TRANSPLANT INTERNATIONAL

Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Danazol induces prolonged survival of fully allogeneic cardiac grafts and maintains the generation of regulatory CD4$^+$ cells in mice

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Keywords
cardiac transplantation, danazol, immunomodulation, mouse, regulatory cells, testosterone derivative.

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Conflicts of interest
Authors declare no conflict of interest.

Received: 6 August 2011
Revision requested: 12 September 2011
Accepted: 15 December 2011
Published online: 13 January 2012

doi:10.1111/j.1432-2277.2011.01427.x

Summary

Danazol, a derivative of testosterone, is useful for treatment of endometriosis as well as pretreatment for *in vitro* fertilization and embryo transfer, although its mechanisms of action are unclear. The aim of this study was to investigate the effect of danazol on alloimmune responses in murine heart transplantation. CBA male mice ($H2^k$) underwent transplantation of C57BL/6 male ($H2^b$) hearts and received a single dose of danazol (0.4, 1.2 or 4 mg/kg/day) by intraperitoneal injection on the day of transplantation and for 6 days thereafter. An adoptive transfer study was performed to determine whether regulatory cells were generated. The median survival time (MST) of allografts in danazol-treated (1.2 and 4 mg/kg/day) mice was 28 and 63 days, respectively, compared with 7 days in untreated mice. Moreover, secondary CBA recipients given whole splenocytes or CD4$^+$ cells from primary danazol-treated (4 mg/kg/day) CBA recipients 30 days after transplantation had prolonged allograft survival (MSTs, 29 and 60 days, respectively). Cell proliferation, interleukin (IL)-2 and interferon-γ were suppressed in danazol-treated mice, whereas IL-4 and IL-10 were up-regulated. Moreover, danazol directly suppressed allo-proliferation in a mixed leukocyte culture. Flow cytometry showed an increased CD4$^+$ CD25$^+$ Foxp3$^+$ cell population in splenocytes from danazol-treated mice. Danazol prolongs cardiac allograft survival and generates regulatory CD4$^+$ cells.

Introduction

Danazol, a 17-ethinyltestosterone derivative, is widely used as a therapeutic agent for endometriosis. There has been considerable research on danazol, since it is currently used as a therapeutic agent in gynecology [1–4], hematology [5,6], and oncology [7]. For example, in aplastic anemia patients when neither hematopoietic stem cell transplantation nor immunosuppressive therapy is available, danazol is recommended as first-line therapy [5]. Moreover, a low to medium dose of danazol is effective in idiopathic thrombocytopenic purpura patients who have become refractory to other therapeutic approaches [8–10].

Danazol is a synthetic anabolic steroid with unique properties similar to corticosteroids, such as inhibition of interleukin (IL)-1 and tumor necrosis factor (TNF)-α production with reduced virilizing and toxic effects [11]. Because of this corticosteroid-sparing effect, danazol has been used successfully in different autoimmune diseases such as idiopathic thrombocytopenic purpura (ITP) [12], systemic lupus erythematosus (SLE) [13,14], and

rheumatoid arthritis (RA) [15]. Moreover, danazol has also been shown to have immunosuppressive effects *in vitro* [16].

In the field of organ transplantation, however, the use of danazol remains controversial despite its immunosuppressive action, and little is known about the mechanisms by which danazol might modulate alloimmune responses. The present study investigated the effect of danazol on alloimmune responses in a murine model of cardiac allograft transplantation.

Materials and methods

Mice

Male C57BL/6 [$H2^b$ (B6)], CBA ($H2^k$), and BALB/c ($H2^d$) mice that were 8–12 weeks of age were purchased from Sankyo Ltd (Tokyo, Japan), housed in conventional facilities at the Biomedical Services Unit of Teikyo University, and used in accordance with the guidelines for animal experimentation approved by the Animal Use and Care Committee of the university and the 'Principles of laboratory animal care' (NIH publication, vol 25, no. 28, revised 1996).

Heart transplantation

Heart transplantation was conducted as described previously [17]. Postoperatively, cardiac graft function was assessed daily by palpating the heart for evidence of contraction. Rejection was defined as complete cessation of the heartbeat and confirmed by direct visualization and histologic examination of the graft.

Treatment with danazol

On the day of cardiac transplantation, transplant CBA recipients were given one dose of 0.4, 1.2 or 4 mg/kg/day of danazol (Mitsubishi Pharma, Osaka, Japan). Subsequently, the recipients were given intraperitoneal danazol injections for 6 days thereafter. Danazol (4 mg) was first dissolved in ethanol (1 ml) (Kanto Chemical, Tokyo, Japan) and then diluted with saline immediately before administration to achieve the desired concentration. Transplant recipients in the control groups were given either no injection (untreated group) or intraperitoneal injections of normal saline with or without ethanol.

