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Administration of an IL-12-Encoding DNA Plasmid Prevents the Development of Chronic Graft-Versus-Host Disease (GVHD)

Tadanobu Okubo,†* Eri Hagiwara,* Shigeru Ohno,* Takashi Tsuji,* Atsushi Ihata,* Atsuhisa Ueda,* Akira Shirai,* Ichiro Aoki,‡ Kenji Okuda,‡ Jun-ichi Miyazaki,§ and Yoshiaki Ishigatsubo*

The transfer of DBA/2 spleen cells into (C57BL/10 × DBA/2)F1 mice induces chronic graft-vs-host disease (GVHD), which is characterized by the production of Th2 cytokines, hypergammaglobulinemia, and immune complex-mediated glomerulonephritis like systemic lupus erythematosus. IL-12 strongly induces the production of Th1 cytokines and reduces Th2 activity in vivo. In this study, the effect of gene therapy on the development of murine chronic GVHD was examined using an IL-12-encoding plasmid (pCAGGSIL-12), with the expectation that it might regulate Th1/Th2 activity and have a beneficial impact on the clinical manifestations of disease. pCAGGSIL-12 or its p40 antagonist plasmid (pCAGGSp40) were injected i.m. every 3 wk in GVHD-induced (C57BL/10 × DBA/2)F1 mice. A total of 100 μg of pCAGGSIL-12 improved the Th1/Th2 balance in vivo, suppressed the production of IgG, and significantly reduced the development of glomerulonephritis. GVHD was exacerbated by injection of the pCAGGSp40 antagonist. Our results demonstrate that GVHD can be treated successfully by the administration of an IL-12-encoding plasmid, and that such therapy does not induce acute GVHD. The Journal of Immunology, 1999, 162: 4013–4017.

The transfer of parental T lymphocytes into non irradiated F1 hosts induces graft-vs-host disease (GVHD) (1, 2). Murine models of GVHD have provided important experimental and clinical information relevant to human disorders such as systemic lupus erythematosus (SLE) (3, 4). (C57BL/10 × DBA/2)F1 (BDF1) mice injected with DBA/2 donor spleen cells develop chronic GVHD characterized by B cell hyperreactivity, autoantibody production, and immune complex-mediated glomerulonephritis (5–8). Recent studies suggest that chronic GVHD is associated with an overproduction of type 2 cytokines such as IL-4 and IL-10 (9, 10).

IL-12 is a heterodimeric cytokine that is composed of two subunits (p35 and p40) and acts to promote both NK cell and CTL activity. In addition, IL-12 induces undifferentiated Th0 cells to commit to the Th1 phenotype and reduces Th2 activity (11, 12). For this reason, IL-12 has been tested as an immunotherapeutic agent for the treatment of a variety of Th2-mediated diseases (13–15). Both anti-IL-12 Ab and the homodimeric p40 subunit of IL-12 act as IL-12 antagonists and have been used to prevent Th1-mediated diseases (16, 17).

Previous studies suggested that the development of chronic GVHD in BDF1 mice could be partially prevented by the administration of mouse IL-12 (rmIL-12) (19). However, it has not been established whether renal disease in chronic GVHD is prevented by IL-12 administration. In part, this may reflect the difficulty of maintaining therapeutic levels of IL-12, which has a relatively short in vivo half-life. Varied effects induced by different protocols of IL-12 administration are also observed (18). Indeed, rmIL-12 therapy converted chronic GVHD into acute GVHD, which is characterized by a Th1-dominated immune response (19, 20).

We hypothesized that the long-term low-level production of IL-12 in vivo might be therapeutically beneficial. Therefore, we administered the pCAGGSIL-12 plasmid (encoding IL-12) to GVHD mice. Results indicate that the IL-12-encoding plasmid (pL2-12) improves Th1/Th2 balance and prevents the development of glomerulonephritis in murine chronic GVHD.

Materials and Methods

Mice

Female BDF1 and DBA/2 mice were obtained from Japan SLC (Shizuoka, Japan) and maintained in our animal facility. Recipient BDF1 mice were 6 wk old; donor DBA/2 mice were retired breeders. Each GVHD group consisted of at least four mice, and three mice were included in each non-GVHD group.

Induction of GVHD

Chronic GVHD was induced by an i.v. injection of 5 × 107 viable DBA/2 spleen cells into BDF1 recipients at days 0 and 7. Non-GVHD controls were injected with the same number of syngenic spleen cells.

