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A novel chronic stress-induced shift in the Th1 to Th2 response promotes colon cancer growth

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ABSTRACT

Epidemiological data have shown that stress and other psychological factors might influence cancer onset and progression. However, to date, the mechanisms are not well understood. In the present study, we used chronic exposure to a scream as a novel form of sound stress to explore the influence of the chronic stress burden on colon cancer progression, and changes in the immune system were observed. Chronic exposure to scream sound stress induced freezing behavior in the mice and decreased the bodyweight gain. It also caused changes in the adrenal gland and increased serum corticosterone and norepinephrine levels. Cytokine microarray analysis showed changes in the levels of Th1 and Th2 cytokines. The chronic scream sound stress caused a shift from the Th1 to the Th2 response both in the circulation and in tumor-infiltrated lymphocytes, and it promoted colon cancer progression significantly. Taken together, chronic scream sound stress can be conveniently used as a novel chronic stress model. Chronic stress contributes to colon cancer progression and induces a Th1/Th2 imbalance in the mouse immune system, which is considered critical during cancer progression.

1. Introduction

Colorectal cancer (CRC) is the third most diagnosed cancer in males and the second in females worldwide, with over 1.2 million new cancer cases and 608,700 deaths in 2008. Recently, the incidence of CRC has rapidly increased in several areas that have historically had a low risk, including China. Smoking, physical inactivity, overweight and obesity, red and processed meat consumption, and excessive alcohol consumption are risk factors for CRC [1]. Moreover, cancer patients are a stressed population [2]. Epidemiological studies indicate that stress, chronic depression, and other psychological factors might influence cancer onset and progression [3–5]. However, to date, the underlying biological mechanisms of stress and cancer have not been well understood, and their clinical significance for human disease remains controversial [6].

Tumor pathology is related to multiple factors such as hereditary, immunologic, psychological, and environmental factors. The immune system recognizes and destroys malignant cells, and it is understood to play a critical role in tumor initiation and progression through the process of cancer immunoediting, composed of the elimination, equilibrium, and escape phases [7]. Innate and adaptive immune cells, such as $CD8^+$ T-lymphocytes, $CD4^+$ T-lymphocytes, NK cells, and macrophages, and cytokines secreted from immune cells, such as interferon gamma (IFN-γ) and interleukin-12 (IL-12), have been confirmed to be involved in eliminating, sculpting, and shaping human carcinoma [7,8]. Moreover, clinical data indicate that a $CD4^+$ T-helper 1(Th1)/Th2 cytokine imbalance is observed in patients with cancer with an elevation of Th2 cytokines [9]. The local activation and regulation of immune cells (tumor-infiltrated lymphocytes, TIL) is also an important function of the immune system during carcinogenesis [10].

Animal models are pivotal for understanding the pathophysiology of stress-induced behavioral alterations and pathological changes. Various acute and chronic stress models have been developed and used in psychoneuroimmunological research on many diseases, including the forced swim test, the tail suspension test, immersion in cold water, electric foot shock, restraint, social isolation, white noise, food deprivation, sleep deprivation, early-life stress, and chronic paradigms such as chronic unpredictable mild stress [11]. Among these models, the chronic stress models are considered more naturalistic and are suggested to more closely resemble the human situation [12]. In this study, we used a novel

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chronic scream sound stress [13] that we created to explore the influence of a chronic stress burden on colon cancer progression in a cancer-loaded mouse model. The role of the stress-induced Th1/Th2 cytokine imbalance in cancer progression was also observed.

2. Materials and methods

2.1. Animals

Male Balb/c mice aged between 4 and 6 weeks, weighing 20–25 g were obtained from the animal experiment center of The Fourth Military Medical University. The mice were housed six per cage in an aseptic room kept at constant temperature (23 ± 2 °C) and humidity (65%), and maintained a 12 h light/dark cycle (light was on from 06:00 h to 18:00 h). Sterilized food and water were available ad libitum. The animal experimental protocols were approved specifically by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University (Permission No. 2011-0110). Every effort was made to minimize the number of animals used and their suffering.

