The restoration of the antitumor T cell response from stress-induced suppression using a traditional Chinese herbal medicine Hochu-ekki-to (TJ-41:Bu-Zhong-Yi-Qi-Tang)

Tieli Li [a], Koji Tamada [a,*], Koichiro Abe [a], Hitoshi Tada [a], Yasuhiro Onoe [a], Katsunori Tatsugami [a], Mamoru Harada [b], Chiharu Kubo [c], Kikuo Nomoto [a]

[a] *Department of Immunology, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582 Japan*
[b] *Department of Virology, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan*
[c] *Department of Psychosomatic Medicine, Faculty of Medicine, Kyushu University, Fukuoka, Japan*

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Abstract

We previously reported that restraint stress impairs the antitumor immune responses through its suppressive effect on the Th1-type cytokine production from CD4$^+$ T cells. In this study, we investigated a potential of Hochu-ekki-to (TJ-41:Bu-Zhong-Yi-Qi-Tang) to restore stress-induced immunosuppression. The oral administration of TJ-41 was able to improve a decreased cellularity in the lymph node and spleen and to improve an inhibition of tumor-specific Th1-type cytokine production, both of which were induced by repeated restraint stress in tumor-bearing mice. The oral administration of TJ-41 also induced a partial recovery of the antitumor cytolytic activity in the stress-burdened tumor-bearing mice. More importantly, the growth of tumors in stress-burdened preimmunized mice was obviously inhibited by TJ-41, and resulted in tumor-free state in 75% of the mice. Regarding the mechanisms by which TJ-41 restored the antitumor responses in stress-burdened mice, we found that the serum levels of corticosterone and interleukin-12 were normalized by TJ-41. In addition, the expression of CD80 and CD86, which both decreased in the stress-burdened mice, was restored to the normal level by TJ-41. Taken together, our results indicate that the oral administration of TJ-41 is able to restore the antitumor T cell responses in stress-burdened tumor-bearing mice by normalizing the serum corticosterone, interleukin-12 and the expression of costimulatory molecules. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Hochu-ekki-to; Stress; Antitumor immunity

1. Introduction

The communication circuit between the immune and neuroendocrine system has been reported to be mediated through ligands and receptors which are shared in both systems [1]. Various types of stress

can activate the sympathetic nerve system and the hypothalamic–pituitary–adrenal axis, leading to the release of catecholamines from sympathetic nerve terminals and the adrenal medulla, while glucocorticoids are released from the adrenal cortex. The elevation of endogenous glucocorticoid due to stress responses has been reported to interfere with immune cell distribution [8], migration [27,29], the activation of cytotoxic T lymphocytes (CTLs) [10] and cytokine production [9,11]. Antitumor immune responses are also inhibited in stressed animals [13,33] probably through such stress-induced immunosuppressive effects. We also recently revealed that restraint stress significantly impairs the antitumor T cell responses through the dysfunction of Th1-type CD4 T cells [25].

Numerous reports have been published describing the biological actions of traditional Chinese herbal medicine on immune responses. Traditional Chinese medicines have been shown to induce the activation of peritoneal macrophages [23] and to enhance the protection against bacterial infections [21,26]. In addition, traditional Chinese medicine has been reported to augment the antitumor immune responses both in vivo and in vitro [14,16]. Although the precise mechanism of these effects still remains unclear, several reports have demonstrated their potential to enhance NK activity [4,18] and IFN-γ production [20]. We also reported that the oral administration of traditional Chinese medicine, Hochu-ekki-to (TJ-41:Bu-Zhong-Yi-Qi-Tang) enhances concomitant immunity against tumor development through an augmentation of the cytostatic activity [15]. These lines of evidence encourage us to examine whether stress-induced suppression in antitumor immune responses can possibly be restored by the administration of traditional Chinese herbal medicines.

In the present study, we demonstrated that the oral administration of TJ-41 restored antitumor immune responses, which were suppressed under the stressed condition, through normalization of serum corticosterone, IL-12 and expression of costimulatory molecules. To our knowledge, this is the first study to examine a potential importance of Chinese herbal medicine in antitumor immune responses under stressed conditions. The further implications of these findings are discussed.

2. Materials and methods

2.1. Mice

Female C57BL/6 (B6) mice were obtained from Japan SLC (Hamamatsu, Shizuoka, Japan) and were used at 8–10 weeks of age. All mice were kept in specific pathogen-free conditions. Care and use of the mice were in accordance with protocols approved by the Kyushu University Faculty of Medicine Laboratory of Animal Experiments.