Immunohistochemical and histologic studies of harvested grafts

Cardiac grafts transplanted into untreated mice and danazol-treated mice were removed 30 days after transplantation and studied immunohistochemically with use of double immunostaining. Fresh 4-μm-thick graft cryosections were fixed in ice-cold acetone and preincubated in Block Ace (Dainippon Pharmaceutical Co. Ltd, Tokyo, Japan). Samples were incubated with anti-CD4 (RM4-5; BD Biosciences, San Jose, CA, USA) and anti-CD8 (53-6.7; BD Biosciences) monoclonal antibody (mAb), or anti-Foxp3 (kindly provided by Professor Kenjiro Matsuno [18], Dokkyo Medical University, Tochigi, Japan) polyclonal antibody; incubated with alkaline phosphatase (ALP)-conjugated anti-rat Ig (712-055-153; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for anti-CD4 and CD8 and with ALP-conjugated anti-rabbit Ig (712-055-152; Jackson ImmunoResearch Laboratories) for anti-Foxp3; and developed blue with Vector Blue (Vector Laboratories, Burlingame, CA, USA). Cryosections were then incubated with rabbit anti-mouse type IV collagen polyclonal antibody (LB1403; Cosmo Bio, Tokyo, Japan) and peroxidase-conjugated anti-rabbit Ig (55693; Mitsubishi Chemical, Tokyo, Japan) and then developed brown with diaminobenzidine (Vector Laboratories).

Cardiac allografts in untreated mice and mice given danazol were removed 30 days after transplantation and studied histologically. Frozen sections (4-μm thick) were cut, mounted on silane-coated slides, and stained with hematoxylin-eosin. The assessment of the infiltrate was based subjectively.

Adoptive transfer studies

Adoptive transfer studies were conducted to determine whether or not regulatory cells were generated in danazol-treated mice. Thirty days after transplantation of B6 hearts into primary CBA recipients treated with danazol for 7 days after grafting, splenocytes (5.0×10^7) from primary recipients with functioning allografts were adoptively transferred into naïve secondary CBA recipients by means of intravenous injection into the penile vein. Each secondary recipient underwent transplantation of a B6 heart immediately after completion of the adoptive transfer. In some experiments, $CD4^+$ cells were purified from the spleens of primary transplant recipients by positive selection using a magnetically activated cell sorter and CD4 microbeads (Miltenyi Biotec, Auburn, CA, USA; purity > 98%), and $CD4^+$ (2.0×10^7) cells were adoptively transferred into naïve secondary recipients, which then immediately underwent transplantation of a B6 heart.

Mixed leukocyte culture

In mixed leukocyte culture (MLC) studies [19], the responder cells were splenocytes from naïve CBA mice or from untreated or danazol-treated CBA mice that had

undergone transplantation of a B6 heart 14 days earlier. The stimulator cells were B6 (allogeneic) or CBA (syngeneic) splenocytes treated with 100 μg/ml mitomycin C (MMC) (Kyowa Hakko, Osaka, Japan) for 30 min at 37 °C. The responder cells (2.5×10^6/ml) were co-cultured with the stimulator cells (5×10^6/ml) in complete medium in a humidified 5% CO_2 atmosphere (CH-16M; Hitachi, Tokyo, Japan) at 37 °C in 96-well, flat-bottomed tissue-culture plates (Iwaki Scitech Division, Tokyo, Japan) for 4 days. Proliferation was assessed using an enzyme-linked immunosorbent assay (ELISA) for bromodeoxyuridine incorporation (Biotrak, version 2; Amersham, Little Chalfont, UK) according to the manufacturer's instructions [20]. In some experiments, the MLC contained naïve CBA responder cells (2.5×10^6/ml) and MMC-treated B6 stimulator (5×10^6/ml) cells. Various amounts of danazol (2, 20 and 200 μg/ml) were added to the MLC to assess the direct effects of this agent on cellular proliferation.

Cytokine assays

On day 4 ELISAs were also performed to assess levels of IL-2, IL-4, IL-10, and interleukin (IFN)-γ in the supernatant of the MLC. The capture mAb (JES5-2A5), detection mAb (JES5-16E3) and recombinant standard for IL-10 were from BD Biosciences. The capture and detection mAbs for IL-2 (JES6-1A12 and JES6-5H4, respectively), IL-4 (BVD-1D11 and BVD-24G2), and IFN-γ (R4-6A2 and XMG1.2) were from Caltag Laboratories (Burlingame, CA, USA). Recombinant standards for IL-2, IL-4 and IFN-γ were from PeproTech (London, UK).

