

Induction of Regulatory T Cells and Prolongation of Fully Allogeneic Cardiac Grafts by Herbal Medicine, Shohangekabukuryo-to

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Abstract

Objective: Shohangekabukuryo-to (Tsumura Japan [TJ]-21) is well known as an herbal medicine with few side effects to treat vomiting, nausea, acute and chronic gastritis, and hyperemesis of pregnancy in Japan. In this study, we performed fully allogeneic cardiac grafts in mice to investigate the effect of TJ-21 on alloimmune responses.

Methods: CBA (H2^k) mice underwent transplantation of a C57BL/6 (H2^b) heart and received oral administration of 0.02, 0.2 and 2 g/kg per day of TJ-21 from the day of operation until 7 days afterward. Adoptive transfer study was performed to determine whether regulatory cells were generated. Histologic and cell proliferation studies, flow cytometry analyses and cytokine measurements were also performed.

Results: Compared to untreated CBA mice rejected C57BL/6 hearts acutely (median survival time [MST], 8 days), MST of allografts from TJ-21-treated mice was prolonged (MST, 77 days). Moreover, adoptive transfer of whole splenocytes from TJ-21-treated allograft CBA recipients had prolongation of allograft survival in secondary recipients (MST, 53 days). Flow cytometry study showed TJ-21-treated mice had an increased CD4⁺CD25⁺Foxp3⁺ cell population. Cell proliferation showed that interleukin-2 (IL-2) and interferon- γ were inhibited in TJ-21-treated mice, whereas IL-4 and IL-10 were increased.

Conclusions: In summary, TJ-21 could induce hyporesponsiveness of fully MHC-mismatched cardiac allografts and generate regulatory cells.

Keywords: Shohangekabukuryo-to (Xiao-Ban-Xia-Jia-Fu-Ling-Tang); Herbal medicine; Regulatory T cells; Cardiac transplantation; Mouse

Introduction

Traditional herbal medicines have been firstly used as far back as 2800 BC [1,2]. In recent years, herbal medicines are rapidly increasing worldwide [3,4]. They have been used to treat a wide variety of diseases, ranging from the common cold to autoimmune diseases [5]. For example, Mao-to (TJ-27) has been used to treat influenza infection [6], Dai-bofu-to (TJ-97) has been used to treat rheumatoid arthritis [7]. In recent studies of our murine model, oral administration of the herbal medicines Tokishakuyaku-san (TJ-23) [8], Sairei-to (TJ-114) [9], or Artemisiae Capillaris Herba [10] was associated with significantly prolonged survival of allogeneic cardiac grafts (median survival times (MSTs) of all groups >100 days) and generation of regulatory cells. Based on these findings, we wondered if there were other several herbal medicines could also affect the immune response.

Shohangekabukuryo-to (TJ-21), one of the traditional Japanese herbal medicines, is composed of 3 herbs: Hoelen, Pinelliae Tuber and Zingiberis rhizoma. In Japan, Hoelen and Pinelliae Tuber have been used to treat dizziness and cough, respectively [11]. Zingiberis has also

been found useful in treating vomiting [12]. Meanwhile the 3 components mixture (TJ-21) has been widely used to treat vomiting, nausea [13], acute and chronic gastritis, as well as hyperemesis of pregnancy [14], with few side effects. However, no investigations have been done on the effect of TJ-21 on alloimmune responses after transplantation. Therefore, the purpose of this study was to determine the effects of TJ-21 and its components on the alloimmune response in a murine model of cardiac transplantation.

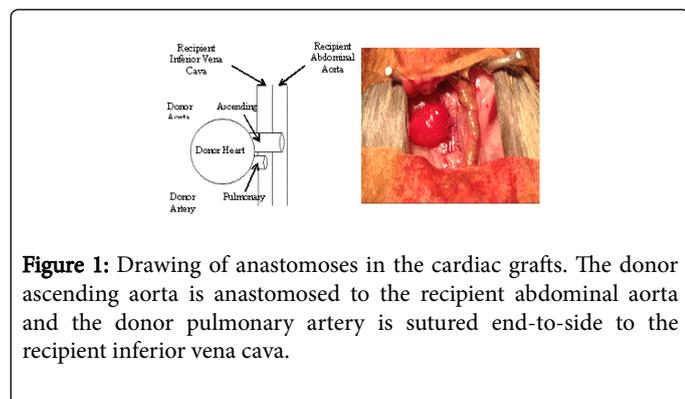
Materials and Methods

Animals

Male C57BL/6 (B6, H2^b) and CBA (H2^k) 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 (Teikyo 13-023).