2.2. Hochu-ekki-to (TJ41)

Hochu-ekki-to, prepared as spray-dried powder of hot-water extract obtained from 10 medical plants in the following ratios: Astragalus Root (4.0), Atractylodes Lancea Rhizome (4.0), Ginseng Root (4.0), Japanese Angelica Root (3.0), Bupleurum Root (2.0), Jujube Fruit (2.0), Citrus Unshiu Peel (2.0), Glycyrrhiza Root (1.5), Cimicifuga Rhizome (1.0) and Ginger Rhizome (0.5), and was kindly provided by Tsumura, Tokyo, Japan. The medicine was suspended in water then boiled 10 min before use. TJ-41 was orally administered by feeding through a water bottle for drinking at a dose of 1000 mg kg^{-1} day^{-1}.

2.3. Stress protocol

The mice were physically restrained in well-ventilated 50 ml polypropylene centrifuge tubes (Corning 25331-50, Corning, NY) every other day, as previously reported [25]. Each restraint stress period lasted 15 h, from 6:00 pm until 9:00 am the next day. The mice were deprived of food and water during each restraint stress period, but were given free access to food and water following each stress period. The stress-free mice were also deprived of food and water during the same period and were used as controls.

2.4. Tumors

B16 is a melanoma line while EL4 is a T cell lymphoma line and both tumor cell lines are of B6 origin. These tumor cell lines were maintained in a complete culture medium. RPMI 1640 (Gibco, Grand

Island, NY) supplemented with 10% heat-inactivated FCS (HyClone, Logan, UT), 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 100 µg/ml gentamycin, 10 mM HEPES and 0.2% sodium bicarbonate was used as the complete culture medium.

2.5. Proliferation assay

B6 mice were burdened with repeated restraint stress from day 0 to day 11 every other day (6 times). Four hours after the 3rd inducement of stress (day 5), the mice were injected s.c. with 1×10^6 B16 melanoma cells into the right flank. After 12 days of tumor inoculation (on day 17), the spleen cells (1×10^6 cell/ml) and the lymph node (LN) cells (5×10^5 cell/ml) were prepared and cultured with mitomycin C (MMC)-treated B16 melanoma cells (2×10^4 cells/ml and 1×10^4 cell/ml, respectively) for 72 h using a 96-well culture plate (Corning) at a volume of 200 µl per well. During the last 8 h of the culture, 37 kBq of [^{3}H]deoxythymidine (dThd) was added to each well. The cultured cells were then harvested and the incorporation of [^{3}H]dThd was counted using the Beta Plate system (Pharmacia LKB Biotechnology, Uppsala, Sweden). MMC treatment of tumor cells was performed using a culture with 100 µg/ml MMC (Kyowa Hakko Kogyo, Tokyo, Japan) at 37°C for 90 min. In some groups, TJ-41 was fed by a water-supplying bottle at 1000 mg kg^{-1} day^{-1} throughout the experiments.

2.6. Cytokine assay

The spleen cells and LN cells from the mice burdened with stress and inoculated with B16 melanoma cells were restimulated with MMC-treated B16 melanoma cells in vitro, under the same conditions as those used in the proliferation assay. After 48 h, the supernatants of the culture were harvested and assayed for the concentration of IFN-γ and IL-2 by ELISA, as previously reported [25]. In some groups, TJ-41 was fed by a water-supplying bottle at 1000 mg kg^{-1} day^{-1} throughout the experiments. For the measurement of serum IL-12, the mice were burdened with stress from day 0 to day 11, inocu-

lated with B16 melanoma cells on day 5 and also provided with TJ-41, as described above. After 12 days of tumor inoculation, the serum concentration of IL-12 was assessed by using a IL-12 p40 ELISA kit (BioSource International, Camarillo, CA).

2.7. CTL assay

B6 mice were burdened with repeated restraint stress from day 0 to day 11 every other day. Four hours after the 3rd inducement of stress, the mice were injected i.p. with 1×10^6 MMC-treated B16 melanoma cells. After 14 days of tumor immunization, the spleen cells (5×10^6 cell/ml) were prepared and cultured with MMC-treated B16 melanoma cells (1×10^5 cells/ml) for 96 h, and then their cytolytic activity was assayed against B16 melanoma and EL4. In some groups, TJ-41 was fed by a water-supplying bottle at 1000 mg kg^{-1} day^{-1} throughout the experiments. The cytolytic activity was measured by a standard 4 h ^{51}Cr-release assay. A graded number of effecter cells were incubated with 1×10^4 cells of ^{51}Cr-labeled target cells in 200 µl of the complete medium for 4 h in a round-bottomed 96-well microtitre plate (Corning). After incubation, 100 µl of the supernatant were harvested. The radioactivity of the supernatant was measured by a gamma-counter (Shimazu, Kyoto, Japan) and the specific release was calculated according to the following formula:

Specific ^{51}Cr release (%)

$$= \frac{\text{test release (cpm)} - \text{spontaneous release (cpm)}}{\text{total release (cpm)} - \text{spontaneous release (cpm)}} \times 100$$

Spontaneous release was determined by the supernatant of the sample that was incubated with no effecter cells, and the total release was determined by the supernatant of the sample that was incubated with 10% Triton X (Wako, Osaka, Japan).