2.2. Chronic scream sound stress procedure

After 7-days acclimatization, mice were randomly divided into the chronic scream sound stress group and the control group with 8 mice in each group. The chronic scream sound stress was produced by exposing the mice to a scream sound (frequencies from 0.5 kHz to 4 kHz) for 6 h daily for 2 weeks, with the starting time of the exposure changed everyday to prevent adaptation. The scream sound was broadcasted through a loudspeaker (Panda CD-100) located at 50 cm above the animal cages, and the intensity was 45–75 dB measured by a sound level meter (Landtek SL-5800). The scream sound was recorded in advance by the following steps: Balb/c mice were subjected to an electric foot shock (1-mA scramble shock, variable-interval schedule, mean intershock interval 30 s and shock duration 30 s) for 30 min in a chamber with a grid floor [14]. The screaming sound the mice produced was simultaneously recorded on CD-ROM in a professional recording room (Xi'an Yin Zhi Xuan). Digital audio production systems, including the Pro Tools HD audio workstation, a Yamaha digital mixer, Genelec monitors, a Neumann U87 AI condenser microphone, and NEVE1 were used to record the scream sound. The mice in the control group were exposed only to the background noise of the animal room. The level of the background noise produced by the ventilation system inside the room and the eating or fighting activities of the mice was 40–45 dB.

2.3. Effect of chronic scream sound stress on colon cancer progression

Colon 26 cells derived from mouse colon cancer was purchased from the Type Culture Collection of the Chinese Academy of Sciences and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2/ 95% air with medium changes every 2 d. The cells in a log phase were used in this study.

Mice were randomly divided into four groups (8 mice each): control group (C), stress group (S), tumor group (T), and tumor-stress group (T-S). After acclimatization, the mice in the S and T-S groups were exposed to the chronic scream sound stress for 2 weeks, while the mice in the C and T groups were exposed to the control conditions. After that, 1×10^5 colon 26 cells (0.1 ml single-cell suspensions) were injected subcutaneously into the right oxters of the mice of the T and T-S groups. As the control, 0.1 ml of PBS was injected in the mice in the C and S groups. The chronic scream sound stress was further applied to the mice in the S and T-S groups for the next 3 weeks. The tumors were measured with calipers, and the tumor size was calculated using the formula: $V = 1/2\ ab^2$, where a represents the largest tumor diameter and b the smallest tumor diameter. On day 25, the mice were sacrificed by cervical dislocation and tumors were harvested and weighed. A portion of the xenografts from the four groups were trimmed and fixed for further analysis or stored at −80 °C for further real-time PCR analysis.

2.4. Assessment of serum norepinephrine and corticosterone levels

The mice were sacrificed by decapitation, and the blood was collected immediately after the stress exposure. The serum was obtained by centrifuge and stored at −80 °C. The concentrations of norepinephrine and corticosterone in the mice serum were measured using a radioimmunoassay according to the manufacturer's protocols.

2.5. Assessment of serum cytokines

RayBio Mouse Cytokine Antibody Arrays (QAM-TH17–1, RayBio) were employed to assay the serum. Eighteen different cytokines were evaluated: interleukin 1 beta (IL-1β), IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17, IL-17F, IL-21, IL-22, IL-23, IL-28, interferon gamma (IFN-γ), macrophage inflammatory protein 3 alpha (MIP-3α), transforming growth factor beta 1 (TGF β1), and tumor necrosis factor alpha (TNF-α). The experiment was repeated in quadruplicate. The levels of IL-4 and IFN-γ were further quantified using traditional ELISAs according to the manufacturer's protocols (R&D).

2.6. Tissue histochemistry

The adrenal glands and the tumor xenografts were fixed, dehydrated and embedded in paraffin. The specimens were serially cut into 5-μm-thick sections and stained with hematoxylin-eosin (H&E) for examination under a light microscope. For IL-4 and IFN-γ immunostaining, after antigen retrieval and blocking, the sections were incubated with the following primary antibodies: anti-mouse IL-4 (1:60, R&D) and anti-mouse IFN-γ (1:50, R&D). Then, they were incubated with a secondary antibody conjugated with horseradish peroxidase (ZSBIO) and developed with ABC (ZSBIO) and diaminobenzidine reagent (BOSTER). Digital images were obtained using a Leica Photo Microscope (Leica, Q550CW).