Plasmids

A plasmid encoding murine IL-12 (designated pCAGGSIL-12) was used in these studies; the construction of this plasmid has been described previously (21). Briefly, mouse IL-12 p35 and p40 cDNAs were inserted into the EcoRI site of the pCAGGS expression vector (22). The expression unit for IL-12 p35, including the CMV immediate-early enhancer chicken β-actin hybrid promoter, IL-12 p35 cDNA, and a rabbit β-globulin poly(A) signal, was excised from pCAGGSp35 and inserted downstream of the expression unit.
unit of pCAGGSp40, pCAGGSp40 alone was used as the antagonistic plasmid. As described previously, the plasmid induced the production of IL-12 mRNA until day 28 postinjection in vivo as detected by RT-PCR; the bioactivity of the culture supernatant of L cells transfected with pCAGGSIL-12 was 200 U/ml in vitro (23). The control (noncytokine-encoding) vector was constructed with pCAGGS alone.

**Plasmid administration**

Mice were injected in the gastrocnemius muscle with either 20–100 μg of pCAGGSIL-12, 100 μg of pCAGGSp40, or control plasmid pCAGGS. A plasmid injection was repeated every 3 wk. The muscle bed was pretreated with 100 μl of 25% sucrose in PBS ~30 min before plasmid injection.

**Enzyme-linked immunosorbent assay (ELISA)**

The 96-microwell plates (Immuno 1, Dynatech Laboratories, Chantilly, VA) were coated for 2 h with goat anti-mouse Ig (Organon Teknika, West Chester, PA), ssDNA, or chicken OVA (Sigma, St. Louis, MO). After washing with PBS and 0.02% Tween 20, plates were blocked with 2% BSA in PBS. Serial dilutions of sera were added for 2 h. After washing with PBS-Tween, plates were overlaid with phosphatase-labeled antimouse IgG, IgG1, or IgG2a (Southern Biotechnology Associates, Birmingham, AL) for 2 h. The concentration of specific Ab bound to the plates was determined by the addition of p-nitrophenylphosphate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and monitored colorimetrically with a standard curve generated using known dilutions of high-titered antisera. Quantitative analyses of serum IFN-γ and IL-4 were performed using commercial ELISA kits (Endogen, Boston, MA) according to the manufacturer’s protocol. The sensitivity of the assays was <1.5 pg/ml for IFN-γ and <5 pg/ml for IL-4. IL-12 heterodimers (p70) were specifically measured using a commercial ELISA kit (Genzyme, Cambridge, MA). The detection limit of the assay was <5 pg/ml. The production of Th2 cytokines dominates the response of mice with chronic GVHD (9, 10). Therefore, serum cytokine levels were compared with the NT and p40 plasmid-injected (p40) groups by 9 wk after GVHD induction. In contrast, mice treated with pCAGGSIL-12 (IL-12) had no proteinuria at 5 wk after GVHD induction, and the incidence was only 25% at 9 wk. The development of proteinuria was accelerated among mice treated with the p40-encoding plasmid.

**Assessment of severity of glomerulonephritis**

Proteinuria in chronic GVHD mice was evaluated by Uristix (Bayer-Sankyo, Tokyo, Japan). Mice were sacrificed at 9 wk after GVHD induction, and their kidneys were fixed or snap-frozen in 1 ml of optimal cutting temperature compound and processed for histology. Snap-frozen kidneys were sectioned by cryostat. Next, kidneys were fixed in cold acetone for 20 min. After washing with PBS, blocking solution (10% normal goat serum, Nichirei, Tokyo, Japan) was added for 30 min. Biotinylated anti-mouse IgG and IgG1 (Vector Laboratories, Burlingame, CA) diluted 1/2000 and IgG1 (Cymbus Biotech, Chandlers Ford, U.K.) diluted 1/200 were added. After washing with PBS, they were treated with streptavidin-FITC (PharMingen, San Diego, CA) for an additional 1 h. Sections were subsequently analyzed by laser fluorescence microscopy (LSM-GB200, Olympus, Tokyo, Japan). The fixed tissues processed for light microscopic study were stained with hematoxylin and eosin.

**Statistical analysis**

Statistical significance was calculated by the Student t test, and ANOVA was performed among three or more groups in each data.

