

Original Article**Immunosuppressive effect of ER-38925, a retinoic acid receptor subtype α -selective agonist, in mouse models of human graft-*vs*-host disease**Takayuki Hida^{1,2}, Kohdoh Shikata¹, Naoki Tokuhara¹, Akira Ishibashi¹, Mitsuo Nagai¹, Toshihiko Yamauchi^{1,*}, Seiichi Kobayashi¹¹ Tsukuba Research Laboratories, Eisai Co., Ltd., Tsukuba-shi, Ibaraki, Japan;² Current address, Morphotek Inc., 210 Welsh Pool Road, Exton, PA, USA.

ABSTRACT: Graft-*vs*-host disease (GVHD) is a devastating disorder that determines the prognosis of patients who receive a bone marrow transplant. GVHD is caused by donor cells responding to host disparate MHC alleles. In this report, we demonstrate that ER-38925, a newly discovered retinoid agonist with selectivity to retinoic acid receptor subtype α (RAR- α), is a potent immunosuppressive agent in mouse models of human GVHD. In a mouse model of lethal acute GVHD (aGVHD), ER-38925 prolonged the lifespan of the recipient mice in a dose-dependent manner. Its effect at 1 mg/kg was almost comparable to that of cyclosporin A at 30 mg/kg. ER-38925 profoundly prevented the development of anti-allogeneic cytotoxic T lymphocyte (CTL) response in the mouse model of aGVHD at 0.1 and 0.3 mg/kg. It strongly inhibited *in vitro* proliferation of alloantigen-stimulated donor T lymphocytes, and RAR- α seemed to play an exclusive role in this effect since inhibition by all-trans retinoic acid, which can activate all subtypes of RAR, was completely reversed by an RAR- α selective antagonist. Moreover, it significantly inhibited the elevation of serum IL-12 and IFN- γ and LPS-induced serum TNF- α elevation, all of which are known to be crucial disease-exacerbating factors in this model and human GVHD, in the mouse model of aGVHD. These results suggest that ER-38925 prevents the development of aGVHD through substantial inhibition of anti-allogeneic responses of donor T lymphocytes. In addition, *in vivo* administration of ER-38925 also blocked serum anti-DNA autoantibody production in a mouse model of human chronic GVHD. This is the first report to clearly show the

remarkable immunosuppressive effects of an RAR- α selective agonist in mouse models of human GVHD. These findings may allow an RAR- α selective agonist like ER-38925 to serve as a novel therapy to prevent both acute and chronic types of human GVHD.

Keywords: Graft-*vs*-host disease, Bone marrow transplantation, Retinoid, Therapy

Introduction

Graft-*vs*-host disease (GVHD) occurs after bone marrow transplantation (BMT) and can be found in acute and chronic forms. In patients who received BMT, acute GVHD (aGVHD) takes place within about 60 days post-transplantation and results in damage to the skin, liver, and gut due to the action of cytotoxic lymphocytes. Chronic GVHD (cGVHD) occurs later and is a systemic autoimmune disease that primarily affects the skin, resulting in the polyclonal activation of B cells and hyperproduction of immunoglobulin (Ig) and various kinds of autoantibodies, including anti-DNA autoantibody. The current method of preventing GVHD is to irradiate donor bone marrow cells or deplete mature T lymphocytes before transferring them to the host. BMT patients are systemically treated with various kinds of immunosuppressive drugs, including glucocorticoid, cyclosporin A, and cyclophosphamide. Despite their undesirable and severe toxicity, none of those therapies completely blocks GVHD development, and more effective and safer remedies are desired for this disease.

GVHD is studied in experimental systems by transferring T lymphocytes into allogeneic or semiallogeneic immunoincompetent recipients. In both types of GVHD model, donor T lymphocytes recognize and respond to the host disparate MHC antigens and

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initiate immunological disorders. In the mouse aGVHD model, animals die within 1-3 weeks after suffering from severe wasting, diarrhea, and skin lesions. In those mice, serum interferon- γ , IL-12 and TNF- α are significantly elevated, as also reported in human GVHD patients. On the other hand, in mouse cGVHD models, host B cells are polyclonally activated and produce excess amount of Ig and autoantibodies through cognate interaction between allospecific donor helper T cells (Th) and host B cells (1,2). Pathological features of those mouse models of GVHD closely resemble the pathological changes in human patients with either acute or chronic GVHD. This enables the use of those animals as models of human GVHD.

Retinoids are vitamin A derivatives used for the treatment of vitamin A deficiency and dermatological disorders as well as for chemoprevention and therapy for certain cancers (3). Retinoids regulate gene expression through the action of retinoic acid receptors (RARs) and retinoid-X receptors (RXRs), both of which belong to the family of nuclear hormone receptors (4). Both RARs and RXRs consist of 3 subtypes, *i.e.* α , β , γ . Those subtypes are expressed in various organs in a specially and/or temporally regulated manner and mediate pleiotropic effects of retinoids, including either beneficial or adverse effects. Many RAR and RXR pan- or subtype specific-modulators have been developed as drugs, mainly targeting cancer and metabolic diseases (5).