2.8. In vivo tumor growth

To examine the tumor growth in vivo, we utilized two different systems, consisting of a primary and secondary tumor growth assay. In the primary tumor

growth assay, B6 mice, which were burdened with repeated restraint stress from day 0 to day 11 every other day were injected s.c. with 1×10^6 B16 melanoma cells at 4 h after the 3rd inducement of stress. In the secondary tumor growth assay, B6 mice which were burdened with repeated restraint stress from day 0 to day 11 every other day were injected i.p. with 1×10^6 MMC-treated B16 melanoma cells at 4 h after the 3rd inducement of stress. After 14 days of tumor immunization, the mice were inoculated s.c. with either 2×10^5 cells of B16 melanoma cells or EL4 lymphoma cells into the right flank. In both assays, TJ-41 was fed by a water-supplying bottle at 1000 mg kg^{-1} day^{-1} in some groups. The growth of the tumor was monitored every 3 or 4 days after tumor inoculation and the product of the two tumor diameters based on measurements with a caliper was thus used to determine the tumor size (mm^2).

2.9. Measurement of corticosterone

The serum level of corticosterone was determined using a radioimmunoassay kit, which was purchased from the Diagnostic Product, Los Angeles, CA.

2.10. Flow cytometric analysis

For the flow cytometry analysis, the stained cells were analyzed by a FACS Calibur flow cytometer with Cellquest software (Becton Dickinson, Mountain View, CA). The supernatants of anti-Fc receptor II/III mAb-producing hybridoma (2.4G2: rat IgG2b) were used to block the non-specific binding. The mAbs used in the flow cytometric analysis were as follows: FITC-conjugated anti-I-A^b mAb (Meiji Institute of Health Science, Tokyo, Japan), biotin-conjugated anti-CD80 mAb (PharMingen, San Diego, CA) and biotin-conjugated anti-CD86 mAb (PharMingen). For the staining of biotin-conjugated mAbs, they were followed by phycoerythrin-conjugated streptavidin (Gibco BRL, Gaithersburg, MD).

2.11. Statistics

The Statistical significance of the data was determined by Student's *t*-test. A *P* value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Restoration of LN and splenic cellularity by TJ-41 in the stress-burdened tumor-bearing mice through the stimulation of proliferative activity

To analyze the effect of TJ-41 on the antitumor immune responses in stress-burdened mice, we first examined the changes of cellularity in the lymphoid organs after TJ-41 administration. Table 1 shows that the oral administration of TJ-41 restored cell numbers of both tumor-draining LN and spleen which were reduced by repeated restraint stress in the tumor-bearing mice. To analyze the mechanism for such effect of TJ-41, we next examined the proliferative activity of LN and splenic lymphocytes in response to stimulation with tumor cells. Although the tumor-reactive proliferation of LN and spleen cells was not suppressed in the stressed mice, such proliferative activity was augmented by the administration of TJ-41 in the mice under stressed conditions but not under stress-free conditions (Fig. 1). These results suggest that the administration of TJ41 allowed the cellularity of the LN and spleen to recover in stress-burdened tumor-bearing mice by stimulating the proliferative activity of lymphocytes.

Table 1

Recovery of LN and spleen cellularity after TJ-41 treatment in stress-burdened tumor-bearing mice

Group	Tumor[a]	Stress[b]	TJ-41[c]	Cell number per mouse[d]	
				LN ($\times 10^6$)	Spleen ($\times 10^7$)
1	+	−	−	3.4 ± 0.2	10.0 ± 0.8
2	+	−	+	3.3 ± 0.4	12.4 ± 0.1
3	+	+	−	1.9 ± 0.5	6.7 ± 0.1
4	+	+	+	$3.2 \pm 0.1^*$	$9.1 \pm 0.4^*$

[a] B6 mice were injected s.c. with 1×10^6 B16 melanoma cells into the right flank 4 h after the third inducement of restraint stress.
[b] B6 mice were burdened with repeated restraint stress every other day for a total of 6 times.
[c] TJ-41 was orally administered by feeding with a water-supplying bottle at a dose of 1000 mg kg^{-1} day^{-1} throughout the experiments.
[d] Twelve days after the inoculation of tumor cells, the cell number of the spleen and tumor-draining LN were analyzed. The values indicate the means $\pm$ S.D. of six mice and the findings are representative of three independent experiments.
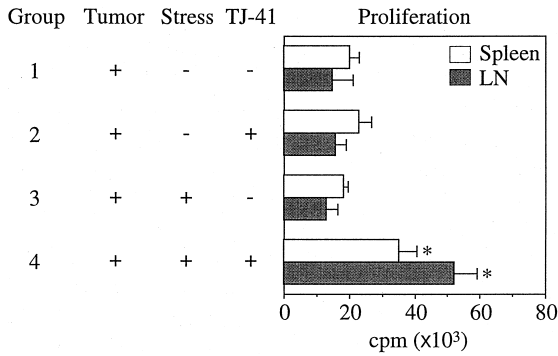$^*P < 0.05$ compared with group 3.