Flow cytometry analysis of CD4, CD25 and Foxp3 expression

Splenocytes were obtained from naïve CBA mice and from danazol-treated and untreated cardiac allograft transplant recipients 1, 2, and 4 weeks after transplantation. The cells were stained with fluorochrome-conjugated anti-CD4, anti-CD25 mAb (RM4-5 and PC61, respectively; BD Biosciences), and anti-mouse Foxp3 mAb (FJK-16s; eBioscience, San Diego, CA, USA), as well as their isotype controls (eBioscience). The stained cells were analyzed using a FACS Canto2 system (BD Biosciences). The percentage of $CD4^+$ $CD25^+$ $Foxp3^+$ in $CD4^+$ cells was determined.

Statistical analysis

Cardiac allograft survival time in two experimental groups was compared using a Mann–Whitney *U* test. In the cell-proliferation, cytokine, and flow cytometry studies, the difference between two groups was assessed using an unpaired Student's *t* test or analysis of variance (ANOVA) with Ryan method. A *P* value of less than 0.05 was regarded as significant.

Results

Survival of fully mismatched cardiac allografts in mice treated with danazol

The CBA recipients of B6 cardiac allografts that had either no treatment ($n = 5$), or intraperitoneal injections of normal saline with ($n = 5$) or without ethanol ($n = 5$) rejected their grafts acutely [median survival times (MSTs), 7, 9, and 7 days, respectively; Table. 1]. In contrast, survival time of B6 grafts in CBA allograft recipients treated with danazol (1.2 and 4 mg/kg) ($n = 5$ and 5, respectively) for 7 days was prolonged to 28 and 63 days (both $P < 0.001$ compared with no treatment, respectively; Fig. 1). However, treatment with 0.4 mg/kg did

Table 1. Cardiac allograft survival in control and danazol-treated mice.

Group	*n*	Individual STs (days)	MST (days)
No treatment	5	6, 7, 7, 7, 8	7
Normal saline with ethanol	5	8, 8, 9, 10, 22	9
Normal saline without ethanol	5	6, 7, 7,7,7	7
Danazol			
4 mg/kg/day, 7 days	5	36, 42, 63, 63, >100	63
1.2 mg/kg/day, 7 days	5	14, 22, 28, 34, 36	28
0.4 mg/kg/day, 7 days	5	7, 7, 9, 10, >100	9

STs, allograft survival times; MST, median allograft survival time.