In addition, retinoids, like 4-hydroxyretinamide, all-trans, and 13-cis retinoic acid were reported to be effective in some immunological disease models like rat adjuvant arthritis (6) and experimental allergic encephalomyelitis (7), both of which are believed to be caused by cell-mediated immunity. Am80, a ligand with an affinity selective for RAR α , was reported to inhibit rat collagen-induced arthritis with a prominent reduction of anti-collagen IgG titers (8) and also inhibit experimental allergic encephalomyelitis in rats (9). In light of these findings, retinoids, and especially RAR α -selective ones, are expected to be a new remedy for intractable human immunological diseases.

ER-38925 is an RAR- α selective retinoid synthesized in the authors' laboratory (10). It has profound immunosuppressive activities in various kinds of models, like mouse cardiac allograft transplantation (11), and mouse lupus nephritis in female (NZB/NZW)F1 mice (12). These findings prompted an investigation of whether ER-38925 would inhibit various kinds of immunological disorders in mouse models of human GVHD.

Materials and Methods

Mice

C57BL/6 (B6; H-2^b), DBA/2 (D2; H-2^d) and B6D2F1

(BDF1; H-2^{b/d}) were purchased from Charles River Japan (Kanagawa, Japan) and maintained in a specific pathogen-free environment in the animal facility at Eisai Tsukuba Research Labs. All mice used in this study were female and 7 to 12 weeks old. All animal experiments were approved by the Animal Care and Use Committee of Eisai.

Drugs

ER-38925 (4-{5-[4,7-dimethyl-benzo[*b*]furan-2-yl]-1*H*-2-pyrrolyl}benzoic acid) was synthesized at Eisai Tsukuba Laboratories with a structure as shown in Figure 1. ER-50891 was also synthesized at Eisai and is an RAR- α selective antagonist as reported previously (13). Am80, a well-known RAR- α selective agonist, was purchased from WAKO Pure Chemical (Osaka, Japan). ER-38925 and Am80 were suspended in 0.5% methyl cellulose solution for *in vivo* administration. Cyclosporin A (CyA, Sandimmun[®] concentrate for infusion, lot.4197, Novartis, Switzerland) was diluted with physiological saline to the appropriate concentration. Recipient mice were administered appropriate doses of ER-38925 and Am80 orally or CyA subcutaneously at a volume of 10 mL of drug solution per kg of body weight.

Induction of aGVHD

Acute GVHD was induced according to the methods previously reported (1). To induce lethal GVHD, BDF1 recipients were lethally irradiated (10 Gy) and intravenously injected with 2×10^7 bone marrow cells and 5×10^7 spleen cells obtained from B6 donors. Parental B6 mice were anesthetized and killed by cervical dislocation prior to removal of the spleen and femur. Dissociated bone marrow and spleen cells were washed and resuspended in D-PBS for intravenous injection into the BDF1 hybrid. After bone marrow transplantation (BMT), recipients were given sterile chow and water, and the survival of each mouse was monitored daily. In other experiments, 5×10^7 B6 spleen cells were intravenously infused into non-irradiated BDF1 mice as a means of examining the induction of cytotoxic T lymphocyte (CTL) against a disparate MHC allele (H-2^d).

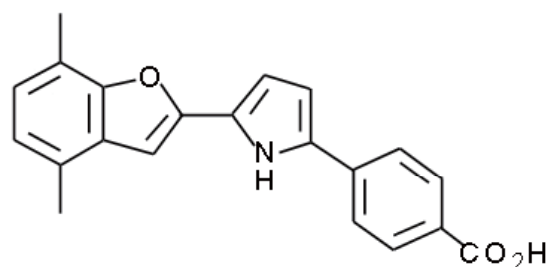


Figure 1. Chemical structure of ER-38925.

Induction of cGVHD

To induce cGVHD, BDF1 recipients were intravenously injected with 5×10^7 DBA/2 spleen cells. Preparation and transfer of the spleen cells were carried out as described in the previous (aGVHD) section. Two weeks after the transfer, mice were bled and their sera were collected for the determination of anti-single stranded DNA (ssDNA) autoantibody titers.

Assessment of CTL activity

Ten to twelve days after the injection of B6 spleen cells, recipients were anesthetized and killed by cervical dislocation prior to removal of the spleen. After dissociation, spleen cells were washed and resuspended in RPMI-1640 at various cell densities. Suspended in 200 μ L of complete RPMI-1640 (supplemented with 10% FCS (lot S07300, Sanko Junyaku, Tokyo, Japan) and 50 μ M 2-mercaptoethanol, 5,000 U/mL penicillin and 5,000 U/mL streptomycin) were 1×10^7 target tumor cells, P-815 (H-2^d, mouse mastocytoma), and EL-4 (H-2^b, mouse T-lymphoma), which were then labeled with 0.1 mCi of ⁵¹Cr (Cat. No. NEZ-030(S), DuPont NEN, Boston, MA) for 1 h at 37°C, 5% CO₂. Cells were then washed thoroughly and resuspended at 1×10^5 cells/mL in complete RPMI-1640. Labeled target cells were plated at 1×10^4 per well on a 96-well round bottom plate with effector cells at an effector/target (E/T) ratio of 80:1, 40:1, 20:1, 10:1, 5:1, and 2.5:1. The plates were incubated at 37°C, 5% CO₂ for 4 h. After incubation, these plates were centrifuged at 1,500 rpm for 5 min, and an aliquot of cell-free supernatant was collected from each well and transferred onto LumaPlates™ (Packard) to determine radioactivity with a TopCount™ Microplate Scintillation Counter (Packard). Percent-specific lysis is defined as $(a-b)/(c-b)$, where *a* equals cpm released by target cells incubated with effector cells, *b* equals cpm released by target cells incubated with medium only (spontaneous release), and *c* equals cpm released by target cells incubated with 1% of Triton X-100 (100% release). No nonspecific cytotoxicity was observed after effector cells were incubated with MHC-matched target cells (EL-4).

