Induction of regulatory T cells and prolongation of survival of fully allogeneic cardiac grafts by administration of Tokishakuyaku-san in mice

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Background. The Japanese herbal medicine Tokishakuyaku-san (TJ-23) has been used to treat neurodegenerative, immune, and respiratory tract diseases, as well as many gynecologic disorders, with few adverse effects. This study investigated the effect of TJ-23 on alloimmune responses in a murine model of cardiac allograft transplantation.

Methods. CBA mice underwent transplantation of a C57BL/6 heart and received oral administration of 2 g/kg per day of TJ-23 or 1 of 16 other commonly used Japanese herbal medicines from the day of transplantation until 7 days afterward. An adoptive transfer study was conducted to determine whether regulatory cells were generated. Histologic and cell proliferation studies, cytokine measurements, and flow cytometry analyses were also performed.

Results. Of the 17 herbal medicines studied, only TJ-23, given in a dose of 2 g/kg per day, induced significantly prolonged allograft survival (median survival time [MST], >100 days). TJ-23 also suppressed proliferation of splenocytes and production of interleukin-2, interleukin-6, and interferon-γ. Adoptive transfer of either whole splenocytes or CD4+ or CD4+ CD25+ cells from TJ-23–treated allograft recipients resulted in indefinite survival of allografts in naive secondary recipients (MST >100 days). Flow cytometry studies showed that the CD4+ CD25+ forkhead/winged-helix (FOXP3)+ regulatory cell population was increased in transplant recipients given TJ-23.

Conclusion. TJ-23 induced hyporesponsiveness to fully allogeneic cardiac allografts and generated CD4+ CD25+ regulatory cells in our model. (Surgery 2011;150:923-33.)

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Japanese herbal medicines have long been used as alternative therapy because of their immunomodulatory effects.1 Recently, surgeons in Japan have begun to include these medicines in postoperative therapy regimens.2 For example, Daikenchu-to has been used by gastroenterologic surgeons to shorten postoperative ileus after abdominal surgery.3 Triptolide, which is present in the Chinese herb Triptergium wilfordii hook F, has been found to have potent immunosuppressive and anti-inflammatory properties4,5 and to prolong cardiac allograft survival in mice.6 Transplantation is the ultimate treatment for patients with total loss of function of a life-sustaining organ. New immunosuppressive drugs have improved allograft survival rates, but long-term administration of these agents may have serious adverse effects, including nephrotoxicity, diabetes, neurotoxicity, and an increased risk of infection and cancer. These complications could be avoided by establishment of a technique for...
creating donor-specific unresponsiveness or immunologic tolerance to donor alloantigens in a transplant recipient.

Studies in experimental models have shown that immunologic tolerance involves both central and peripheral mechanisms. Peripheral tolerance can be achieved by such mechanisms as anergy, deletion, ignorance, and active immune regulation. Among the mechanisms of peripheral tolerance, active suppression by regulatory T cells is likely to have a crucial role in maintaining tolerance to transplants; other mechanisms may not suppress newly generated alloreactive lymphocytes. Once donor-specific regulatory cells have been induced and survive in the recipient of a graft, it may be possible to modify the life-long use of nonspecific immunosuppressive agents. An approach to immunosuppression that involves donor-specific regulatory cells would also have the advantage of being physiologic. Several types of regulatory cells are produced endogenously by the immune system to establish unresponsiveness to self-antigen. Therefore, identification of agents that promote induction and maintenance of regulatory cells may have implications for the development of new tolerogenic strategies in transplantation.

In a murine model, we previously demonstrated the efficacy in inducing donor-specific regulatory cells and prolonging allograft survival of the following commonly used agents: Antithrombin III, selective cyclooxygenase 2 inhibitor, sarpogrelate hydrochloride, ranitidine, eicosapentaenoic acid, and ursodeoxycholic acid. The principal potential clinical advantage of these agents over conventional immunosuppressive agents is that they have been proven to be relatively harmless to patients.

We recently used the same murine model to demonstrate that administration of Sairei-to, a commonly used Japanese herbal medicine, prolongs allograft survival and generates regulatory cells.