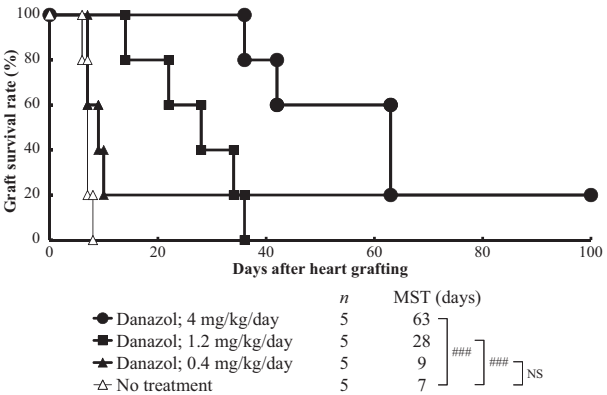
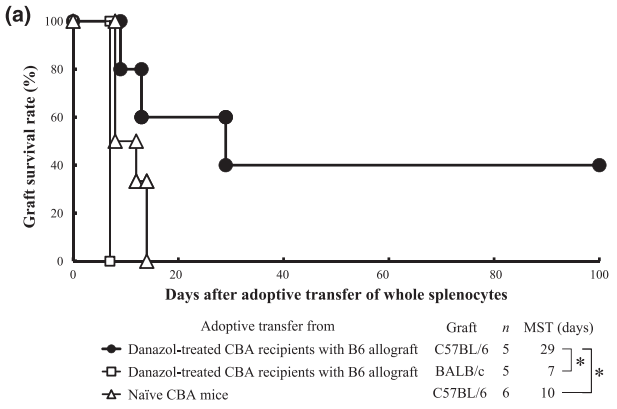
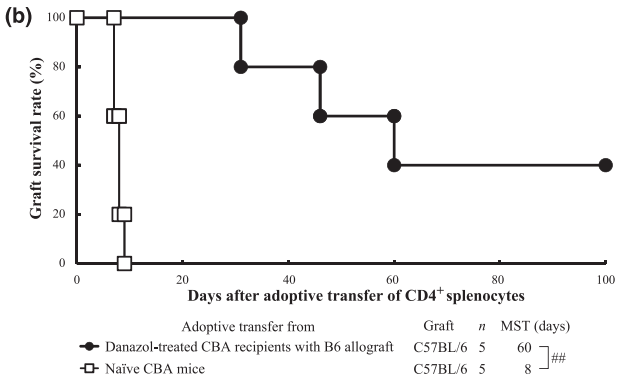
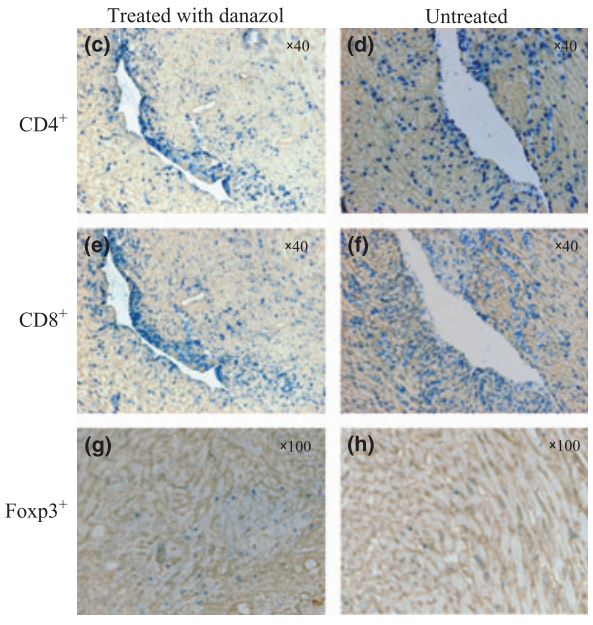
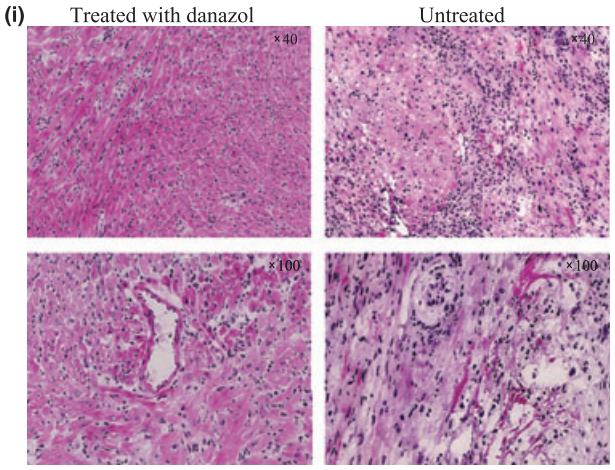


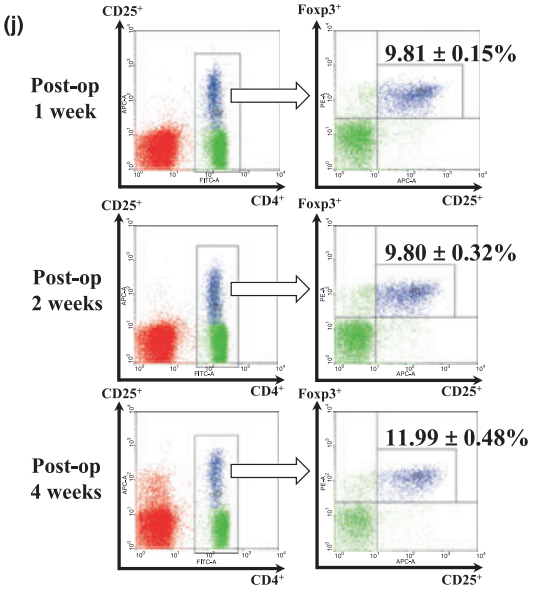
Figure 1 Cardiac allograft survival. CBA recipients of a B6 heart were given no treatment, one of three doses of danazol (0.4, 1.2 and 4 mg/kg/day) or saline with or without ethanol on the day of transplantation and for 6 days thereafter. MST, median survival time. ##$P < 0.005$ and ###$P < 0.001$ for difference between two groups.