2.7. Real-time PCR analysis

Total RNA was extracted from the mouse xenografts using the TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. Samples containing 0.5 μg of the total RNA were reverse-transcribed by oligo d(T) using the PrimeScript RT reagent Kit (Takara). The resulting complementary DNA was then subjected to real-time quantitative PCR (IQ-5 System, BIO-RAD Inc., USA) according to the manufacturer's directions. The primer sequences are as follows: IL-4, 5′-ACAGGAGAAGGGACGCCAT-3′ and 5′-GAAGCCCTACAGACGAGCTCA-3′; IFN-γ, 5′-TCAAGTGGCATAGATGTGGAAGAA-3′ and 5′-TGGCTCTGCAGGATTTTCATG-3′. To control for sample-to-sample variation, primers for GAPDH (5′-TTCACCACCATGGAGAAGGC-3′ and 5′-GGCATGGACTGTGGTCATGA-3′ were used. The data were analyzed using the $2^{(-\Delta\Delta Ct)}$ method.

2.8. Statistical analysis

The statistical significance of the differences between groups was determined with Student's two-tailed *t*-test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Chronic scream sound exposure caused a stress burden in Balb/c mice

The mice that were exposed to 6 h of the scream sound everyday showed reduced aggression and less activity. They had a lower bodyweight gain than the control mice (Fig. 1A). From day 0 to days 1, 7, and 14, the weight of the control mice was increased from 20.45 *g* to 20.91 *g*, 23.12 *g*, and 23.77 *g*, respectively. However, the weight of the stressed mice increased from 19.28 *g* to 19.34 *g*, 21.06 *g*, and 22.76 *g*, which was significantly less than the increase in the control mice. In response to stressors, the stress system is activated to maintain homeostasis, including the perception of threat by the central nervous system and activation of an autonomic nervous system and hypothalamic–pituitary-adrenal (HPA) axis [15]. The adrenal gland is an essential stress-responsive organ that is a part of the HPA axis. During the 2 weeks of stress, the zona fasciculate (ZF) cells in the adrenal cortex were relatively enlarged and showed an irregular shape compared with the control. The cytoplasm of ZF cells were lighter than those of the control, and they were filled with large lipid droplets (Fig. 1B). The serum concentrations of norepinephrine and corticosterone were significantly increased by 14.76% and 12.17%, respectively, in the mice exposed to the scream sound compared with the control mice (Fig. 1C). These data suggest that chronic exposure to a scream sound induces hyper functioning of the adrenal gland and increased hormone secretion. That is, chronic exposure to a scream sound activates the HPA axis and causes a stress burden in mice.

3.2. Chronic scream sound stress caused a shift from the Th1 response to the Th2 response in the immune system

It is known that stress has profound inhibitory effects on the immune response [16]. Cytokines are important for the generation of cell-mediated immunity. To explore the changes in cytokines caused by chronic stress, we utilized a RayBio Mouse Cytokine Array to assay the serum from the mice (Fig. 2A). The microarray