Fig. 1. The increased proliferation of spleen cells and tumor-draining LN cells after the oral administration of TJ-41 in stress-burdened mice. B6 mice were burdened with or without repeated restraint stress from day 0 to day 11 every other day. Four hours after the 3rd inducement of stress, the mice were injected s.c. with 1×10^6 B16 melanoma cells into the right flank. After 12 days of tumor inoculation, the spleen cells and the LN cells were prepared and cultured with MMC-treated B16 melanoma cells for 72 h. During the last 8 h of culture, 37 kBq of [^{3}H]deoxythymidine (dThd) was added to each well. In some groups, TJ-41 was fed by a water-supplying bottle at 1000 mg kg^{-1} day^{-1}. The values are the mean $\pm$ S.D. of triplicate wells, and the findings are representative of three separate experiments. $^*P < 0.05$ compared with the TJ-41-untreated group.

3.2. Restoration of tumor-reactive Th1-type cytokine production by TJ-41 in the stress-burdened tumor-bearing mice

We recently reported that the stressed condition impairs the antitumor T cell responses mainly through the suppression of Th1-type cytokine production from CD4$^+$ T cells [25]. Since our group also reported that the oral administration of TJ-41 can augment the concomitant immunity against tumors [15], we next examined the effect of TJ-41 on cytokine production under stressed conditions. As shown in Fig. 2, the tumor-reactive production of IFN-γ and IL-2, which was suppressed in stress-burdened mice, was restored after the oral administration of TJ-41 in both spleen and LN cells. The TJ-41 administration also showed a potential to increase the cytokine production even in stress-free mice. We also examined the IL-4 production in these groups, but the IL-4 level was extremely low in all groups (data not shown).

3.3. Partial restoration of tumor-specific CTL activity by TJ-41 in the stress-burdened tumor-immunized mice

In our previous report, the stress-induced inability to generate antitumor CTLs from the tumor-immunized mice depends on their suppressed production of Th1-type cytokines [25]. We therefore next examined the effect of TJ-41 on the generation of antitumor CTLs from the mice under stressed conditions. The tumor-specific CTL activity against B16 melanoma induced from the immunized spleen cells was significantly suppressed under stressed conditions in comparison to stress-free conditions (Fig. 3). However, the oral administration of TJ-41 partially
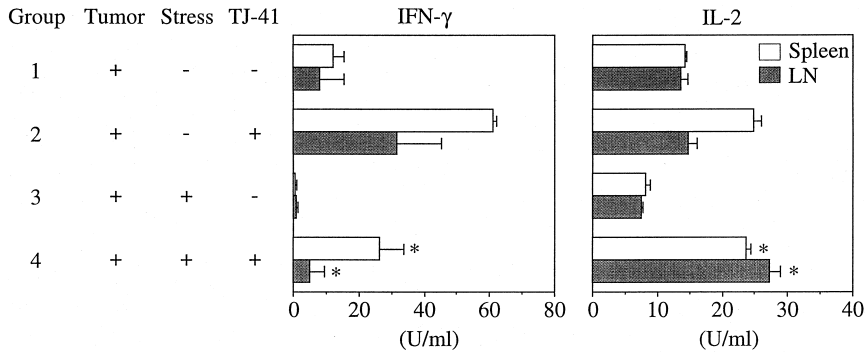

Fig. 2. Restoration of the cytokine production from the spleen cells and tumor-draining LN cells after the oral administration of TJ-41 in stress-burdened mice. The spleen cells and LN cells from the mice, which were burdened with or without stress and inoculated with B16 melanoma cells, were restimulated with MMC-treated B16 melanoma cells in vitro, as the same condition of Fig. 1. After 48 h, the supernatants of the culture were harvested and assayed to determine the concentration of IFN-g and IL-2 by ELISA. In some groups, TJ-41 was fed by a water-supplying bottle at 1000 mg kg^{-1} day^{-1}. The values are the mean $\pm$ S.D. of triplicate wells, and the findings are representative of three separate experiments. $^*P < 0.05$ compared with the stress-burdened TJ-41-untreated group.
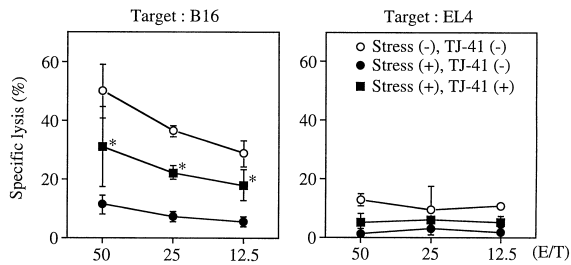
Fig. 3. Partial restoration of the CTL activity after the oral administration of TJ-41 in stress-burdened mice. B6 mice were burdened with or without repeated restraint stress from day 0 to day 11 every other day. Four hours after the 3rd inducement of stress, the mice were injected i.p. with 1×10^6 MMC-treated B16 melanoma cells. After 14 days of tumor immunization, the spleen cells were prepared and cultured with MMC-treated B16 melanoma cells for 96 h, and then their cytolytic activity against B16 melanoma and EL4 was determined by a 4 h ^{51}Cr-release assay. In some groups, TJ-41 was fed by a water-supplying bottle at 1000 mg kg^{-1} day^{-1}. The values are the mean $\pm$ SD of triplicate wells, and the findings are representative of three separate experiments. *$P < 0.05$ compared with the stress-burdened TJ-41-untreated group.