In the current study, we administered 17 Japanese herbal medicines individually to mice with fully mismatched cardiac grafts to determine whether any of the agents affected the immune response. Only 1 of these medicines, Tokishakuyaku-san (TJ-23), yielded promising results and was investigated further.

TJ-23 is composed of 6 herbs: Paeoniae radix, Poria sclerotium, Atractylodis lanceae rhizoma, Alismatis rhizoma, Cnidii rhizome, and Angelicae radix. TJ-23 has been used to treat neurodegenerative, immune, and respiratory tract diseases, as well as many gynecologic disorders, with few side effects. It has also been reported to provide protective effects against certain types of neurodegeneration.

TJ-23 is an effective free radical scavenger and antioxidant that enhances the activity of mitochondrial-fraction superoxide dismutase. The Ministry of Health, Labour and Welfare in Japan has approved TJ-23 for use in treating several gynecologic disorders, including menopausal syndrome, dysmenorrhea, luteal insufficiency, amenorrhea, and oversensitivity to cold.

In studies in vitro, TJ-23 modulated the release of Th1/Th2 cytokines, suggesting that it may have the potential to normalize cytokine balance and to be useful in managing autoimmunity-related recurrent abortion. Clinically, TJ-23 therapy resulted in improvements in symptom scores and outcomes of pulmonary function tests in adults and children with asthma. In rats with preeclampsia, TJ-23 attenuated the hypertension and intrauterine growth retardation induced by NO-nitro-l-arginine methyl ester.

We describe a study of the possible mechanisms of the effect of TJ-23 on the alloimmune response in our murine model of cardiac allograft transplantation.

MATERIALS AND METHODS

Mice. Male C57BL/6 (H2b), CBA (H2k), and BALB/c (H2d) 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 Teikyo University.

Heart transplantation. All transplant procedures were performed with the mice under general anesthesia. Fully vascularized heterotopic hearts from C57BL/6 or BALB/c donors were transplanted into CBA mice by using microsurgical techniques. Postoperatively, graft function was assessed daily by palpation 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 commonly used Japanese herbal medicines. CBA recipients of a C57BL/6 heart were either not treated, given distilled water (control group), or given 2 g/kg per day of 1 of 17 commonly used Japanese herbal medicines (Table I) from the day of transplantation to 7 days afterward. The medicine was dissolved in distilled water and given orally with use of a metal tube (Thomas Scientific, Swedesboro, NJ). Because TJ-23 was found to have an immunomodulatory effect, some transplant recipients were treated with 0.2 or 0.02 g/kg per day of this agent to investigate the lowest dose that would produce this effect. In
efforts to identify a specific component of TJ-23 responsible for the immunomodulatory effect, other recipients were given either 2 g/kg per day of only 1 of the 6 herbal constituents of TJ-23 or 1 component of TJ-23 with the dose selected according to the percentage of TJ-23 accounted for by that component. *Paeoniae radix*, *Poria sclerotium*, *Atractylodis lanceae rhizoma*, and *Alismatis rhizoma* each comprise 18% of TJ-23 and *Cnidii rhizoma* and *Angelicae radix* each comprise 14%; therefore, the first 4 components were each given in a dose of 0.36 g/kg per day, whereas the dose for the latter 2 was 0.27 g/kg per day (Table II). Moreover, to investigate whether all 6 components of TJ-23 were essential in the induction of prolonged survival of cardiac allografts, we made 6 mixtures in which 1 component of TJ-23 was omitted (a different component from each mixture; Table III). We also tested 4 types of combination of *Paeoniae radix*, *Poria sclerotium*, and *Cnidii rhizome* mixtures. All the herbal medicines and materials used in the study were gifts of Tsumura (Tokyo, Japan; the “TJ” numbers are Tsumura commercial designations).

### Histologic studies of harvested grafts

Cardiac allografts transplanted into untreated mice and mice given TJ-23 were removed either 30 or 100 days after transplantation and studied histologically. The specimens were immersion fixed in 5% neutrally buffered formalin and embedded in paraffin by using routine procedures. Paraffin sections (4 μm thick) were cut, mounted on saline-coated slides, and stained with hematoxylin and eosin. Transplant vasculopathy was assessed after elastica–van Gieson staining.