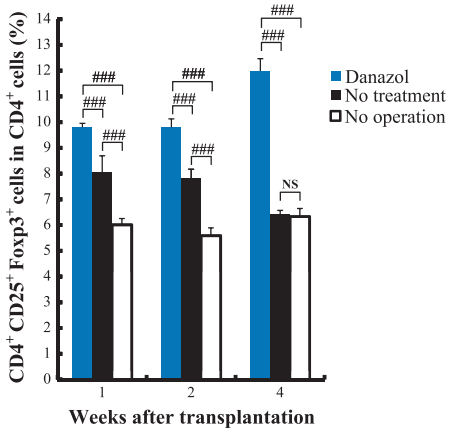


Figure 2 Evidence of generation of regulatory cells in CBA allograft recipients treated with danazol. (a, b) Cardiac allograft survival after adoptive transfer of whole splenocytes (a) or $CD4^+$ cells (b). MST, median survival time. $*P < 0.05$ and $\#\#P < 0.005$ for difference between two groups. (c–h) Results of double immunostaining of cardiac allografts obtained 30 days after transplantation from untreated and danazol-treated mice. Fresh 4-μm-thick graft cryosections were incubated with anti-CD4 (c, d) or anti-CD8 (e, f) monoclonal antibody or anti-Foxp3 polyclonal antibody (g, h). In Fig. 2c–f, the left-hand panels show photomicrographs from mice treated with danazol, and the right-hand panels show photomicrographs from untreated mice (magnification ×40). In Fig. 2g and h, the left-hand panel shows a photomicrograph from a danazol-treated mouse (magnification ×100), and the right-hand panel shows a photomicrograph from an untreated mouse (magnification ×100). (i) Histologic studies of harvested cardiac allografts stained with hematoxylin-eosin. In Fig. 2i, the left-hand panels show photomicrographs from mice treated with danazol (magnification ×40 and ×100), and the right-hand panels show photomicrographs from an untreated transplant recipient (magnification ×40 and ×100). (J) CD4, CD25, and Foxp3 expression in splenocytes as determined by flow cytometry 1, 2, and 4 weeks after transplantation (Post-op). The right-hand graph shows the percentage of $CD4^+$ $CD25^+Foxp3^+$ cells in the $CD4^+$ cells as determined by flow cytometry. Data are mean ± SD ($n = 5$ mice in each group). $\#\#\#P < 0.001$ for difference between two groups. NS, not significant.

not increase graft survival (MST, 9 days; Fig. 1). These results indicate that treatment with danazol induces hyporesponsiveness to cardiac allografts in a dose-dependent manner.

Histologic features of grafts from mice treated with danazol

Histologic examinations of cardiac allografts obtained 30 days after transplantation showed preserved graft structure in danazol-treated recipients, whereas allografts from untreated recipients showed myocyte damage, edema, and more aggressive inflammatory infiltrate in a process of acute rejection (Fig. 2i).

Generation of regulatory cells in mice treated with danazol

In the present investigation, adoptive transfer studies were conducted to determine whether or not generation of regulatory cells was involved in the induction of hyporesponsiveness in the danazol-treated mice. We found that naïve secondary CBA allograft recipients given adoptive transfer of splenocytes ($n = 5$) and $CD4^+$ cells ($n = 5$) from danazol-treated primary CBA recipients 30 days after heart transplantation had significantly prolonged survival of B6 hearts (MSTs, 29 and 60 days, respectively; $P < 0.05$ and $P < 0.005$ compared with the transfer of splenocytes and $CD4^+$ cells from naïve CBA mice; Fig. 2a and b). In contrast, naïve secondary CBA recipients given adoptive transfer of splenocytes ($n = 6$) and $CD4^+$ cells ($n = 5$) from naïve CBA mice rejected B6 hearts acutely (MSTs, 10 and 8 days, respectively). Moreover, when whole splenocytes from danazol-treated primary CBA transplant recipients with functioning B6 allografts were adoptively transferred into naïve secondary CBA recipients ($n = 5$) that then immediately underwent transplantation of a BALB/c heart, the BALB/c allografts were rejected acutely (MST, 7 days; Fig. 2a). These data indicate that treatment with danazol generated regulatory cells in the primary allograft recipients and that one of the regulatory populations consisted of $CD4^+$ cells, which may have been donor specific.

The immunohistochemical and histologic studies showed that cardiac allografts from untreated transplant recipients had aggressive infiltration of $CD4^+$ (Fig. 2c and d), $CD8^+$ (Fig. 2e and f) and severe myocardial damage (Fig. 2i), whereas allografts from danazol-treated recipients had sparser cell infiltration and less myocardial damage than untreated mice. Furthermore, cardiac allografts from danazol-treated recipients had more $Foxp3^+$ cells than those from untreated mice (Fig. 2g and h). Flow cytometry studies showed that the population of $CD4^+$ $CD25^+$ $Foxp3^+$ cells in the $CD4^+$ cells was increased in the spleens of danazol-treated recipients compared with those of naïve or untreated CBA mice (Fig. 2j). These data suggest that the $CD4^+$ regulatory cells contained a population that was $CD4^+$ $CD25^+$ $Foxp3^+$.