**Results**

**Effect of pIL-12 on the incidence of proteinuria in chronic GVHD**

Severe glomerulonephritis characterized by 3+ proteinuria developed 3–9 wk after DBA/2 spleen cells were injected into BDF1 recipients (8, 24). Mice treated with a control plasmid (NT; IL-12 noncoding plasmid) developed glomerulonephritis with the same kinetics: 50% of these animals had proteinuria by 6 wk, and all were proteinuric by 9 wk (Fig. 1). In contrast, only 25% of mice that received 100 μg of pCAGGSIL-12 (IL-12) developed proteinuria by 9 wk after GVHD induction. Unlike the beneficial effect of pCAGGSIL-12, mice injected with 100 μg of a plasmid encoding the IL-12 antagonist p40 had accelerated proteinuria (Fig. 1).

**pIL-12 suppresses Ig production**

Previous studies showed that serum IgG titers correlated with the severity of glomerulonephritis in murine chronic GVHD (24). In the current work, hypergammaglobulinemia was most prominent at 3–5 wk after cell transfer (Fig. 2 and Ref. 25). By comparison, the serum IgG titer among recipients of pCAGGSIL-12 was significantly lower than that of NT in samples taken at 3–5 wk after GVHD induction (p < 0.05, Fig. 2). No such effect was seen following administration of the p40-encoding plasmid (Fig. 2). Consistent with expectations, serum IgG titers correlated closely with the development and severity of proteinuria.

**pIL-12 suppresses anti-DNA Ab**

To determine whether the increase in IgG was due to the specific activation of autoreactive B cells, we analyzed serum autoantibodies to DNA and OVA. The serum titers of anti-DNA Ab in the NT and p40 groups were significantly lower than that seen for the non-GVHD group at 3–5 wk after cell transfer (p < 0.05) (Fig. 3); however, the titer for the IL-12 group was not significantly different from that of the non-GVHD group during same period. There was no difference in the titer of anti-OVA Ab between each group (data not shown).

**Effect of pCAGGSIL-12 on cytokine and Ig production in vivo**

The production of Th2 cytokines dominates the response of mice with chronic GVHD (9, 10). Therefore, serum cytokine levels were examined in BDF1 mice treated with pCAGGSIL-12. Of interest,
IL-4 levels were significantly lower in animals treated with pCAGGSIL-12 when compared with the NT group at 3–5 wk after GVHD induction \( \left( p < 0.05, \text{Fig. 4A} \right) \). Although serum IFN-\( \gamma \) levels were higher in the pCAGGSIL-12-treated mice, this effect did not reach statistical significance during this period (Fig. 4B). The ratio of Th1 cytokine to Th2 cytokine was significantly altered by pIL-12.

Changes in the balance between IgG2a and IgG1 Ab levels in vivo provide evidence of the biological effect of changes in the cytokine milieu. Consistent with the ability of IL-4 to induce B cells to switch from IgM to IgG1 production, mice with GVHD had significantly increased serum levels of IgG1 compared with normal mice. This effect was markedly reduced by pIL-12 at 3–6 wk after GVHD induction \( \left( p < 0.02, \text{Fig. 5A} \right) \). Moreover, serum titers of IgG2a were significantly higher in the IL-12 plasmid-treated animals during this period \( \left( p < 0.05, \text{Fig. 5B} \right) \), perhaps reflecting the impact of locally increased IFN-\( \gamma \) production.

Impact of pCAGGSIL-12 on Ig deposits in the glomeruli in GVHD mice

Untreated GVHD is characterized by Ig deposition in the glomerular basement membrane, culminating in the development of life-threatening glomerulonephritis (26–28). We used immunofluorescent staining to monitor IgG deposition. As seen in Fig. 6, mice treated with pCAGGSIL-12 had reduced Ig deposition in their glomeruli at 6 wk after GVHD induction. The deposition of IgG1 Abs was particularly reduced.

Discussion

This report examines the impact of an IL-12-encoding DNA plasmid on the development of chronic GVHD in a murine model. We found that 100 \( \mu \)g of pCAGGSIL-12 prevented the development of both the immunological and clinical manifestations of chronic GVHD. The administration of pCAGGSIL-12 altered the balance between Th2 and Th1 cytokine production, suppressed hypergammaglobulinemia, and prevented the development of proteinuria and glomerulonephritis in chronic GVHD. Previous studies have shown that the i.p. administration of rmIL-12 could convert chronic GVHD into acute GVHD (19). However, ours is the first study to demonstrate that GVHD can be successfully treated by administration of a pIL-12, and that such therapy does not induce acute GVHD.