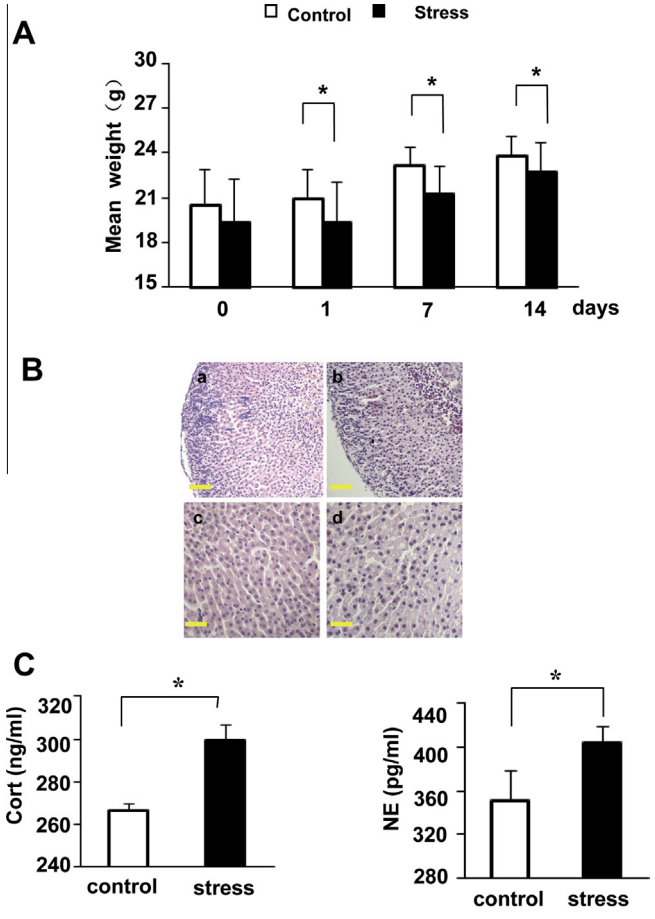

Fig. 1. Chronic exposure to scream sound induces stress in mice. (A) Male Balb/c mice in the control and the stress (scream sound treated for 6 h daily) group were housed for 2 weeks. The body weights were measured every other day. (B) After that, the mice were sacrificed, and the adrenal glands were removed, fixed, and sliced into sections. Then, the adrenal sections were stained with H&E (control, a and c; stress, b and d. bar in a and b, 60 μm; bar in c and d, 30 μm.). (C) The serum was obtained from the mice. The levels of norepinephrine and corticosterone were measured using radioimmunoassay. The data are shown as the mean ± SEM. $n = 8$ Mice per group. $^{*}P < 0.05$. The experiments were repeated three times with reproducible results.

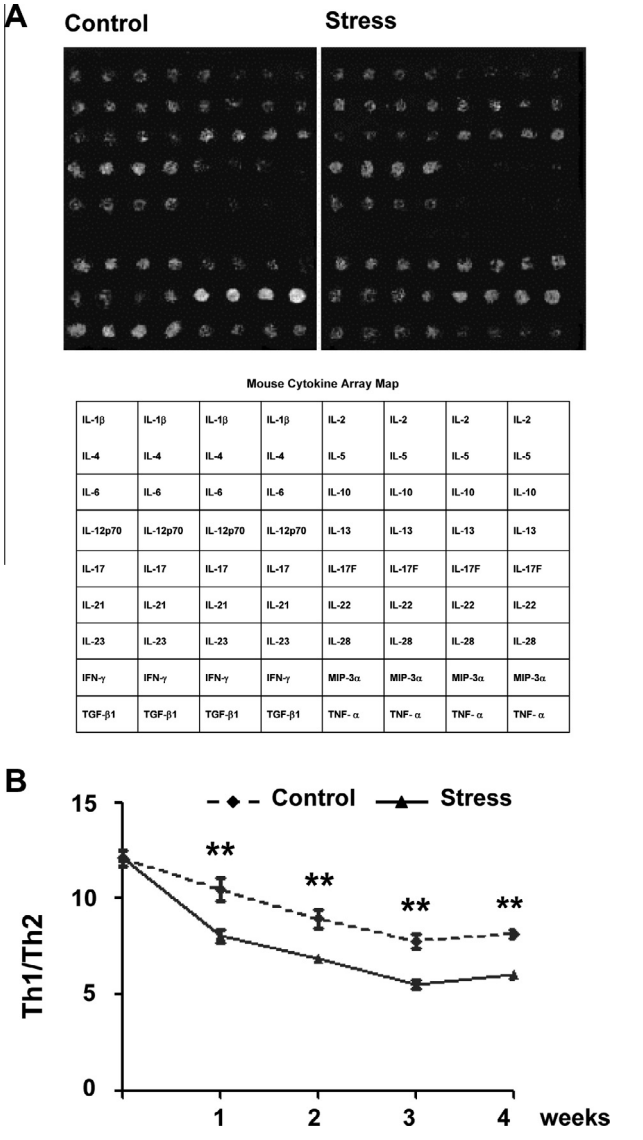

Fig. 2. Chronic scream sound stress causes cytokine changes in mice. (A) Male Balb/c mice in the control and stress groups were treated as described in Fig. 1 for 2 weeks. After that, the serum was analyzed using a slide-based mouse cytokine array kit. (B) Mice in the control and the stress groups were housed for 4 weeks. Every week, mice from each group were sacrificed and serum was obtained. The concentrations of IL-4 and INF-γ in the mice serum were measured using ELISA. The data are shown as the mean ± SEM and are representative of three independent experiments. $n = 6$ Mice per group. $^{*}P < 0.05$.

intensities of IL-4, IL-5, IL-6, IL-10, and IL-13, which are Th2 cytokines, were stronger in the serum from the chronic stress-treated mice than the controls. Conversely, the microarray intensities of TNF-α and IL-2, which are Th1 cytokines, showed reduced expression in the serum from the stressed mice. The microarray signal intensities were quantified as shown in Fig. S1. To evaluate the cytokine expression in detail, we detected IFN-γ, a representative Th1 cytokine, and IL-4, a representative Th2 cytokine, using ELISA in a time-dependent manner (Fig. 2B). Chronic exposure to scream sound stress led to a significant decrease in the ratio of IFN-γ to IL-4 (IFN-γ/IL-4) throughout the 4-week exposure to stress. Therefore, the chronic scream sound stress caused a shift from the Th1 response to the Th2 response in the mouse immune system.