Heart transplantation

All transplant procedures were performed with the mice under general anesthesia. Fully vascularized heterotopic hearts from B6 donor were transplanted into CBA mice by using microsurgical techniques (Figure 1) [15].



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.

Administration of TJ-21 and Its 3 components

Naive CBA recipients of a B6 heart were given no treatment, distilled water (control group), or oral administration of 0.02, 0.2 and 2 g/kg/day of TJ-21 from the day of transplantation to 7 days afterward.

To identify a specific component of TJ-21 responsible for the immunomodulatory effect, other recipients were given 2 g/kg/day of only 1 of the 3 herbal components of TJ-21.

Hoelen, Pinelliae Tuber and Zingiberis Rhizoma comprise 40%, 48% and 12% of TJ-21 respectively; therefore, they were each given in a dose of 0.8 g/kg, 0.96 g/kg and 0.24 g/kg per day (Table 1).

Agent administered	Dose (g/kg per day)	Individual STs (days)	MST (days)
Hoelen	2	6, 16, 16, 18, 18, 18, 20	18
Pinelliae Tuber	2	7, 8, 16, 18, 18, 19	17
Zingiberis Rhizoma	2	6, 6, 10, 12, 14	10
Hoelen	0.8	7, 7, 9, 25, 42	9
Pinelliae Tuber	0.96	6, 7, 14, 17, 27	14
Zingiberis Rhizoma	0.24	11, 11, 24, 30, 62	14

Table 1: Allograft survival times (ST) in mice given oral administration of components of Shohangekabukuryo-to (TJ-21). MST: Median survival time.

Moreover, we made 3 mixtures in which 1 component of TJ-21 was omitted to investigate whether all 3 components of TJ-21 were essential

in the induction of prolonged survival of cardiac allografts (a different component from each mixture; Table 2).

Oral administration (2 g/kg per day)	No.	Individual STs (days)	MST (days)
TJ-21 missing Hoelen	5	10, 15, 15, 26, 50	15
TJ-21 missing Pinelliae Tuber	5	10, 14, 23, 48, 50	23
TJ-21 missing Zingiberis Rhizoma	5	9, 13, 13, 21, >100	13

Table 2: Allograft survival (ST) of administration of 1 component omitted Shohangekabukuryo-to (TJ-21). MST: Median survival time.

All the herbal medicines were dissolved in distilled water and given orally with use of a metal tube (Thomas Scientific, Swedesboro, NJ). The medicines were made as frozen dry powder gifted by Tsumura (Tokyo, Japan).

with functioning allografts were adoptively transferred into naive CBA mice (secondary recipients) through the penile vein. After the adoptive transfer, the secondary recipients underwent transplantation of a B6 heart, immediately.

Adoptive transfer studies

Adoptive transfer studies were conducted to determine whether regulatory cells were generated after treatment with TJ-21. Accordingly, 30 days after CBA recipients (primary recipients) underwent transplantation of a B6 cardiac allograft and treatment with TJ-21 (2 g/kg/day), splenocytes (5.0×10^7) from primary recipients

Histologic studies of harvested grafts

Cardiac grafts transplanted into untreated mice and mice given TJ-21 were removed 30 days after transplantation and studied immunohistochemically and histologically. Immunohistochemical and Histologic staining were performed as described previously [16]. Hematoxylin and eosin (HE) staining was assessed by grading with a

semiquantitative scale for the amount of mononuclear cell infiltration (0, no infiltration; 1, faint and limited infiltration; 2, moderate infiltration; 3, severe infiltration) [17,18]. In immunohistochemical (IHC) study, the number of infiltrating Foxp3⁺ cell in TJ-21-treated or untreated mice was counted. All graft heart slides were assessed blindly by unrelated one researcher.