### Adoptive transfer studies

Adoptive transfer studies were conducted to determine whether regulatory cells were generated after treatment with TJ-23. Thus, 30 days after CBA recipients (primary recipients) underwent transplantation of a C57BL/6 cardiac allograft and were given TJ-23 (2 g/kg per day), splenocytes (5 × 10⁷) from primary recipients with functioning allografts were adoptively transferred into naive CBA mice (secondary recipients). The secondary recipients underwent transplantation of a C57BL/6 heart immediately after the adoptive transfer. In some experiments, CD4⁺ cells were purified from the spleens of primary recipients by positive selection using a magnetically activated cell sorter and CD4 microbeads (Miltenyi Biotec, Auburn, CA; purity >98%), and 2 × 10⁷ of the CD4⁺ cells were adoptively transferred into naive secondary recipients, which then immediately underwent transplantation of a C57BL/6 or BALB/c heart. In other experiments, CD4⁺ CD25⁺ cells were purified from the spleens of primary recipients given TJ-23 by using a magnetically activated cell sorter and a mouse CD4⁺ CD25⁺ regulatory T-cell isolation kit (Miltenyi Biotec). CD4⁺ CD25⁺ cells (10⁶) were then adoptively transferred into naive secondary recipients, which then immediately underwent transplantation of a C57BL/6 heart.

### Table I. Allograft survival times (ST) in mice given oral administration of 2 g/kg per day of an herbal medicine

<table>
<thead>
<tr>
<th>Herbal medicine</th>
<th>Individual STs (days)</th>
<th>MST (d)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (untreated)</td>
<td>6, 7, 7, 8, 8</td>
<td>7</td>
<td>NS</td>
</tr>
<tr>
<td>Sho-sciryu-to (TJ-19)</td>
<td>7, 7, 7</td>
<td>7</td>
<td>NS</td>
</tr>
<tr>
<td>Shikunshino-to (TJ-75)</td>
<td>7, 7, 7, 11, 11</td>
<td>7</td>
<td>NS</td>
</tr>
<tr>
<td>Sho-saiko-to (TJ-9)</td>
<td>7, 7, 7, 7, 15</td>
<td>7</td>
<td>NS</td>
</tr>
<tr>
<td>Unse-in (TJ-57)</td>
<td>7, 7, 16</td>
<td>7</td>
<td>NS</td>
</tr>
<tr>
<td>Keishibukuru-gan (TJ-25)</td>
<td>7, 7, 7, 15, 33</td>
<td>7</td>
<td>NS</td>
</tr>
<tr>
<td>Shimotsu-to (TJ-71)</td>
<td>8, 8, 8, 8, 8</td>
<td>8</td>
<td>NS</td>
</tr>
<tr>
<td>Gorei-san (TJ-17)</td>
<td>7, 8, 8, 8, 8, 28</td>
<td>8</td>
<td>NS</td>
</tr>
<tr>
<td>Oren-gedoku-to (TJ-15)</td>
<td>7, 7, 7, 10, 12</td>
<td>8.5</td>
<td>NS</td>
</tr>
<tr>
<td>Juzen-taiho-to (TJ-48)</td>
<td>7, 7, 7, 7, 9, 13, 17, 22, 24, &gt;100</td>
<td>9</td>
<td>NS</td>
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<tr>
<td>Hochueeki-to (TJ-41)</td>
<td>7, 7, 9, 10, 10, 14, 16</td>
<td>10</td>
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<tr>
<td>Ryokankomyoshingenin-to (TJ-119)</td>
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<td>Ninjinoyei-to (TJ-108)</td>
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<td>Maobushiaishin-to (TJ-127)</td>
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<td>Shigyaku-san (TJ-35)</td>
<td>22, 22, 30</td>
<td>22</td>
<td>&lt;.05</td>
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<td>Tokishiyaktakagoshuyuokyo-to (TJ-38)</td>
<td>8, 8, 8, 22, 26, 67, &gt;100</td>
<td>22</td>
<td>&lt;.01</td>
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<tr>
<td>TJ-23</td>
<td>24, 50, 60, &gt;100, &gt;100, &gt;100, &gt;100</td>
<td>&gt;100</td>
<td>&lt;.005</td>
</tr>
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*Compared with untreated group on Mann-Whitney testing.  
MST, Median allograft survival time; NS, not significant.
Flow cytometry analysis. CD4, CD25, and forkhead/winged-helix (FOXP3) expression in splenocytes was determined by flow cytometry. Thirty days after cardiac allograft transplantation, splenocytes from recipients treated with TJ-23, untreated recipients, and naïve CBA mice were stained with fluorochrome-conjugated anti-CD4 or anti-CD25 monoclonal antibody (mAb; RM4-5 and PC61, respectively; BD Biosciences, San Jose, CA) or anti-mouse FOXP3 mAb (FJK-16s; eBio-science, San Diego, CA), as well as their isotype controls. The stained cells were analyzed by using a FACSCalibur system (BD Biosciences).