Anti-ssDNA IgG

For anti-ssDNA, ELISA plates (Costar, Cambridge, MA, Cat #3590) were first coated at room temperature with 10 mg/mL poly-L-lysine (Sigma, St. Louis, MO) for 0.5 h and then incubated overnight at 4°C with ssDNA preparation (heat-inactivated calf thymus DNA) at 5 μ g/mL. After wells were washed with washing buffer (0.05% Tween 20-0.1 M phosphate buffer (pH 7.4)) and blocked with 50% FCS-PBS (37°C, 2 h), 50 μ L of serially diluted serum sample were added and

plates were incubated at 37°C for 2 h. After washing, biotinylated goat anti-mouse IgG (Amersham) diluted with ELISA buffer (10% normal rabbit serum, 0.05% Tween 20-PBS (-)) at 1:1000 was added and plates were incubated further (37°C, 2 h). Anti-DNA IgG was detected with horseradish peroxidase-labeled streptavidin (Amersham, Buckinghamshire, England) and developed with o-phenylenediamine dihydrochloride. About 30 min later, the reaction was stopped by adding 50 μ L of sulphuric acid, and plates were read at an OD of 490 nm with a Micro Plate Reader. The amount of anti-ssDNA activity in each sample was expressed as units/mL in reference to standard serum obtained from an old female (NZB/NZW)F1 mouse, which was defined as having 1,000 U/mL of activity.

Serum cytokines

For the detection of serum cytokines, B6 spleen cells were transferred to BDF1 mice, which were treated with the drugs from the day of disease induction. Two weeks later, half of these mice were bled from the retro-orbital vein under diethyl ether anesthesia, and their sera were obtained for the detection of IL-12 and IFN- γ . The other half were intravenously injected with 30 μ g of lipopolysaccharide (LPS) and sera were obtained 3 h later for the detection of TNF- α . ELISA kits for mouse IL-12(p40), IFN- γ , and TNF- α were all purchased from BioSource International (Camarillo, CA), and assays were carried out according to the manufacturer's instructions.

aGVHD-type mixed lymphocyte reaction

Single cell suspensions of B6 and BDF1 spleen were prepared as described above. BDF1 spleen cells were growth-arrested by treating with mitomycin C (Sigma) prior to incubation. Suspended in 180 μ L of complete RPMI-1640 medium were 2×10^5 of B6 and 1×10^5 of mitomycin C-treated BDF1 spleen cells, which were then incubated at 37°C, 5% CO₂ for 5 days. Drugs were dissolved and diluted at appropriate concentrations in complete RPMI-1640 containing 0.1% DMSO and added to the culture in a volume of 20 μ L at the beginning of the culture. After incubation, each well was pulsed with 0.5 μ Ci of ³H-thymidine and further incubated for 16 h. Radioactivity incorporated in each well was determined with a beta-plate counter (Pharmacia, Uppsala, Sweden). Results are expressed as the mean cpm for triplicate cultures.

Statistics

In a lethal aGVHD model, the difference between the control and drug-treatment group in days of survival was analyzed with a Log-rank test. In cytokine

production assays, the difference between vehicle- and drug-treated groups was analyzed with a Mann-Whitney test. In cGVHD, the difference between dosage groups in serum anti-ssDNA titer was analyzed with one-way ANOVA followed by a Dunnett-type multiple comparison. A probability value of 5% (two-sided) was considered statistically significant. All statistical analyses were conducted using the SAS6.12 software package (SAS Institute Japan, Tokyo, Japan).

Results

Effect of ER-38925 on survival in the aGVHD model

Vehicle-treated BDF1 mice that were lethally irradiated and infused with bone marrow and spleen cells of B6 mice developed lethal aGVHD and began to die as of 12 days after the cell transfer. In this group, all mice were dead by day 22 after the cell transfer. Mice treated with ER-38925 at 1, 3, and 10 mg/kg had a significantly prolonged lifespan compared with the vehicle control ($p < 0.05$ by Log-rank test, Figure 2). During 4-week treatment, no mice in ER-38925 groups, except for one at 1 mg/kg, died from aGVHD. After the termination of the treatment, however, mice in those groups eventually died from aGVHD and no animal survived longer than 70 days post-disease induction. Reproducibility of the improvement in this parameter as a result of ER-38925 at 1 mg/kg was observed in an additional experiment (data not shown). In addition, ER-38925 at 0.3 mg/kg also significantly improved the survival of aGVHD mice in a separate experiment ($p < 0.05$ by Log-rank test, data not shown). ER-38925 at 1 mg/kg, p.o. again prolonged the survival of aGVHD mice almost as potently as CyA at 30 mg/kg, s.c., and both drugs had a

statistically significant effect ($p < 0.05$ by Log-rank test, Figure 3).