Flow cytometry analysis

CD4, CD25, and Foxp3 expression in splenocytes was determined by flow cytometry. 30 days after cardiac allograft transplantation, splenocytes from recipients treated with TJ-21, untreated recipients, and naive 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; eBioscience, San Diego, CA), as well as their isotype controls. The stained cells were analyzed by using a FACS Canto2 system (BD Biosciences). We analyzed stained cells with a FACS Canto2 system (BD Biosciences) to determine the percentage of CD4⁺ CD25⁺ Foxp3⁺ cells in CD4⁺ cells.

Cell proliferation and cytokines

In mixed leukocyte culture (MLC) studies [19], the responder cells were splenocytes from naive CBA mice or from untreated or TJ-21-treated CBA mice that had undergone transplantation of a B6 heart 14 days earlier. The stimulator cells were B6 (allogeneic) splenocytes treated with 100 µg/mL mitomycin C (Kyowa Hakko, Osaka, Japan) for 30 minutes at 37°C. The responder cells (2.5 × 10⁶ /mL) were co-cultured with the stimulator cells (5.0 × 10⁶ /mL) in complete medium in a humidified 5% carbon dioxide atmosphere (CH-16M; Hitachi, Tokyo, Japan) at 37°C in 96-well, round-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 [20].

An ELISA was also performed to assess levels of interleukin (IL)-2, IL-4, 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 mAbs 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). Recombinant standards for IL-2, IL-4, and IFN-γ were from PeproTech (London, UK).

Statistical analysis

Cardiac allograft survival in groups of mice was compared by using Mann-Whitney U testing (Graphpad Prism; Graphpad, CA). In the cell proliferation, cytokine, and flow cytometry studies, 2 groups were compared by using unpaired Student t-test or analysis of variance (ANOVA) with Ryan method. P<0.05 was considered significant.

Results

Survival of cardiac allografts in mice treated with TJ-21 and each component

CBA mice with no treatment or oral administration of distilled water rejected B6 allografts acutely (median survival times [MSTs], 8

days, Figure 2a). CBA recipients given 2 g/kg/day of TJ-21 had prolonged cardiac allograft survival (MSTs, 77 days, P<0.01 versus untreated and distilled water-treated group, respectively, Figure 2a). 0.2 g/kg/day of TJ-21 was less effective in prolongation of grafts survival (MSTs, 13 days, P<0.05 versus distilled water-treated group, Figure 2a).

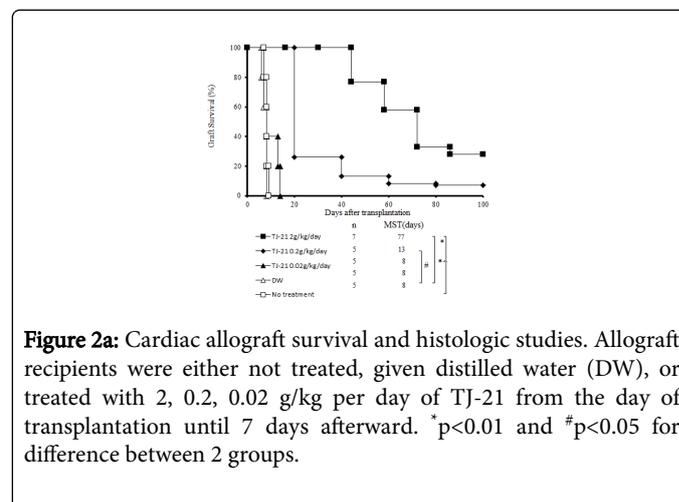


Figure 2a: Cardiac allograft survival and histologic studies. Allograft recipients were either not treated, given distilled water (DW), or treated with 2, 0.2, 0.02 g/kg per day of TJ-21 from the day of transplantation until 7 days afterward. *p<0.01 and #p<0.05 for difference between 2 groups.