Mixed leukocyte culture studies and enzymelinked immunosorbent assays. In mixed leukocyte culture (MLC) studies, responder cells were splenocytes from naïve CBA mice or from untreated or TJ-23–treated CBA mice that had undergone transplantation of a C57BL/6 heart 14 days earlier. The stimulator cells were C57BL/6 (allogeneic) or CBA (syngeneic) splenocytes treated with 100 µg/mL mitomycin C (Kyowa Hakko, Osaka, Japan) for 30 minutes 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% carbon dioxide 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 by using an enzyme-linked immunosorbent assay (ELISA) for bromodeoxyuridine incorporation (Biotrak, version 2; Amersham, Little Chalfont, UK) according to the manufacturer’s instructions.

An ELISA was also performed to assess levels of interleukin (IL)-2, IL-4, IL-6, IL-10, and interferon (IFN)-γ in the supernatant of the MLC on day 4. The capture mAb (JES5-2A5), detection mAb (JES5-16E3), and recombinant standard for IL-10 were from BD Biosciences. The capture and detection mAb for IL-2 (JES6-1A12 and JES6-5H4, respectively), IL-4 (BVD-1D11 and BVD-24G2, respectively), and IFN-γ (R4-6A2 and XMG1.2, respectively) were from Caltag Laboratories (Burlingame, CA). The capture mAb (MP5-20F3), detection mAb (MP5-32C11), and recombinant standard for IL-6 were from Beckman Coulter (Fullerton, CA). Recombinant standards for IL-2, IL-4, and IFN-γ were from PeproTech (London, UK); those for IL-6 were from Endogen (Rockford, IL).

Statistical analysis. Cardiac allograft survival in groups of mice was compared by using Mann–Whitney testing. In the cell proliferation, cytokine, and flow cytometry studies, 2 groups were compared by using unpaired Student t tests. Graphpad
Prism statistical software (Graphpad, San Diego) was used for all analyses. \( P < .05 \) was considered significant.

**RESULTS**

**Effects of herbal medicines on cardiac allograft survival.** Control CBA mice given either no treatment or distilled water rejected C57BL/6 cardiac allografts acutely (median survival times [MSTs], 7 and 8 days, respectively; Fig 1). All CBA recipients treated with 2 g/kg per day of TJ-23 had prolonged graft survival (MST, >100 days; \( n = 7 \); \( P < .01 \) vs distilled water-treated controls; Fig 1). Treatment with 0.2 g/kg of TJ-23 was less effective to prolong allograft survival (MST, 27 days; \( n = 8 \); \( P < .05 \) vs control group; Fig 1); whereas treatment with 0.02 g/kg of TJ-23 did not affect allograft survival (MST, 8; \( n = 5 \); \( P \) value not significant vs control group; Fig 1). These data suggest that treatment with TJ-23 induced hyporesponsiveness to fully mismatched cardiac allografts in a dose-dependent manner. Table 1 shows the individual survival times and MSTs of allografts in mice given 1 of the 17 herbal medicines tested. TJ-19, TJ-75, TJ-9, TJ-57, TJ-25, TJ-71, TJ-17, TJ-15, and TJ-48 had no effect on allograft survival, whereas TJ-119, TJ-108, TJ-127, TJ-96, TJ-35, and TJ-38 had a modest effect.