but significantly allowed the tumor-specific CTL activity to recover in such stress-burdened tumor-immunized mice. The CTL activity against EL4, which might represent the non-specific cytolytic activity of the cultured spleen cells, showed a similar tendency, but no significant difference was observed. We also examined the effect of TJ-41 on the NK activity of spleen cells from the stress-burdened mice, but no difference was observed in the mice either with or without poly (I:C) injection (data not shown). These results thus suggest that the oral administration of TJ-41 can restore the tumor-specific CTL activity generated from the stress-burdened tumor-immunized mice.

3.4. Enhancement of protective immunity against tumor by TJ-41 in the stress-burdened mice

We further determined the effect of TJ-41 on the growth of tumors in stressed mice using two different systems, consisting of a primary and secondary tumor growth assay (see Section 2.8). In the previous study, we have reported that our stress protocol significantly promotes the tumor growth in the secondary growth assay, but not in the primary growth

assay, due to the impaired CTL activity [25]. The oral administration of TJ-41 showed no significant effect for the primary tumor growth in the mice under either stressed conditions or stress-free conditions (Fig. 4A). On the other hand, the secondary growth of B16 melanoma was significantly suppressed by the administration of TJ-41 in the stress-burdened, B16 melanoma-immunized mice (Fig. 4B).
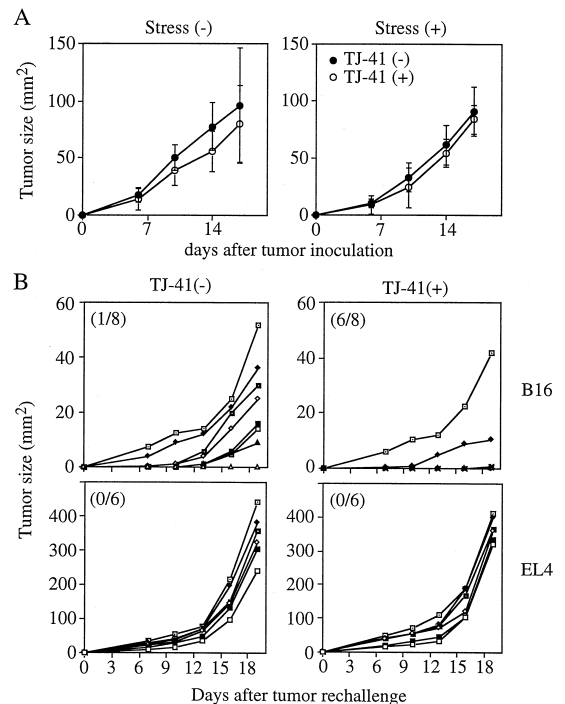

Fig. 4. Enhancement of protective immunity against B16 melanoma after the oral administration of TJ-41 in stress-burdened mice. (A) B6 mice were burdened with or without repeated restraint stress from day 0 to day 11 every other day. Four hours after the 3rd inducement of stress, the mice were injected s.c. with 1×10^6 B16 melanoma cells into the right flank. (B) B6 mice were burdened with repeated restraint stress from day 0 to day 11 every other day. The mice were injected i.p. with 1×10^6 MMC-treated B16 melanoma cells at 4 h after the 3rd inducement of stress. After 14 days of tumor immunization, the mice were inoculated s.c. with either 2×10^5 cells of B16 melanoma cells or EL4 lymphoma cells into the right flank. In some groups, TJ-41 was fed by a water-supplying bottle at 1000 mg kg^{-1} day^{-1}, and the growth of tumor was monitored every 3 or 4 days after tumor inoculation. The numbers in parentheses in (B) indicate the ratio of tumor-free mice/total mice at 30 days after tumor inoculation. The results are shown as means $\pm$ S.D. in (A) and as an individual size of each mice in (B), and the findings are representative of two separate experiments.

There was no significant effect of TJ-41 against the growth of the control tumor, EL4. We further monitored the rate of tumor-rejected mice in the secondary tumor growth assay, and found that the administration of TJ-41 increases the percentage of tumor rejection from 12% to 75% at 30 days after tumor inoculation (Fig. 4B). These results suggest that TJ-41 has the potential to enhance the protective immunity against tumors in stress-burdened mice.