The components of TJ-21 induced only modest prolongation on cardiac grafts in mice (Table 1). Moreover, when CBA mice with cardiac grafts were orally administered 1 component missing mixtures, none of the graft survival was prolonged as long as the real TJ-21 (Table 2).

Histologic features of allografts from recipients treated with TJ-21

Histologic examinations of cardiac allografts obtained 30 days after transplantation showed cell infiltrated but significantly preserved structure with a few myocardial injuries in transplant recipients given TJ-21, whereas allografts from untreated recipients showed severe myocyte damage and edema of the acute rejection process (Figure 2b). Moreover, in each section of HE staining there was a significant difference by grading with a semiquantitative scale [21].

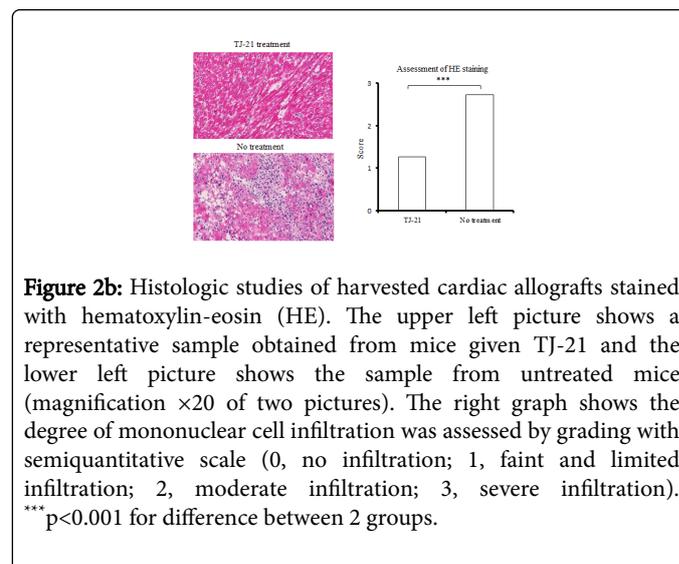


Figure 2b: Histologic studies of harvested cardiac allografts stained with hematoxylin-eosin (HE). The upper left picture shows a representative sample obtained from mice given TJ-21 and the lower left picture shows the sample from untreated mice (magnification ×20 of two pictures). The right graph shows the degree of mononuclear cell infiltration was assessed by grading with semiquantitative scale (0, no infiltration; 1, faint and limited infiltration; 2, moderate infiltration; 3, severe infiltration). ***p<0.001 for difference between 2 groups.

Generation of regulatory cells in mice treated with TJ-21

In adoptive transfer study, we found that naïve secondary CBA allograft recipients given adoptive transfer of splenocytes from TJ-21 treated CBA recipients 30 days after heart transplantation graft had significantly prolonged survival of B6 hearts (MSTs, 53 days, $P < 0.01$ versus the transfer of splenocytes from naïve CBA mice, Figure 3a).

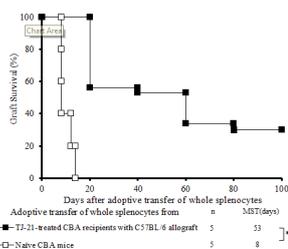


Figure 3a: Evidence of generation of regulatory cells in CBA allograft recipients treated with TJ-21. (A) Cardiac allograft survival after adoptive transfer of whole splenocytes. MST: median survival time; * $p < 0.01$ for difference between 2 groups.

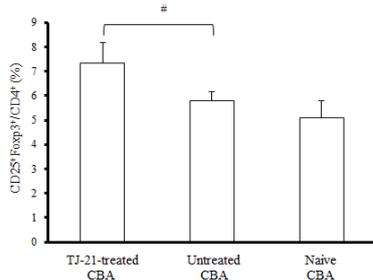


Figure 3b: CD4, CD25, and Foxp3 expression in splenocytes, as determined by flow cytometry. Data are mean \pm SD (=5 mice in each group). # $p < 0.05$ for difference between 2 groups.