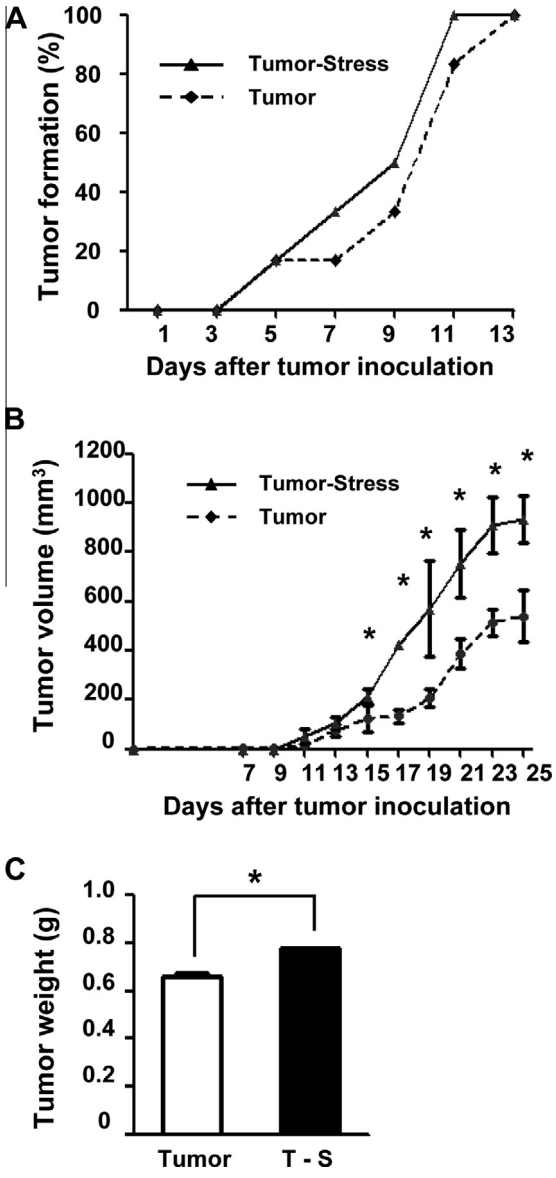

Fig. 3. Chronic scream sound stress promotes colon cancer growth *in vivo*. (A) Male Balb/c mice were randomly divided into four groups: control group (C), stress group (S), tumor group (T), and tumor-stress group (T-S). After 2 weeks of exposure to scream sound stress, mice in the S and T-S groups were injected with 1×10^5 colon 26 cells subcutaneously. As the control, PBS was injected in the mice in the C and S groups. Chronic scream sound stress was further applied on the mice of group S and T-S. The tumor formation rate was observed and calculated during the first 13 days after tumor inoculation. (B) For 3 weeks after tumor inoculation, the tumor size was measured and calculated. (C) On day 25, the mice were sacrificed and the tumors were harvested and weighed. The data are shown as the mean ± SEM. $n = 8$ Mice per group. $^*P < 0.05$. The experiments were repeated three times with reproducible results.

3.3. Chronic scream sound stress promoted colon cancer progression

We then explored the influence of chronic scream sound stress on colon cancer progression. Mice were treated as described in Materials and methods. Although tumors were formed in both groups, they emerged earlier in the T-S group than in the T group, and as a result, the formation rate of colon cancer during the first 2 weeks was higher in the T-S mice (Fig. 3A). The same volume of PBS as a control was injected into the C and S groups, and there was no tumor formation in these mice. The tumors grew faster and larger in the T-S group compared with the T group (Fig. 3B). On day 25 after the tumor inoculation, the mean volume of the tumors in the T-S mice was approximately 1.74 times larger than the tumors in the T mice. The mice were sacrificed, and the tumors were harvested (representative photo is shown in Fig. S2) and weighed. The mean weight of the xenografts in the T-S mice was increased by 17.76% compared with the T mice (Fig. 3C). H&E staining of the xenograft sections showed that the necrotic area in the T-S mice was significantly larger than that in the T mice (Fig. S3). Additionally, the nuclei-cytoplasm ratio of the tumor cells was increased; nuclear heterochromatin and mitotic phase were more frequent