3.5. Normalization of the serum level of corticosterone and IL-12 by TJ-41 in the stress-burdened tumor-bearing mice

Up to now, we demonstrated that the oral administration of TJ-41 restored the antitumor immune responses both in vitro and in vivo, which were suppressed in the stress-burdened mice. In order to investigate mechanisms of TJ-41, we first examined the influence of TJ-41 on the serum level of corticosterone, because the increase of glucocorticoid secretion under the stressed conditions is known to induce variable kinds of immunosuppression in the tumor-bearing host [31]. As shown in Fig. 5A, our stress protocol induced the elevation of serum corticosterone, consistently with our previous report [25]. Interestingly, the oral administration of TJ-41 significantly decreased the serum corticosterone level in the stress-burdened mice, although no remarkable change was observed in the mice with stress-free condition. We further examined the kinetics of serum corticosterone and the relationship to the restoration
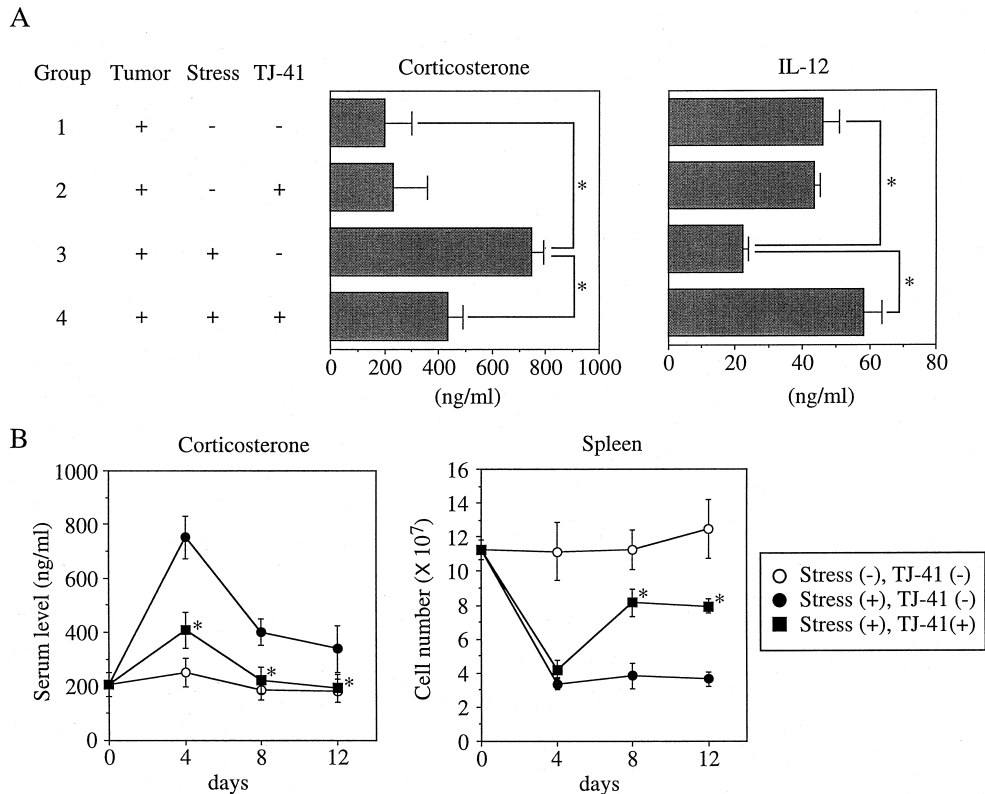

Fig. 5. Normalization of serum corticosterone and IL-12 by TJ-41 in the stress-burdened mice. B6 mice were burdened with or without repeated restraint stress from day 0 to day 11 every other day, and TJ-41 was fed at 1000 mg kg^{-1} day^{-1} in some groups. In (A), 4 h after the 3rd inducement of stress, the mice were injected s.c. with 1×10^6 B16 melanoma cells into the right flank. After 12 days of tumor inoculation, the serum was collected and the levels of corticosterone and IL-12 were determined by radioimmunoassay and ELISA, respectively. In (B), the serum level of corticosterone and the cell number of spleen were assessed on day 0, 4, 8 and 12. The values are means ± S.D. of at least five mice. *$P < 0.05$ between compared two groups in (A) and between closed circle and closed square in (B).

of cellularity in lymphoid organs by TJ-41. The serum corticosterone increased as early as on day 4 after the beginning of the stress and maintained the significantly high level at least up to on day 12 (Fig. 5B). The TJ-41 treatment in the stress-burdened mice, however, was able to decrease serum corticosterone on day 4 and normalize on day 8 and 12. Consistently with such kinetics of corticosterone, the splenic cellularity in the TJ-41-treated stress-burdened mice was significantly recovered on day 8 and 12 in comparison with TJ-41-untreated stress-burdened mice (Fig. 5B).