Effect of ER-38925 on cytotoxic T lymphocyte (CTL) induction in aGVHD mice

aGVHD is associated with the marked induction of anti-allogeneic donor CTL, and such CTLs are thought to play a crucial role in the induction of various disorders in aGVHD. The effect of ER-38925 on the lethality of aGVHD may result from the inhibition of CTL in this model. To assess this possibility, CTL activity was compared in control and drug-treated aGVHD mice. aGVHD was induced by injection of B6 spleen cells into non-irradiated BDF1 recipients. As shown in Figure 4a, on 12 day following the transfer of donor cells, a vigorous anti-allogeneic (anti-H-2^d) CTL response was detected. Treatment of mice with ER-38925 at 0.1 and 0.3 mg/kg from the day of transfer prevented the induction of this CTL response. ER-38925 suppressed the response by about 50% and 80% at 0.1 and 0.3 mg/kg, respectively. This inhibitory effect of ER-38925 at 0.3 mg/kg was fully consistent with its improvement of the survival of aGVHD mice, as mentioned before. Administration of CyA also prevented CTL induction in aGVHD models, and the inhibition of CyA at 10 mg/kg was almost comparable to that of 0.1 mg/kg of ER-38925 (Figure 4a, b). All-trans retinoic acid (ATRA) inhibited CTL response at 10 and 30 mg/kg (Figure 4b). Moreover, oral administration of another RAR- α selective retinoid, Am80, at 1 mg/kg also prevented CTL generation (Figure 4c). Hence, the inhibition of anti-allogeneic CTL induction in the aGVHD model seems to be a common feature for various types of retinoids.

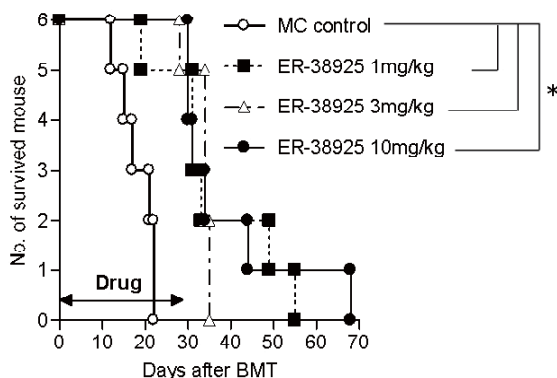


Figure 2. Effect of ER-38925 on the survival of aGVHD mice. Lethal aGVHD was developed in lethally irradiated female BDF1 mice by transferring bone marrow and spleen cells of B6 mice. Mice were administered either vehicle (0.5% MC) or ER-38925 (1, 3 or 10 mg/kg) for 4 weeks from the day of disease induction. Each group consisted of 6 animals at the beginning of the study. Respective lines indicate survival for each treatment group. ER-38925 prolonged the lifespan of aGVHD mice starting at 1 mg/kg. A statistically significant difference was found between the control and each ER-38925 treatment group. * $p < 0.05$ by Log-rank test.

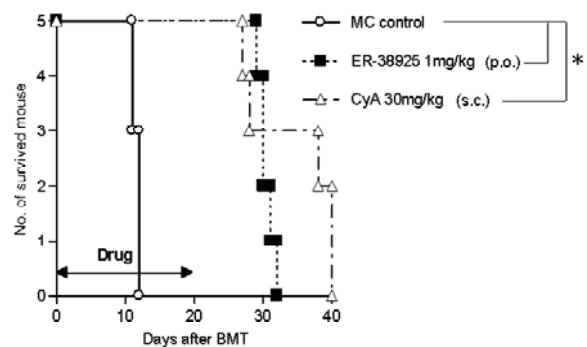


Figure 3. Effect of ER-38925 and CyA on the survival of aGVHD mice. aGVHD were induced in female BDF1 mice as described in Figure 2, and those mice were divided into 3 groups and administered vehicle (0.5% MC), ER-38925 (1mg/kg, p.o.), or CyA (30 mg/kg, s.c.) respectively, for 20 days from the day of disease induction. Each group consisted of 5 animals at the beginning of the study. Respective lines indicate survival for each treatment group. Both ER-28925 and CyA significantly improved survival at the dose tested. * $p < 0.05$ by Log-rank test.

Effect of ER-38925 on alloantigen-induced proliferation of donor spleen cells

In aGVHD, parental T lymphocytes recognize and respond to disparate MHC alleles resulting in a massive proliferative response and cytokine secretion. To ascertain the mechanisms by which retinoids inhibit CTL induction in the aGVHD model, the effect of compounds on the anti-allogeneic proliferation of donor

spleen cells was studied. As shown in Figure 5, B6 donor spleen cells profoundly proliferated *in vitro* in the presence of host (BDF1) spleen cells (open circles in Figure 5a, b). ATRA and ER-38925 inhibited this response in a concentration-dependent manner, and their IC₅₀ values in this assay were 0.62 and 1.64 nM, respectively (Figure 5a). The next assay investigated whether inhibition of these retinoids is mediated via RAR- α or not. ER-50891 is a retinoid synthesized in this laboratory and is defined as an RAR- α specific

