected with the IL-12- or IL-18-encoding plasmids alone, while it is completely absent in those of mice injected with both IL-12- and IL-18-encoding plasmids together. Similarly, blocking of endogenous IL-18 by administration of the natural antagonist IL-18-binding protein has been shown to reduce experimentally induced acute lung inflammation in rats (40). However, in lpr mice, deletion of either IL-12R or the IL-18R α-chain, thus modulation of one of both cytokines alone, led to only moderate amelioration of the lung pathology (23, 26). This also favors the notion that both IL-18 and IL-12, which act synergistically, need to be affected in order to interfere with the autoimmune disease of lpr mice.

Auto-antibodies, e.g., recognizing cellular DNA, are at the basis of immune complex-mediated organ failure, including kidney and lung, of lpr mice. In the IL-12- and the IL-18Rα knockout lpr mice (23, 26), genetic models which eliminate the respective cytokine activity by 100%, only moderate reductions of anti-dsDNA IgG levels were observed. Thus, in our therapeutic model, where the cytokine activities most probably are affected less effective, one would expect a less pronounced effect. Indeed, in comparison with empty vector-treated controls, neither the single (IL-12 or IL-18 cDNA) nor the combined (IL-12/IL-18 cDNA) treatments lead to a statistically significant reduction of the anti-dsDNA IgG serum level. It remains to be determined whether a qualitative rather than a quantitative difference exists between the four treatment groups. Moreover, additional genetic requirements seem to be responsible for the manifestation of clinical symptoms in lpr mice (41).

In conclusion, the results presented here underscore the important role of the combination of the two cytokines IL-12 and IL-18 in the development and progression of the lupus-like syndrome of lpr mice. Furthermore, they demonstrate that in comparison with both empty vector-injected mice and mice treated with one of the two cytokine cDNAs alone, the i.m. injection of a combination of cDNAs coding for IL-12 and IL-18 significantly improved pathological symptoms of SLE-prone lpr mice with respect to three hallmarks of the disease: lymphoaccumulation, renal function and lung infiltration. Given the facts that injection of recombinant IL-12 or IL-18 proteins in lpr mice worsened the lupus-like disease (21, 42), that genetic inhibition of IL-12 or IL-18 function in lpr mice ameliorated it (23, 26) and that elevated IL-12 and IL-18 serum levels are found in SLE patients (43–46), the cDNA injection procedure presumably induces anti-IL-12 and anti-IL-18 activities. The exact nature of this activity is not quite clear yet. Inhibitory IL-12p40 homodimers might have been formed; however, anti-cytokine antibodies, generated due to the autoimmune background of the lpr mice, are the most likely mechanism (25, 32, 47–49).

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References


Abbreviations

BrdU bromodeoxyuridine
FasL Fas-ligand
i.m. intramuscular
lpr MRL/Mp-Tnfrsf6<sup>β<sup>
POD peroxidase
SLE systemic lupus erythematosus
TNFα tumor necrosis factor-α
TPA 12-O-tetradecanoylphorbol-13-acetate
IL-12/IL-18 cDNA plasmid injection in MRL/lpr


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