Cell proliferation and cytokine production in mice treated with danazol

Maximum proliferation of naïve CBA splenocytes (responder cells) against B6 splenocytes (stimulator cells) treated with MMC occurred on the fourth day of the MLC. Proliferation of splenocytes from CBA transplant recipients treated with danazol was markedly suppressed compared with that of splenocytes from untreated recipients (Fig. 3a). Moreover, the addition of danazol to an allogeneic MLC inhibited proliferation of CBA responder cells against B6 stimulator cells in a dose-dependent manner (Fig. 3b).

Levels of IL-4 (Fig. 3c) and IL-10 (Fig. 3d) in splenocytes from danazol-treated mice were significantly higher than those in splenocytes from untreated CBA mice ($P < 0.001$ and $P < 0.05$, respectively). In contrast, levels of IL-2 (Fig. 3e) and IFN-γ (Fig. 3f) were considerably decreased in danazol-treated recipients compared with those in untreated CBA mice ($P < 0.001$ and $P < 0.001$, respectively).

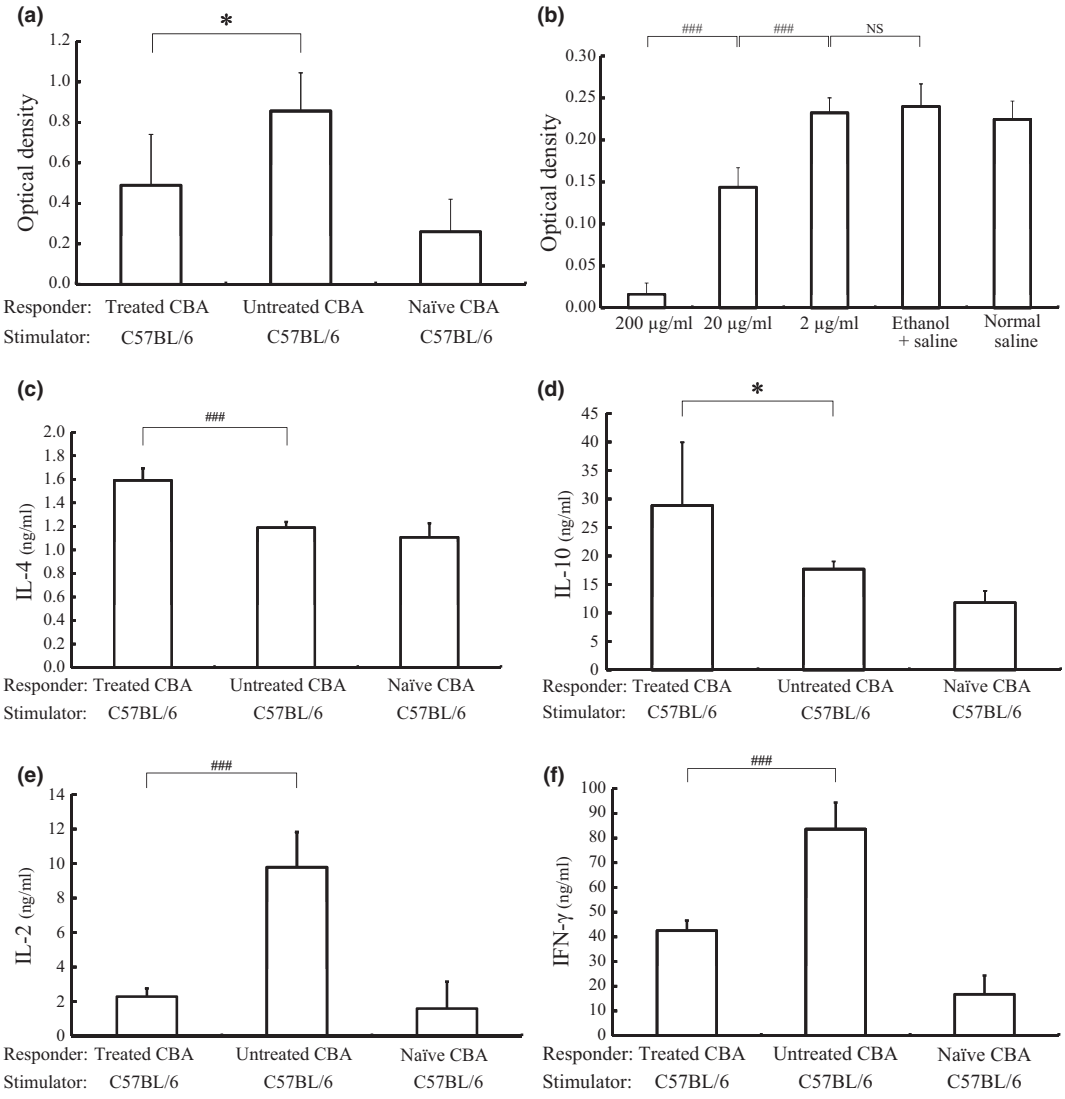


Figure 3 Evidence of induction of alloproliferative hyporesponsiveness by danazol. (a) Results of cell-proliferation assays in mixed leukocyte cultures (MLCs). The data shown are mean ± SD values. *$P < 0.05$ for difference between two groups. (b) Direct effect of danazol on alloproliferation in the MLC. The allogeneic mixed leukocyte reaction (allo) showed cellular proliferation of CBA responder cells against B6 stimulator cells. ###$P > 0.001$ for difference between two groups. NS, not significant. (c–f) MLC studies of cytokines. Levels of interleukin (IL)-4 (c), IL-10 (d), IL-2 (e), and interferon (IFN)-γ (f) in the MLC were assessed by enzyme-linked immunosorbent assay (ELISA). The data shown are mean ± SD values obtained in one representative experiment because similar results were achieved in three independent experiments. *$P > 0.05$ and ###$P > 0.001$ for difference between two groups.

Discussion

The influences of androgens on T cells are complex and inadequately studied in both humans and animals. However, danazol has been clinically used as a therapeutic agent for not only endometriosis but also hematologic [5,6,8–10] and renal disorders [21–23]. The results of these previous investigations indicate that danazol treatment can influence the immune system, probably through immunomodulation and immunosuppression. In our study, we investigated the possible effect of danazol on the alloimmune response in a murine model of heart transplantation.