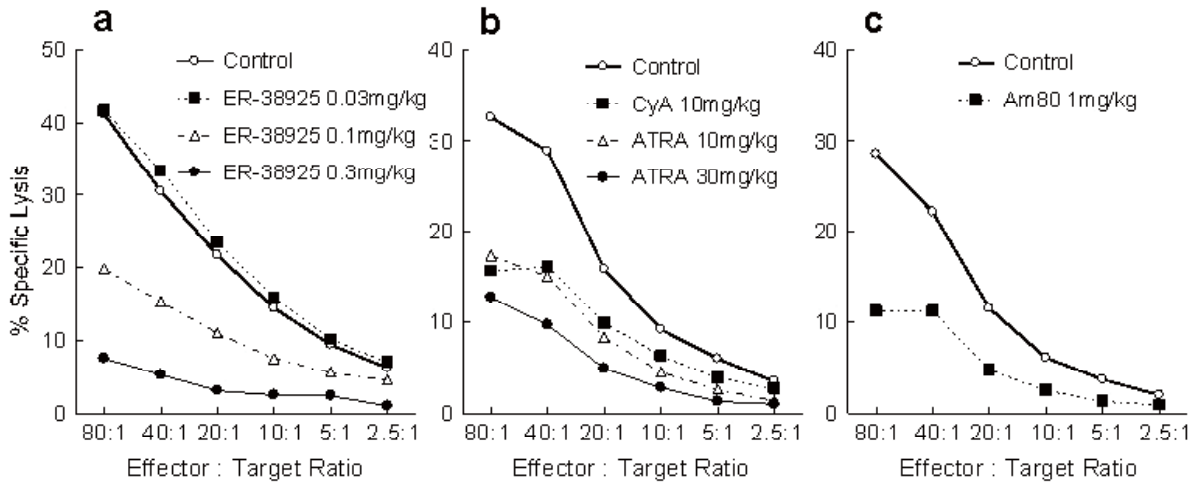


Figure 4. Effect of ER-38925, ATRA and CyA on the CTL induction in aGVHD mice. Anti-alloantigen (H-2^d) specific CTL was induced in female BDF1 mice by transferring spleen cells of B6 mice. Mice were divided into groups consisting of 5 animals and administered with drugs at various doses from the day of cell transfer, as indicated in Figure 4a, 4b, and 4c. Control mice received the vehicle only (0.5% MC). Twelve days after the cell transfer, anti-allogeneic CTL activity was determined by measuring the cytotoxicity against P815 target cells (H-2^d). Each point represents the mean CTL activity of 5 animals. **a.** ER-38925 inhibited the response starting at 0.1 mg/kg. **b.** CyA and ATRA inhibited the response at 10 mg/kg and 10 mg/kg, respectively. **c.** Am80 inhibited the response at 1 mg/kg.

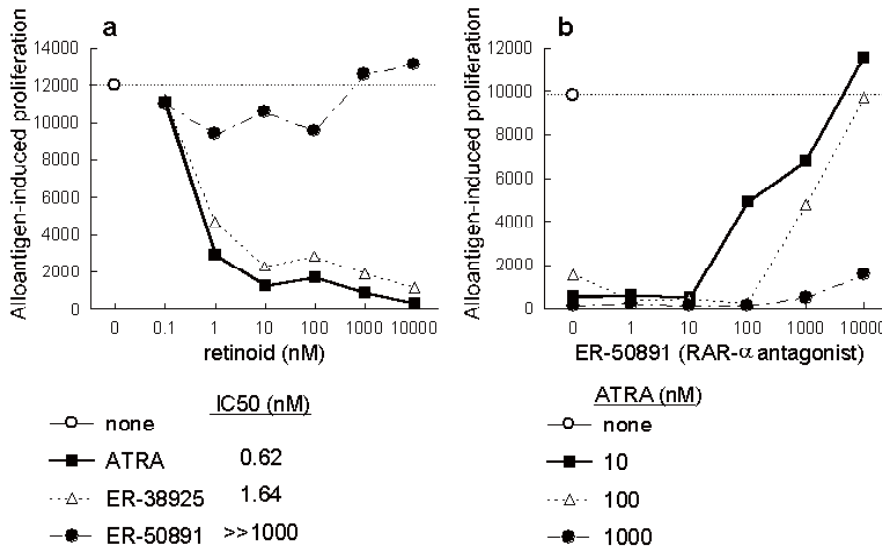


Figure 5. *In vitro* effect of retinoids on alloantigen induced proliferation of donor spleen cells. Anti-alloantigenic (anti-H-2^d) proliferation of donor spleen cells was induced by coculturing B6 spleen cells (2×10^5) and mitomycin C-treated BDF1 spleen cells (1×10^5). Each compound was added at the beginning of the culture. Five days later, each culture was pulsed with 0.5 mCi of ³H-thymidine for the final 16 h. Each point represents the mean of radioactivity incorporated into triplicate cultures. **a.** ATRA and ER-38925 inhibited the response in a concentration-dependent manner, while ER-50891, an RAR- α selective antagonist, did not, up to the highest concentration tested. **b.** ATRA inhibited the response almost completely starting at 10 nM. Inhibition of the response by ATRA at 10 and 100 nM was completely reversed by ER-50891 at 10,000 nM.

antagonist, as reported previously (13). This compound did not alter the response up to 10,000 nM (Figure 5a). The inhibition of ATRA at either 10 or 100 nM was reversed by ER-50891 in a concentration-dependent manner and completely recovered in the presence of 10,000 nM of ER-50891 (Figure 5b). Hence, the RAR- α receptor seems to play a crucial role in the suppression of alloantigen-induced proliferation of donor lymphocytes by retinoids in the aGVHD model.