We next examined the serum level of IL-12, which is known as a potent inducer of Th1-type

cytokines [35], in the mice under the stressed condition with or without TJ-41 treatment. As shown in Fig. 5A, the serum IL-12, which significantly decreased by the stressed condition, was restored to the normal level by the treatment with TJ-41. Taken together, these results suggest that TJ-41 allowed the antitumor immune responses to restore by normalizing the serum level of corticosterone and IL-12 under the stressed conditions.

3.6. Recovered expression of costimulatory molecules by TJ-41 in the stress-burdened mice

Since glucocorticoids have been reported to induce an immunosuppressive state through a down-regulation of the expression level of costimulatory molecules on dendritic cells [28], we further examined the effect of TJ-41 on the expression of CD80 and CD86. As shown in Fig. 6, the expression levels of both CD80 and CD86 on MHC class II-positive cells decreased in the spleen of the stress-burdened mice. However, the oral administration of TJ-41 restored the reduced expression of CD80 and CD86 to the level of the mice with a stress-free condition. These results suggest that the oral administration of TJ-41 restored the antitumor immune responses through the normalization of the costimulatory function of antigen presenting cells (APC).

4. Discussion

Cancer patients are supposed to be associated with various types of stress, including not only physical stress but also psychological stress. Since the stressed condition has been reported to induce immunosuppression in antitumor responses, the restoration of such an immunosuppressive state is considered important. In spite of several reports which demonstrate the potential of traditional Chinese herbal medicine to enhance antitumor activity, there have so far been no investigations elucidating the effect of traditional Chinese herbal medicine on stress-induced immunosuppression in a tumor-bearing state. In this study, we found that the oral administration of TJ-41 restored the cellularity of lymphoid organs, cytokine production and CTL activity against tumor cells, which had been inhibited in stress-burdened tumor-bearing mice. In addition,
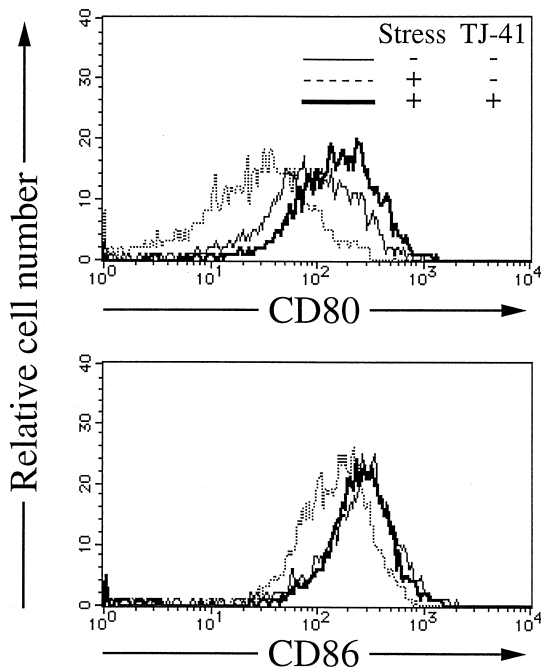

Fig. 6. Restoration of CD80 and CD86 expression by TJ-41 in the stress-burdened mice. The B6 mice were burdened with or without repeated restraint stress from day 0 to day 11 every other day. Four hours after the 3rd inducement of stress, the mice were injected i.p. with 1×10^6 MMC-treated B16 melanoma cells, and after 14 days of tumor immunization, the spleen cells were prepared and cultured with MMC-treated B16 melanoma cells for 48 h. The cells were then harvested and analyzed for the expression level of CD80 and CD86 by a flow cytometer under the gate of I-A^b-positive cells. In some mice, TJ-41 was fed by a water-supplying bottle at 1000 mg kg^{-1} day^{-1}. Similar results were obtained in five mice of each group, and the representative data of these mice are shown.

we revealed the effects of TJ-41 to normalize the level of serum corticosterone, IL-12 and the expression of costimulatory molecules on APC. We consider these results to therefore support the usefulness of TJ-41 in the treatment of cancer in combination with other immunotherapies.

Our stress protocol induced a decreased cellularity in tumor-draining LN and the spleen in tumor-bearing mice (Table 1), which is consistent with our previous findings [25]. Since the proliferative activity of lymphocytes was not suppressed by the stressed condition (Fig. 1), the reason for the decrease in cellularity is thus considered to be due to some other mechanisms such as apoptosis or change of cell distribution. Indeed, endogenous glucocorticoids induced by the stressed conditions can mediate apoptosis in peripheral lymphocytes as well as thymocytes [5]. In this study, we suggest that the oral administration of TJ-41 is able to restore the cellularity of lymphoid organs through following possible mechanisms. First, TJ-41 stimulated the proliferative activity of lymphocytes under the stressed condition (Fig. 1). Second, the endogenous corticosterone in serum was returned to the normal level by TJ-41 correspondingly with the splenic cellularity (Fig. 5B). Third, TJ-41 recovered the surface expression of costimulatory molecules (Fig. 6) which presumably inhibit apoptosis in lymphoid organs since the costimulatory signal is known to increase such anti-apoptotic proteins as Bcl-XL [2]. Our findings thus suggest multiple pathways of TJ-41 to restore the lymphoid cellularity although further investigations would be needed to explore the precise mechanisms.