In our murine model, 1 week of treatment with danazol significantly prolonged survival of fully mismatched cardiac allografts. Danazol treatment also generated $CD4^+$ regulatory cells in allograft recipients, and these cells had suppressive activity in MLC. Furthermore, IL-2 and IFN-γ were suppressed in danazol-treated mice, whereas IL-4 and IL-10 from danazol-treated recipients were up-regulated.

Flow cytometry studies showed an increased $CD4^+$ $CD25^+$ $Foxp3^+$ cell population in splenocytes from those mice.

There are several possible mechanisms by which treatment with danazol might have increased allograft survival in our model. One mechanism is that danazol treatment resulted in generation of regulatory cells. We previously found that some anti-inflammatory or immuno-modulatory agents induce hyporesponsiveness to fully allogeneic grafts by means of generation of regulatory cells [24–26], and also other reports showed similar results [27]. Recent studies in murine heart transplantation models have shown that the expansion and generation of regulatory T cells were involved in immunologic tolerance through the central and peripheral mechanisms [28–30]. Induction of hyporesponsiveness to an allograft is a dynamic, multistep process involving many mechanisms, including immune regulation, deletion, anergy, and ignorance [31]. Among these, immune regulation—control of alloimmune responses by regulatory cells—is considered one of the most important. Active suppression by regulatory cells is involved in the induction and maintenance of self-tolerance [32] and unresponsiveness to allografts [33]. In our adoptive transfer studies (Fig. 2a), most naïve secondary CBA transplant recipients given splenocytes from danazol-treated primary CBA recipients with functioning B6 cardiac allografts had significantly prolonged survival of their B6 cardiac grafts. Furthermore, adoptive transfer of $CD4^+$ cells from danazol-treated primary transplant recipients resulted in an even greater prolongation of allograft survival in the secondary recipients (Fig. 2b). These data suggest that treatment with danazol generated regulatory cells in the primary recipients and that the regulatory population contained $CD4^+$ cells. In addition, flow cytometry studies showed that the percentage of $CD4^+$ $CD25^+$ $Foxp3^+$ cells in $CD4^+$ cells increased in the primary allograft recipients (Fig. 2j).