Effect of ER-38925 on cytokine production in aGVHD hosts

A well-known fact is that various kinds of cytokines are produced in aGVHD hosts and work as disease-exacerbating factors in both human GVHD patients

and murine GVHD models. In particular, cytokines produced by type 1 T-helper cells (Th1), *i.e.* IL-12, IFN- γ , and TNF- α are known to play a crucial role in the development of aGVHD and are regarded as promising targets for disease intervention (14-17). As shown in Figure 6, as of 14 days after disease induction BDF1 mice with aGVHD had elevated serum IL-12 (p40) and IFN- γ when compared with intact controls that received spleen cells from a syngeneic strain (BDF1). The elevation of IL-12 in aGVHD mice was statistically significant. aGVHD control mice had much higher serum IFN- γ than intact controls, although this difference was not statistically significant due to a large data variation among the control animals. Compared to the aGVHD control, ER-38925 decreased serum IL-12 by about 30%, and the decrease was statistically

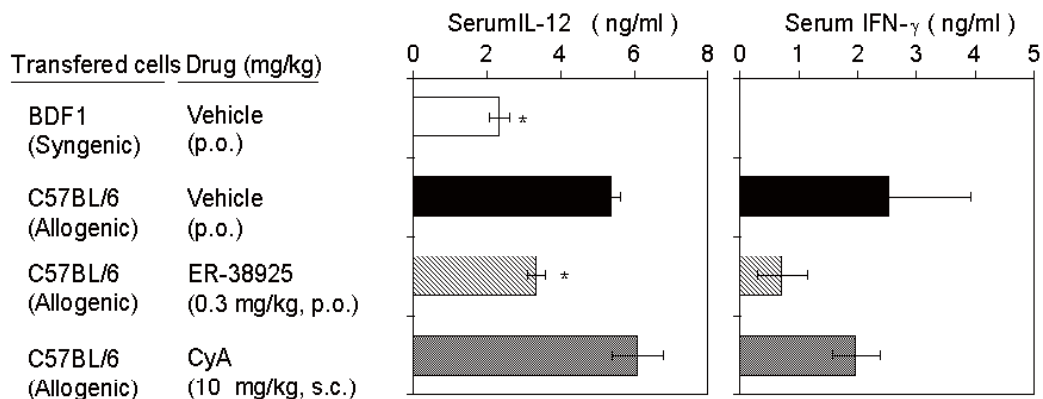


Figure 6. Effect of retinoids on cytokine production in aGVHD mice. Sera were collected from BDF1 mice with aGVHD at day 14 post-disease induction. Serum IL-12 (p40) and IFN- γ content was determined by ELISA as described in the Methods section. Each mouse was administered either ER-38925, CyA, or the vehicle once daily from the day of cell transfer. The dose and route of administration for each drug are indicated in the figure. BDF1 mice transferred with spleen cells from the syngeneic strain served as an intact control (open column). Each column represents the mean \pm S.E.M. of 5 animals. A significant difference from the aGVHD control as indicated by a Mann-Whitney test is shown in the figure, * $p < 0.05$. ER-38925 inhibited the elevation of serum IL-12 and IFN- γ in the aGVHD model while CyA did not.