Previous efforts to study the effect of exogenous cytokines on disease have faced difficulty in maintaining steady-state protein levels in vivo. This was particularly true of cytokines such as IL-12, which have a relatively short \( t_1/2 \) in vivo. Interpretation of previous studies involving the administration of exogenous rIL-12 has been complicated. For example, in nonobese diabetic mice, insulin-dependent diabetes mellitus was accelerated by the daily administration of rmIL-12 (29) but was ameliorated by intermittent administration (30). In a murine model of collagen-induced arthritis (CIA) (31, 32), low-dose IL-12 injection accelerated CIA (33), whereas high-dose IL-12 administration (34) or IL-12-deficient
mice (35) suppressed the disease. The explanation for these opposite effects of IL-12 is not yet understood (18), but may reflect the effect of bolus cytokine administration. To overcome this problem, we used the pCAGGSIL-12 plasmid encoding IL-12. Recent reports demonstrate that IL-12 accelerates disease progression in a CIA model using gene transfer of IL-12 by adenoviral vector (36). Consequently, gene transfer of IL-12 was proved to have a long-term effect on the disease in vivo. We believe that the ability of pCAGGSIL-12 to prevent chronic GVHD is attributable to the steady prolonged production of IL-12 by transfected cells. In this context, previous studies have shown that muscle cells at the site of plasmid administration can produce IL-12 mRNA for >3 wk (23), although those authors failed to detect bioactive IL-12 (p70) directly in vivo. Consistent with such an interpretation, the development of GVHD was not significantly altered by treating mice with control plasmid or with lower doses of pCAGGSIL-12 (≤20 µg/mouse). Unfortunately, serum levels of bioactive IL-12 (p70) in normal and transfected mice were below those detectable by ELISA, and we were unable to analyze this issue directly. Moreover, although pCAGGSIL-12 treatment significantly reduced IL-4 levels, the rise in IFN-γ induced by this plasmid did not reach statistical significance. IL-12 has a negative effect on IL-4 secretion that is independent of its ability to induce IFN-γ secretion (12, 13, 32). We consider it possible that the reduction in IL-4 associated with pCAGGSIL-12 administration had a beneficial effect in preventing GVHD. Alternatively, it is possible that IFN-γ production was increased at the site where the host was responding to transfected DBA/2 spleen cells, but that this local effect was not associated with a systemic rise in cytokine levels.

However, the ratio between serum IL-4 and IFN-γ levels was significantly altered. Evidence that this change in cytokine balance affected immune homeostasis is shown by the increase in serum IgG2a and the decrease in serum IgG1 levels as well as by the suppression of hypergammaglobulinemia in GVHD mice. Our findings are consistent with the view that the balance between Th1 and Th2 cytokines, rather than the absolute among any single cytokine, influences the type of immune response in vivo. This paradigm holds true for many disease states. SLE, for example, is characterized by a generalized increase in the production of type 2 cytokines and by a systemic decrease in the production of type 1 cytokines (37). This array of abnormalities could have a single common source, but more likely represents the outcome of multiple interacting immune elements, including those that lead to disease and to the attempts of the host to restore homeostasis.

We postulate that IL-12 plays a key role in determining the cytokine balance in GVHD. This conclusion is supported by the finding that GVHD is exacerbated by the administration of a plasmid encoding the IL-12 antagonist, p40. Previous studies showed that the biological effect of IL-12 is opposed by p40 (16, 17). Recently, Bagenstose et al. showed that IL-12 could regulate the production of autoantibodies in mercury-induced autoimmunity (38). Interestingly, serum levels of IL-4 and IgG1 were increased in that model of mercury-induced autoimmunity as well as in the chronic GVHD model we studied. In contrast to our findings, IL-12 treatment did not reduce renal Ig deposits in the mercury model (38). However, those studies were conducted by the administration of rmIL-12 i.p. for four consecutive days, a protocol that was ineffective in chronic GVHD. Thus, it remains possible that use of a pIL-12 and the resultant steady-state cytokine production may be beneficial in the treatment of multiple autoimmune diseases. Collectively, our data suggest that IL-12 has a therapeutic effect on chronic GVHD. Administering a pIL-12 circumvents the need for multiple daily injections of expensive cytokine and appears to provide the steady-state levels of IL-12 that are needed to prevent this disease. It is possible that pCAGGSIL-12 may also prove useful in the treatment of other diseases that are characterized by an increased type 2 to type 1 cytokine balance, such as SLE. Indeed, as our ability to modulate the immune environment using cytokine-expressing plasmid grows, so too should our ability to design rational therapies to treat certain infection and allergy diseases.

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