The results of experiments in which 1 only component of TJ-23 was administered are shown in Table II. Used alone in either a dose of 2 g/kg per day or a dose selected according to the relative amount of each TJ-23 constituent, none of the components of TJ-23 prolonged allograft survival. Moreover, when mice with cardiac allografts were orally administered 1 component missing mixtures, none of the graft survival was prolonged as long as the real TJ-23 had induced (Table III). These results showed that all of the components were indispensable for TJ-23 to induce the significant prolongation of allografts survival.

Interestingly, when *Paeoniae radix*, *Poria sclerotium*, and *Cnidii rhizome* were used together, the MST was 10 days. When *Poria sclerotium* combined with *Cnidii rhizome* were used, the MST was 9 days. When *Paeoniae radix* combined with *Poria sclerotium* were used, the MST was 10.5 days. When *Paeoniae radix* combined with *Cnidii rhizome* were used, the MST was 84.5 days (\( P < .05 \) with control untreated group), which was the most close to the results of the whole TJ-23 treated mice. These results indicated that the combination of *Paeoniae*
radix and Cnidii rhizome seemed to play the major role in inducing indefinite prolongation of allograft survival by TJ-23 treatment.

Histologic features of allografts from recipients given TJ-23. Histologic examinations of cardiac allograft samples obtained 30 days after transplantation showed significantly preserved graft structure in transplant recipients given TJ-23 (Fig 2, A), whereas samples from untreated recipients showed myocyte damage, edema, and aggressive inflammatory infiltrates characteristic of the acute rejection process (Fig 2, B). Allograft samples obtained 100 days after transplantation from recipients treated with TJ-23 showed a few myocardial injuries, with infiltrating leukocytes and mild obliteratorive vasculopathy (Fig 2, C and D).

Generation of regulatory cells in mice treated with TJ-23. We previously found that some anti-inflammatory or immunomodulatory agents induce unresponsiveness to fully allogeneic grafts by means of generation of regulatory cells.10,11 To determine whether induction of regulatory cells was involved in the prolongation of allograft survival by TJ-23 treatment in the current investigation, we conducted adoptive transfer studies. As shown in Fig 3, A, most naïve secondary CBA recipients of C57BL/6 hearts given adoptive transfer of splenocytes from primary CBA transplant recipients treated with TJ-23 had indefinite survival of the allografts (MST, >100 days; n = 6; P < .01 vs controls). In contrast, secondary CBA recipients given adoptive transfer of splenocytes from naïve CBA mice (controls) rejected C57BL/6 hearts acutely (MST, 12 days; n = 5). Naïve secondary CBA recipients given adoptive transfer of splenocytes from primary recipients treated with TJ-23 also rejected third-party (BALB/c) cardiac allografts (MST, 13 days; n = 7; P < .01 vs C57BL/6 allograft results).

When CD4+ cells purified from spleens of primary CBA transplant recipients treated with TJ-23 were adoptively transferred into naïve secondary CBA recipients, all C57BL/6 allografts in the secondary recipients survived indefinitely (Fig 3, B; MST, >100 days; n = 8; P < .01 vs controls).

Fig 2. Histologic studies of harvested cardiac allografts stained with either hematoxylin and eosin (A, B, C) or elastica-van Gieson stain (D; original magnification, ×75). (A) C57BL/6 cardiac graft sample obtained 30 days after transplantation from a CBA mouse given 2 g/kg per day of TJ-23 from the day of transplantation until 7 days afterward. (B) C57BL/6 graft sample obtained 30 days after transplantation from an untreated CBA transplant recipient. (C, D) C57BL/6 cardiac graft samples obtained 100 days after transplantation from CBA mice given 2 g/kg per day of TJ-23.
Evidence of generation of regulatory cells in CBA allograft recipients treated with 2 g/kg per day of TJ-23.