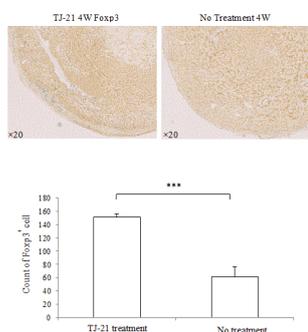


Figure 3c: Infiltration of Foxp3⁺ cells in cardiac grafts 30 days after grafting from untreated or TJ-21-treated recipients (magnification $\times 20$). The down graph shows the count of infiltrating Foxp3⁺ cells in cardiac allografts from untreated or TJ-21-treated recipients. *** $p < 0.001$ for difference between 2 groups.

In flow cytometry study, the percentage of population of CD25⁺Foxp3⁺ cells in CD4⁺ cells was increased in the spleens of recipients given TJ-21 compared with those of untreated CBA recipients ($P < 0.05$; Figure 3b). There is no significant difference in the percentage of population of CD4⁺CD25⁺Foxp3⁺ cells in CD4⁺ cells between the naïve CBA with and without TJ-21 (Figure 3b).

Additionally, IHC study showed that the number of Foxp3⁺ cells in cardiac grafts from TJ-21-treated CBA recipients obviously increased more than that in untreated recipients ($P < 0.05$; Figure 3c).

Cell proliferation and cytokine production in mice treated with TJ-21

Maximum proliferation of naïve CBA splenocytes (responder cells) against B6 splenocytes (stimulator cells) treated with mitomycin C occurred on the day of MLC. Proliferation of splenocytes from CBA recipients given TJ-21 was significantly suppressed compared with that of splenocytes from untreated mice ($P < 0.05$, Figure 4a).

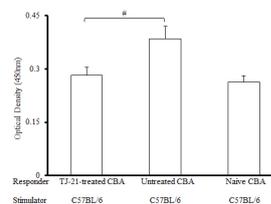


Figure 4a: Evidence of induction of alloproliferative hyporesponsiveness in CBA recipients of allograft treated with TJ-21. Results of cell-proliferation assays in mixed leukocyte cultures (MLCs). The data shown are mean \pm SD values derived from samples from 6 mice in each group. # $p < 0.05$ for difference between 2 groups.

Levels of IL-2 (Figure 4b) and IFN- γ (Figure 4c) in splenocytes from mice treated with TJ-21 were significantly lower than those in splenocytes from untreated CBA mice.

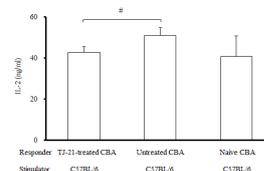


Figure 4b: Levels of cytokines in MLCs. Levels of interleukin (IL)-2 (B) in the MLCs were assessed by enzyme-linked immunosorbent assays. Data are shown as mean \pm SD values derived from samples from 6 mice in each group. # $p < 0.05$ for difference between 2 groups.

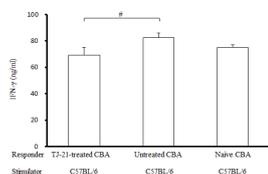


Figure 4c: Levels of cytokines in MLCs. Levels of interleukin interferon- (C), in the MLCs were assessed by enzyme-linked immunosorbent assays. Data are shown as mean \pm SD values derived from samples from 6 mice in each group. # $p < 0.05$ for difference between 2 groups.

In contrast, levels of IL-4 (Figure 4d) and IL-10 (Figure 4e) were increased in recipients treated with TJ-21 compared with untreated CBA mice.

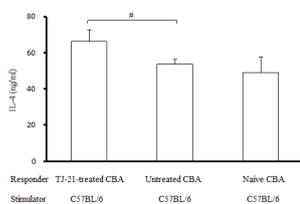


Figure 4d: Levels of cytokines in MLCs. Levels of IL-4 (D) in the MLCs were assessed by enzyme-linked immunosorbent assays. Data are shown as mean \pm SD values derived from samples from 6 mice in each group. # $p < 0.05$ for difference between 2 groups.