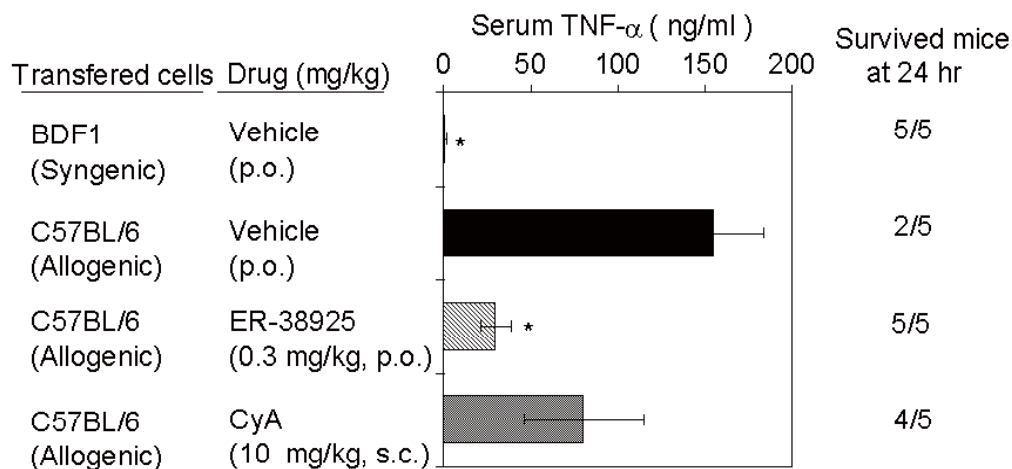


Figure 7. Effect of retinoids on TNF- α production in aGVHD mice. BDF1 mice with aGVHD were intravenously challenged with LPS (30 μ g/mouse) at day 14 post-disease induction. Three hours later, serum was collected from each mouse, and its TNF- α content was determined by ELISA. The number of mice alive at 24 h post-LPS injection is indicated on the right of each column. Mice were treated with drugs or the vehicle in the same manner as described in Figure 6. Each column represents the mean \pm S.E.M. of 5 animals. A significant difference from the aGVHD control as indicated by a Mann-Whitney test is shown in the figure, * $p < 0.05$. ER-38925 significantly inhibited LPS-induced serum TNF- α elevation and improved the survival after LPS challenge in the aGVHD model. These effects were more significant than those of CyA at 10 mg/kg.

significant ($p < 0.05$ by Mann-Whitney test, Figure 6). It also reduced serum IFN- γ in aGVHD mice by about 60%, but this effect was not statistically significant, again due to a large data variation among aGVHD controls. In contrast to ER-38925, CyA had no effect on serum IL-12 content, and it decreased serum IFN- γ only moderately, with no statistical significance to the decrease. In addition to the above Th1-type cytokines, aGVHD mice are known to produce TNF- α when challenged with microorganic components like lipopolysaccharide (LPS). This phenomenon may be mediated by activated monocytes or macrophages primed by IFN- γ and represent the accelerated vulnerability to septic shock after systemic infection in GVHD patients (18). The current aGVHD mice also produced a large amount of TNF- α in sera after challenge with LPS, and 3 out of 5 animals died from septic shock within 24 h, although neither response was observed in intact controls used in the same assay (Figure 7). Mice treated with ER-38925 at 0.3 mg/kg produced less TNF- α in sera, and this effect was statistically significant ($p < 0.05$ by Mann-Whitney test, Figure 7). Additionally, none of these mice died after the LPS challenge. The effect of ER-38925 on these parameters was more prominent than CyA at 10 mg/kg.

Effect of ER-38925 on cGVHD

cGVHD is characterized by a systemic autoimmune disease that primarily affects the skin and results in the

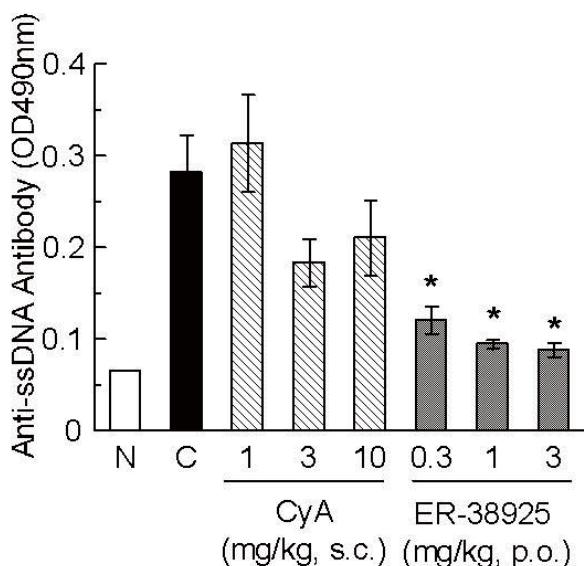


Figure 8. Effect of ER-38925 and CyA on autoantibody induction in a cGVHD model. cGVHD was developed in female BDF1 mice by transferring spleen cells of DBA2. After the cell transfer, those mice were divided into groups each consisting of 5 animals and administered the vehicle (0.5% MC, p.o.), ER-38925 (0.3, 1 or 3 mg/kg, p.o.) or CyA (1, 3 or 10 mg/kg, s.c.) from the day of cell transfer. Intact control mice were left untreated throughout the study period. Two weeks later, mice were bled and serum anti-ssDNA titer was determined by specific ELISA as described in the Methods section. A significant difference between the control and drug treatment group was noted in One-way ANOVA, * $p < 0.05$. ER-38925 inhibited the elevation of serum anti-ssDNA titer in a dose-dependent manner in the cGVHD model while CyA did not.

polyclonal activation of B cells and hyperproduction of Ig, including IgE and anti-DNA autoantibodies (1,19). To investigate the effect of ER-38925 on cGVHD, parental DBA/2 spleen cells were transferred to BDF1 hybrids. In vehicle-treated cGVHD hosts, the serum anti-ssDNA autoantibody titer was substantially elevated. ER-38925 significantly inhibited the elevation of anti-ssDNA starting at a dose of 0.3 mg/kg ($p < 0.05$ by One-way ANOVA, Figure 8). The effect of ER-38925 was much more marked than that of CyA since the latter did not significantly inhibit this parameter even at 10 mg/kg.

Discussion

The present study clearly shows that retinoids, and especially RAR- α selective retinoids, can inhibit mouse GVHD responses both *in vitro* and *in vivo*. ER-38925 significantly prolonged the lifespan of aGVHD hosts starting at 1 mg/kg (Figure 2), and this effect was almost comparable to 30 mg/kg of CyA (Figure 3). Moreover, it potently inhibited anti-ssDNA autoantibody production in the cGVHD model (Figure 8). To the extent known, this report is the first to clearly indicate the remediating effect of a retinoid on various GVHD-related disorders.