(A) Graft survival after adoptive transfer of whole splenocytes. CBA mice (primary recipients) underwent transplantation of a C57BL/6 cardiac graft and were treated with 2 g/kg per day of TJ-23. Thirty days later, splenocytes \((5 \times 10^7)\) from primary recipients with functioning allografts were adoptively transferred into naïve CBA mice (secondary recipients), which were then given a C57BL/6 (donor-specific) or BALB/c (third-party) cardiac allograft. Secondary recipients in the control group underwent adoptive transfer of splenocytes from naïve CBA mice. The difference in graft survival between the 2 groups was significant. \(\# P < .01\) for difference between 2 groups.

(B) Graft survival after adoptive transfer of CD4\(^+\) cells. CBA mice (primary recipients) underwent transplantation of a C57BL/6 cardiac graft and were treated with 2 g/kg per day of TJ-23. Thirty days later, CD4\(^+\) cells were purified from the spleens of primary recipients with functioning allografts by positive selection using a magnetically activated cell sorter and CD4 microbeads. CD4\(^+\) cells \((2 \times 10^7)\) were then adoptively transferred into naïve secondary recipients, which then immediately underwent transplantation of a C57BL/6 (donor-specific) or BALB/c (third-party) heart. Secondary recipients in the control group underwent adoptive transfer of CD4\(^+\) cells from naïve CBA mice. \(\# P = .01\) for difference between 2 groups.

(C) CD4, CD25, and FOXP3 expression in splenocytes, as determined by flow cytometry. Thirty days after cardiac allograft transplantation, splenocytes from TJ-23–treated recipients, untreated recipients, and naïve CBA mice were stained with fluorochrome-conjugated anti-CD4 or anti-CD25 mAb or antimouse FOXP3 mAb, as well as their isotype controls. The stained cells were then analyzed by using a FACSCalibur system. \(\# P < .01\) for difference between 2 groups.

(D) Graft survival after adoptive transfer of CD4\(^+\) CD25\(^+\) cells. CBA mice (primary recipients) were treated with 2 g/kg of TJ-23 and underwent transplantation of a C57BL/6 cardiac graft. Thirty days later, CD4\(^+\) CD25\(^+\) cells were purified from the spleens of primary recipients with functioning allografts by using a magnetically activated cell sorter and a murine CD4\(^+\)CD25\(^+\) regulatory T-cell isolation kit. CD4\(^+\) CD25\(^+\) cells \((10^6)\) were then adoptively transferred into naïve secondary recipients, which then immediately underwent transplantation of a C57BL/6 heart. Secondary recipients in the control group underwent adoptive transfer of CD4\(^+\) CD25\(^+\) cells from naïve CBA mice. \(\# P < .01\) for difference between 2 groups.
Adoptive transfer of CD4+ cells from naïve CBA mice (controls) did not induce graft prolongation (MST, 8 days; n = 5). Naïve CBA recipients given adoptive transfer of CD4+ cells from primary recipients treated with TJ-23 also rejected BALB/c allografts (MST, 10 days; n = 7; P < .01 vs C57BL/6 allograft results). These data indicate that administration of TJ-23 for 8 days generated regulatory cells in the recipients and that 1 of the regulatory populations was CD4+ cells that might have been donor specific.

The flow cytometric studies of the phenotype of the generated regulatory cells showed that the population of CD4+ CD25+ cells was increased in the spleens of CBA recipients of C57BL/6 hearts treated with TJ-23 compared with those of either naïve CBA mice or untreated CBA transplant recipients (data not shown). The population of CD4+ CD25+ FOXP3+ cells was also increased in mice given TJ-23 (Fig 3, C).

When CD4+ CD25+ cells (10^6) purified from splenocytes from TJ-23–treated CBA recipients with functioning C57BL/6 allografts 30 days after transplantation were adoptively transferred into naïve CBA mice (secondary recipients) immediately before transplantation of a C57BL/6 heart, all allografts in the secondary recipients had significantly prolonged survival (Fig 3, D; MST, >100 days; n = 5; P < .01 vs controls). In contrast, naïve secondary CBA recipients that underwent adoptive transfer of CD4+ CD25+ cells from naïve CBA mice eventually rejected their C57BL/6 allografts (MST, 8.5 days; n = 6).