Regarding tumor-reactive cytokine production, TJ-41 showed a potential to augment IFN-γ and IL-2 production in both tumor-draining LN cells and spleen cells (Fig. 2). TJ-41 exhibited such an enhancement of Th1-type cytokines even in stress-free mice, thus suggesting its ability to preferentially induce Th1-type responses regardless of the stress. Indeed, TJ-41 has been reported to enhance the protection against infection with *Listeria monocytogenes* which is controllable under Th1-type conditions [26]. Moreover, we demonstrated that TJ-41 is capable of recovering serum IL-12 to the normal level under the stressed condition (Fig. 5A), while IL-12 is so far known as the most important factor to initiate Th1-type cytokines [35]. On the other hand,

effects of TJ-41 on Th2-type cytokines was not apparent in this study because our protocol, utilizing the immunization with inactivated tumor cells, is predominantly related to the Th1-type response. However, our group previously reported the administration of TJ-41 to suppress IL-4 production and the serum IgE level in mice immunized by antigens mixed with aluminum hydroxide [19]. Taken together, TJ-41 appears to preferentially shift the Th1/Th2 balance to the Th1-type responses. In addition, the effect of TJ-41 to decrease the level of serum corticosterone (Fig. 5A) is thus also considered to contribute to the regulation of the Th1/Th2 balance, since glucocorticoids are reported to induce Th2-type cytokines [6,7,34].

We demonstrated that the administration of TJ-41 can partially restore the suppressed antitumor CTL activity in the stress-burdened mice (Fig. 3). This restoration of CTLs would depend on the enhancement of Th1-type cytokines by TJ-41, because we previously reported that the in vitro addition of IL-2 can restore the antitumor CTLs in stress-burdened mice [25]. In addition, the normalization of serum IL-12 (Fig. 5A) and the expression of costimulatory molecules on APC (Fig. 6) also participate in CTL restoration since these factors are important for the effective induction of antitumor CTLs [3,12]. In recent studies, the interactions between CD40 on APC and CD40 ligand on T cells are reported to be important for the regulation of costimulatory molecules on APC and induction of CTLs [30,36], thus suggesting a worthwhile examination about the influence of TJ-41 on CD40 ligand. As a result of the restoration of CTL activity, the secondary tumor growth in the mice treated with TJ-41 was inhibited and surprisingly resulted in a 75% tumor rejection in the stressed mice (Fig. 4B). This effect of TJ-41 thus appears to differ from the direct suppression of tumor cells, because they showed no effect against primary tumor growth (Fig. 4A). Moreover, this result also suggest that TJ-41 can predominantly modulate adaptive immune responses by T cells and B cells rather than innate immune responses by NK cells and macrophages.

In spite of the immunomodulatory properties of TJ-41 as described above, it remains unclear which components in TJ-41 are predominantly responsible for the effects in this study. In addition, we could not

verify the immunological effects of TJ-41 on B cell, macrophages and NK cells since we focused on T cell functions in this study. By reference to previous papers, an acidic polysaccharide derived from ginseng roots generates $CD8^+$ killers through the activation of macrophages and $CD4^+$ T cells [24]. Ginseng is also reported to enhance NK activity both in human and mouse studies [22,32], although no significant increase of NK activity was observed in stress-burdened TJ-41-treated mice in this study (data not shown). In addition, glycyrrhiza root is reported to stimulate peritoneal macrophages to produce TNFa in cyclophosphamide-treated immunocompromised mice [17]. Regarding our present results, we consider two fundamental pathways by which TJ-41 modulates the immune responses in the stressed conditions. One is a direct stimulation against immune cells including T cells and APC, which is presumably mediated through receptors for biologically active components in TJ-41 such as acidic polysaccharides. The other is an indirect pathway through the regulation of neuroendocrine system, demonstrated as the normalization of corticosterone in this study. Since glucocorticoids are reported to inhibit Th1-type responses, IL-2 production and expression of costimulatory molecules [6,7,28,34], the latter pathway would be compatible to the present results.

In conclusion, we herein demonstrated that the oral administration of TJ-41 was able to restore the cellularity of lymphoid organs and the antitumor immune responses in stress-burdened mice through the normalization of the serum corticosterone, IL-12 and the expression of costimulatory molecules. TJ-41 is thus considered to be a beneficial medicine for restoring the immunological status in patients suffering from cancer with physical and psychological stress.

Acknowledgements

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