In addition to this possible mechanism for danazol-induced hyporesponsiveness in our model, the balance between Th-1/Th-2 cytokines may have a strong influence on the function of regulatory cells. Recent studies have showed that the development of Th-2 cytokine secretion induced by IL-33 [34] and administration of not anti-IL-17 but anti-IL-6 mAb [35] promoted Treg expansion and ultimately prolonged murine cardiac allograft survival. In the present study, notable expression of Th-cytokines, a decrease of type 1 (IL-2 and INF-γ) and an increase of type 2 cytokines (IL-4 and IL-10) was detected in danazol-treated mice. Moreover, our MLC finding of up-regulation of IL-10 production by splenocytes in danazol-treated allograft recipients suggests that IL-10 could have contributed to the generation of regulatory cells. IL-10 has anti-inflammatory and suppressive effects on most hematopoietic cells, and plays a crucial role not only in the function of regulatory cells but also in their generation [36]. We previously demonstrated the importance of IL-10 in generating regulatory cells in our murine cardiac transplantation model [37]. Thus, it is likely that in the present study, up-regulation of IL-10 with danazol resulted in induction of $CD4^+$ regulatory cells. Moreover, an anti-inflammatory effect may be induced through regulatory cells. Our immunohistochemical and histologic studies of allografts obtained from danazol-treated mice showed sparser myocardial infiltration of $CD4^+$/$CD8^+$ leukocytes and minimal myocardial damage, respectively. In the light of these findings, it appears possible that danazol-induced regulatory cells may inhibit immune responses against allografts.

A second possible mechanism for danazol-induced hyporesponsiveness is the immunosuppression effects by the specific structure of danazol, a synthetic anabolic steroid with unique properties similar to corticosteroids. Actually, danazol has a corticosteroid-sparing effect and has been used successfully in the treatment of refractory autoimmune diseases such as ITP [12], SLE [13,14] and RA [15] as well as intractable immune-mediated diseases such as Henoch-Schönlein purpura [22] and IgA nephropathy [21,23]. Hill *et al.* [16] found that danazol administration *in vitro* significantly inhibited lymphocyte proliferation in MLC. Furthermore, Mori *et al.* [11] reported that danazol inhibited inflammatory cytokines such as IL-1 and TNF production by monocytes in a dose-dependent manner. More recently, testosterone has been reported to exert a protective effect on experimental autoimmune encephalomyelitis by the induction of a Th-2 bias in autoantigen-specific T lymphocytes [38]. Consistent with these reports, in our study, histologic studies of allografts obtained from danazol-treated recipient showed only minimal leukocyte infiltration (Fig. 2i) and danazol inhibited allo-proliferation of naïve CBA responder cells in a dose-dependent manner in the MLC with naïve B6 stimulator cells (Fig. 3b). Taken together with these previous reports, our results indicate that danazol may have the ability to inhibit activation of alloreactive T cells directly, either through the balance between Th-1 and Th-2 cytokines or some other unknown mechanisms.

The effect of immunosuppression by danazol functioning as a sex hormone is a third possible mechanism for the danazol-treated hyporesponsiveness observed in our study. Since danazol is a modified testosterone, it has some properties similar to testosterone and causes hypoestrogenemia. Many previous studies have shown suppressive effects of testosterone on the immune system [39–43]. In particular, two previous studies reported that testosterone prolonged skin graft survival time in rats [44] and became a new factor in the differentiation of regulatory T cells [45]. In our present study, danazol may

indirectly function like testosterone and prolong cardiac allograft survival time as well as generate regulatory cells.

In conclusion, treatment with danazol prolongs allograft survival and generates regulatory $CD4^+$ cells that directly inhibit alloimmune responses. Furthermore, danazol functions indirectly much like testosterone.

Authorship

MN, QZ, MU: designed the study. QZ, HB, TH, MU: performed the experiments. MN, TW, MU: wrote the article. XJ, AA, MU: analyzed the data.

Funding

Japan Gore-Tex, Tokyo, Japan, provided funding for editorial assistance.

Acknowledgements

The authors thank Prof. Kenjiro Matsuno, Mr. Hisashi Ueta, and Ms. Junko Sakumoto, Department of Anatomy (Macro), Dokkyo University, Tochigi, Japan and Prof. Kouji Matsushima and Dr. Satoshi Ueha, Department of Molecular Preventive Medicine and SORST, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan for technical assistance with the immunohistochemistry studies and Ms. Hisae Iinuma and Ms. Junko Tamura, Department of Surgery, Teikyo University School of Medicine, Tokyo, Japan for technical assistance with hematoxylin-eosin staining. We are also grateful to Renée J. Robillard, MA, ELS, for editorial assistance. This study was supported by Mitsubishi Pharma Corporation.

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