**Cell-proliferation assay and production of cytokines.** Maximum proliferation of naïve CBA splenocytes (responder cells) against C57BL/6 splenocytes (stimulator cells) treated with mitomycin C occurred on day 4 of the MLC. Proliferation of splenocytes from CBA recipients given TJ-23 was significantly reduced compared with that of splenocytes from untreated mice (Fig 4, A; P < .01).

Levels of IFN-γ (Fig 4, B), IL-2 (Fig 4, C), and IL-6 (Fig 4, D) in splenocytes from mice treated with TJ-23 were significantly lower than those in splenocytes from untreated mice. There were no differences between untreated and treated mice in the production of IL-4 or IL-10 (data not shown).

**DISCUSSION**

This study found that of 17 herbal medicines tested (Table I), only 1, TJ-23, was effective in inducing hyporesponsiveness to fully allogeneic cardiac grafts in mice and that it did so in a dose-dependent manner. Treatment with TJ-23 also generated regulatory T cells that may have been donor specific, and these cells demonstrated suppressive activity in MLCs. Finally, splenocytes from mice given TJ-23 showed downregulation of IL-2, IL-6, and IFN-γ in MLCs. Our results suggest several possible mechanisms, functioning either alone or together, for the induction by TJ-23 of prolonged survival of fully mismatched cardiac allografts in our murine model.

One possible mechanism is that treatment with TJ-23–induced regulatory cells. Active suppression by regulatory cells has been found to be 1 of the important mechanisms of induction and maintenance of self-tolerance32 and unresponsiveness to allografts.7 In our adoptive transfer study, administration of either whole splenocytes or CD4+ splenocytes from allograft recipients treated with TJ-23 induced indefinite survival of allografts in secondary recipients that had the same strain of donors as the primary recipients; but, this treatment did not significantly prolong survival of allografts from third-party donors. These results indicate that TJ-23 treatment generated regulatory cells, that the regulatory population contained CD4+ cells, and that the regulatory cells might have been donor specific.

Moreover, the flow cytometry analysis found that the population of CD4+ CD25+ cells (data not shown) and CD4+ CD25+ FOXP3+ cells in the CD4+ cell population was increased in transplant recipients given TJ-23 compared with untreated recipients and naïve mice. In addition, in our adoptive transfer study, administration of CD4+ CD25+ splenocytes from primary allograft recipients treated with TJ-23 also induced indefinite survival of allografts in secondary recipients. This result confirmed that the regulatory population generated by TJ-23 treatment contained CD4+ CD25+ cells. Previous studies showed that CD4+ CD25+ regulatory T cells have an important role in transplantation tolerance.33 Furthermore, the transcription factor scurfin, which is encoded by the FOXP3 gene,34 is a specific marker for regulatory activity35 and is essential for the development and function of regulatory CD4+ CD25+ T cells.36 Thus, treatment with TJ-23 may have increased the population of regulatory cells in the spleens of allograft recipients, thereby contributing to the prolongation of graft survival. However, the precise mechanisms by which TJ-23 treatment might induce regulatory cells remain unknown.

Another possible mechanism for the prolonged allograft survival in TJ-23–treated mice is that TJ-23 had a protective effect on myocardial cells. Our histologic examinations showed that, compared
with cardiac allografts from untreated mice, allografts obtained 30 and 100 days after transplantation from mice given TJ-23 had only a few myocardial injuries, with infiltrating leukocytes and mild obliterative vasculopathy. Ueda et al.19 previously found that TJ-23 has free radical scavenging activity. Oxidative stress, in which reactive oxygen species (free radicals) are generated extra- or intracellularly and exert toxic effects on cells, has been demonstrated to have an important role in cardiac ischemic and reperfusion injuries.37 Moreover, ischemia reperfusion injury is considered to result in delayed graft function and reduced long-term patency of transplanted