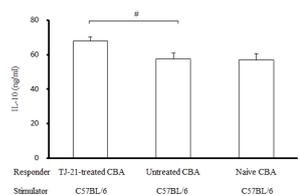


Figure 4e: Levels of cytokines in MLCs. Levels of interleukin IL-10 (E) in the MLCs were assessed by enzyme-linked immunosorbent assays. Data are shown as mean \pm SD values derived from samples from 6 mice in each group. # $p < 0.05$ for difference between 2 groups.

Discussion

This study demonstrated that TJ-21 could prolong survival of fully allogeneic cardiac grafts in mice and effect alloimmune responses in our heart transplantation model. There are several mechanisms by which treatment with TJ-21 has induced increased allograft survival in our model. The first mechanism of our research is that TJ-21 induced regulatory cells. Active suppression of regulatory cells has been found to be involved in the mechanisms for induction and maintenance of self-tolerance [22] and prevention of vasculopathy in cardiac allografts [23,24] and unresponsiveness to allografts [25]. Additionally, we have ever submitted many manuscripts about the potential of

immunomodulation and immunosuppressive effect on herbal medicines [10,26]. Our adoptive transfer study showed that administration of whole splenocytes from allograft recipients treated with TJ-21 had significantly prolonged survival of their B6 cardiac allograft (Figure 3a). In our current studies, the population of CD4⁺CD25⁺Foxp3⁺ cells in the spleens from TJ-21-treated CBA recipients obviously increased in flow cytometry (Figure 3b) and TJ-21-treated recipients clearly had more Foxp3⁺ cells than untreated recipients (Figure 3c). Moreover, allo-proliferation of splenocytes from TJ-21-treated CBA recipients was visibly suppressed in MLC (Figure 4a). These data suggest that treatment with TJ-21 generated regulatory cells, including CD4⁺CD25⁺Foxp3⁺ cells, in primary CBA recipients.

A second possible mechanism for TJ-21 prolonged survival of allogeneic cardiac grafts in mice is that the balance between Th-1 and Th-2 cytokines secretion may affect the regulatory cells' function strongly. In our previous studies, we have demonstrated that inducible regulatory T cells (Tregs) might enable the change of Th-1 and Th-2 cytokines [27]. Moreover, some studies demonstrated that administration of traditional herbal medicines could change Th-1 and Th-2 cytokines in mice [28,29]. In our current study, a distinguished increase of Th-2 cytokine (IL-4 and IL-10) and a decrease of Th-1 cytokine (IL-2 and IFN- γ) were detected in the TJ-21-treated CBA recipients (Figures 4b-4e), suggesting that administration of TJ-21 might generate regulatory cells by Th-1/Th-2 cytokine changes.

The third possible mechanism is that TJ-21 treatment resulted in suppression of the infiltration and proliferation of leukocytes. Our histologic studies of allografts obtained from TJ-21-treated recipients showed much less leukocyte infiltration and maintenance of myocardial structure than those from no treated recipients (Figure 2b). Moreover, our MLC and ELISA studies showed that TJ-21-treated allograft recipients showed down-regulation of leukocyte proliferation and the production of IL-2, IFN- γ . IFN- γ has been found to be one of key effectors in cardiac graft arteriosclerosis and participate in cell-mediated vascular injury which could cause indirect myocyte injury [30,31]. According to this mechanism, TJ-21 would have protected allografts against immune injury.

Interestingly, we found that no single component of TJ-21, used either alone or in an increased dose, prolonged allograft MSTs (Table 1) to the extent that TJ-21 itself did. Moreover, no TJ-21 formula that was missing one of the 3 components increased MSTs (Table 2) as much as TJ-21 did. Only the complete TJ-21 combination of all 3 components induced significant prolongation of allograft survival. Actually, our previous investigations about Tokishakuyaku-san (TJ-23) and Sairei-to (TJ-114) had the similar results [8,32]. These results indicate that interaction among the components was significant in prolonging allograft MSTs. A traditional assumption in herbal medicine, herbal formulas have an overall effect that is different from the effects of their components, could explain these possibilities [32].

Conclusion

From this finding, we demonstrated that treatment with TJ-21 induced generated regulatory cells and prolonged survival of fully allogeneic cardiac grafts in mice.

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