The authors' RAR- α selective retinoid effectively inhibited anti-allogeneic CTL induction in aGVHD hosts starting at 0.1 mg/kg (Figure 4). Since ATRA and Am80 also inhibited this response, this effect seems to be common among various types of retinoids. Retinoid-induced inhibition of CTL induction in aGVHD hosts seems to be mediated, at least partly, through the massive inhibition of donor cell proliferation since ER-38925 and ATRA inhibited the alloantigen-induced *in vitro* proliferation of B6 donor spleen cells in a concentration-dependent manner (Figure 5a). Moreover, inhibition of this response by ATRA was totally reversed by an excessive amount of an RAR- α selective antagonist, ER-50891 (Figure 5b). Therefore, RAR- α seems to play a pivotal role in the anti-proliferative effect of retinoids on alloantigen-stimulated murine lymphocytes, and may also play a role in the inhibition of CTL induction by retinoids.

IFN- γ , a representative Th1 cytokine, is critical to promoting CTL induction in murine GVHD models (20). Hayes and his colleagues observed increased IFN- γ and decreased IL-4 and IL-5 secretion in vitamin A-deficient mice, and such an imbalance was reversed by the administration of retinoic acid (21-23). They also reported that retinoic acid inhibits IFN- γ production by a Th1 cell line via a CD28 co-stimulatory signal blockade (24). Retinoid-mediated suppression of human (25) and murine (26) IFN- γ production is exerted at the transcriptional level. Consistent with these reports, ER-38925 reduced the serum IFN- γ level that was upregulated in the aGVHD model (Figure 6).

In addition, retinoids have recently been reported to inhibit IL-12 production by activated macrophages through functional interactions between their receptors (RXR and RAR) and NF- κ B, a crucial transcription factor for IL-12 gene expression (27). Fully consistent with this report, elevation of serum IL-12 was markedly suppressed in ER-38925-treated aGVHD mice (Figure 6). The inhibitory effect of ER-38925 on serum IL-12 and IFN- γ upregulation was observed at 0.3 mg/kg, the dose also effective in inhibiting CTL induction. Taken together, these findings indicate that the current retinoid must have inhibited Th1 differentiation in the aGVHD models and thereby reduced the secretion of Th1 cytokines like IL-12 and IFN- γ . These effects must have contributed to its potent suppression of CTL induction (Figure 4) since both cytokines have been reported to be critical to allo-specific CTL induction (20,28,29).

TNF- α is the other disease-exacerbating factor of GVHD in humans and rodents (17,18), and its serum level is known to correlate well with the severity of human GVHD (30,31). LPS is a component of endogenous bowel flora and a potent inducer of pro-inflammatory cytokine release from monocytes or macrophages. Translocation of LPS across damaged gut mucosa to systemic circulation is believed to take place during both experimental and clinical GVHD, and the serum level of LPS was elevated in BMT patients with GVHD (32). Once translocated into systemic circulation, LPS triggers monocytes or macrophages that have been primed by IFN- γ produced by activated T cells to produce a cytopathic amount of inflammatory cytokines like TNF- α or IL-1 (33). Together with activated T cells, those cytokines cause organ damage in GVHD hosts (34). This pathway must also be crucial in human GVHD, since the severity of GVHD is effectively reduced by germ-free conditions or antibiotic therapy in BMT recipients (35). In this study, ER-38925 reduced the production of TNF- α in LPS-injected aGVHD hosts and thereby improved their survival (Figure 7). This result suggests that ER-38925 inhibited the production of IFN- γ by activated donor T cells and thereby prevented the activation of monocytes or macrophages in aGVHD hosts (see Figure 6). This effect, together with ER-38925's potent inhibition of CTL activity, must largely contribute to its disease remediation in the aGVHD model.

In addition to aGVHD, ER-38925 intensely inhibited autoantibody production in cGVHD mice. Its effect excelled over that of CyA. The mechanism(s) by which ER-38925 suppress autoimmune disease in cGVHD remains to be elucidated. Nevertheless, anti-DNA production is also reported to be abrogated in IFN- γ R knock-out (NZB \times NZW)F1 mice, which are known to be a model of human SLE (36). Hence, ER-38925 may inhibit anti-ssDNA production via massive inhibition of IFN- γ production in cGVHD. This possibility warrants future study.

While the immunosuppressive mechanism of RAR- α selective retinoids is not precisely understood at the cellular level, recent studies indicate that retinoids induce the CD4⁺CD25⁺ regulatory T cell (Treg) subset (37,38) in mice in an RAR- α -dependent manner (39,40). Research has also suggested that Treg plays an important role in determining the prognosis of patients who receive a haematopoietic cell transplant. Namely, Treg decreases the incidence and severity of mouse GVHD (41,42) and promotes donor bone marrow engraftment by protecting these cells from host rejection (43) in mouse models. These findings suggest that ER-38925 suppressed GVHDs by inducing Treg cells in the semiallogeneic GVHD models used in this study. This possibility remains to be elucidated in future studies.

The present results suggest that retinoids, and especially RAR- α selective retinoids including ER-38925, may serve as a new remedy for preventing and/or treating GVHD in human BMT patients.

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