**Fig 4.** Evidence of induction of alloproliferative hyporesponsiveness by TJ-23. (A) Results of cell proliferation assay in a MLC. Untreated CBA mice and CBA mice treated with 2 g/kg per day of TJ-23 were given cardiac grafts from C57BL/6 mice. Fourteen days later, splenocytes (2.5 × 10^6 cells/mL) from the CBA transplant recipients (responder cells) were cocultured with splenocytes from C57BL/6 mice (stimulator cells; 5 × 10^6 cells/mL). Data are shown as mean values ± standard deviation derived from samples from 5 mice in each group. (B, C, D) Levels of cytokines in MLCs. CBA mice were given cardiac grafts from C57BL/6 mice and treated with 2 g/kg per day of TJ-23. Fourteen days later, splenocytes (2.5 × 10^6 cells/mL) from the CBA transplant recipients (responder cells) were cocultured with splenocytes from C57BL/6 mice (stimulator cells; 5 × 10^6 cells/mL) for 4 days. Levels of IFN-γ (B), IL-2 (C), IL-4 (data not shown), IL-6 (D), and IL-10 (data not shown) in the MLCs were assessed by ELISA. Data are shown as mean values ± standard deviations derived from samples from 5 mice in each group. There were no differences between groups in the production of IL-4 or IL-10.
organs (kidneys). Therefore, treatment with TJ-23 may protect cardiac allografts from myocardial injuries by means of free-radical scavenging activity.

A third possible mechanism is that TJ-23 treatment resulted in suppression of the infiltration and proliferation of leukocytes. Our MLC and ELISA studies showed that allograft recipients given TJ-23 showed downregulation of leukocyte proliferation and the production of IL-2, IFN-γ, and the anti-inflammatory cytokine IL-6. IFN-γ increases expression of class II antigens on endothelial cells and participates in cell-mediated vascular injury, an important cause of indirect myocyte injury. IFN-γ has also been found to be a key effector in cardiac graft arteriosclerosis. Therefore, downregulation of IFN-γ production induced by TJ-23 may have been involved in mitigation of allograft rejection in our model. According to this scenario, TJ-23 would have protected the grafts against immune injury by means of its anti-inflammatory effects and by suppressing the infiltration and proliferation of leukocytes and cytokines.

Finally, in a study by Aberle et al, TJ-23 directly enhanced ventricular contraction in isolated cardiomyocytes. This effect of TJ-23 might have contributed to the survival of cardiac allografts in our investigation, although we had not detected the cardiac contractile functions of the allografts in this study.

Interestingly, we found that other herbal medicines that have some of the same components as TJ-23 did not have the same effect in our model. For example, like TJ-23, TJ-19, TJ-57, TJ-25, TJ-71, TJ-48, TJ-108, TJ-35, and TJ-38 each contain Paeoniae radix. Atractylodis lanceae rhizoma is a component not only of TJ-23 but also of TJ-75, TJ-17, TJ-48, and TJ-41. Alismatis rhizoma is a constituent of both TJ-23 and TJ-17. However, only treatment with TJ-23 prolonged survival of allografts for a MST of >100 days. Moreover, none of the individual components of TJ-23 induced prolongation of allograft survival to the same extent as did the complete TJ-23 formula. These results suggest that interaction among the components of TJ-23 was important in producing the results obtained with the medicine and that induction of hyporesponsiveness by TJ-23 depends on its specific combination of constituents. These possibilities are consistent with the traditional assumption in Japanese herbal medicine that, unlike modern Western pharmaceutical agents, herbal formulas have an overall effect that is different from the effects of their components.

In summary, in our murine model, TJ-23, but not the other Japanese herbal medicines tested, induced prolonged survival of fully allogeneic cardiac grafts and generated CD4+ CD25+ regulatory cells. Our findings indicate that TJ-23 inhibited allograft rejection by means of an immunomodulatory ability that the other medicines do not possess. In addition, our study shows that an understanding of traditional herbal medicines may illuminate new pathways for exploration in efforts to achieve immunomodulation in clinical transplantation.

The authors thank Renée J. Robillard, MA, ELS, for editorial assistance.

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