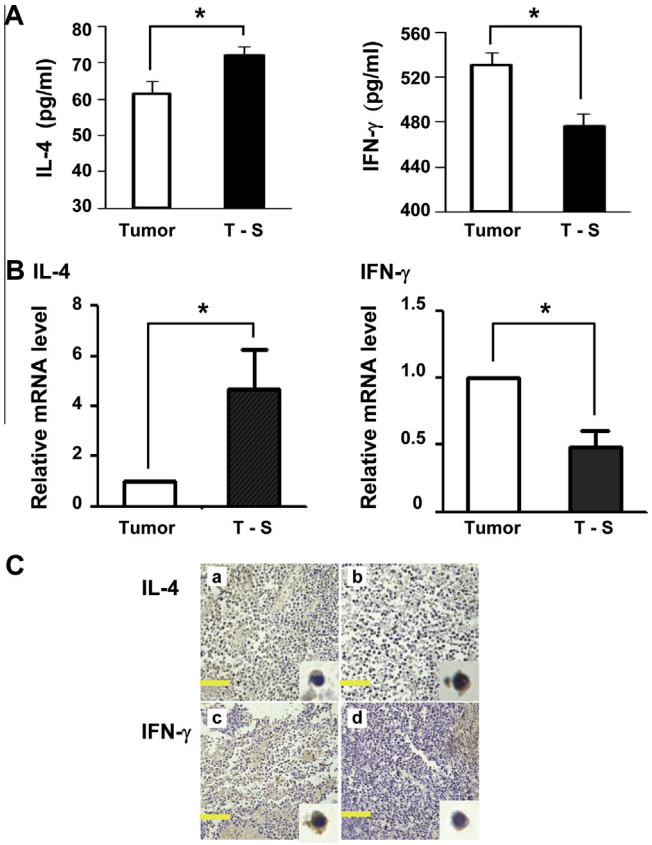

Fig. 4. Chronic scream sound stress causes cytokine changes during colon cancer growth *in vivo*. (A) Mice in the T and T-S groups were treated as in Fig. 3. 2 Weeks after tumor inoculation, the serum was obtained and the concentrations of IL-4 and IFN-γ were measured using ELISA. (B) The tumors were excised, and the total RNA was isolated from each xenograft. After reverse transcription, real time PCR of IL-4 and IFN-γ in the mouse serum was performed using GAPDH as a control. (C) Tumor sections were analyzed by immunohistochemistry with anti-IL-4 and anti- IFN-γ separately (group T, a and c; group T-S, b and d. Bar, 20 μm.). The inserts are magnifications of the tumor-infiltrated lymphocytes in each image. The data are shown as the mean ± SEM and are representatives of three independent experiments. $n = 6$ Mice per group. $^*P < 0.05$.

in the xenografts from the T-S mice. Moreover, after inoculation with colon 26 cells at a suboptimal dose of 2×10^4 cells, the tumor formation rate in the T-S mice was approximately 2 times larger than the tumor formation rate in the T mice on day 21 (Fig. S4). These results demonstrate that chronic exposure to scream sound stress promotes colon cancer progression *in vivo*.

Finally, the changes in cytokine expression during colon cancer progression due to chronic exposure to scream sound stress were investigated. The level of serum IL-4 in the T-S mice was increased by 14.68% relative to the unstressed T mice. Conversely INF-γ was decreased by 10.41% relative to the unstressed mice (Fig. 4A). In xenografts from the T-S mice, the mRNA level of these 2 cytokines also showed an identical result. The level of IL-4 increased by 365.07%, and INF-γ decreased by 51.45% relative to the T group (Fig. 4B). Immunohistochemical analysis showed more intense staining for IL-4 and less staining for INF-γ in the xenograft sections from the T-S mice compared with the xenograft sections from unstressed mice (Fig. 4C). The inserts in each image show a representative image of the infiltrated lymphocytes in the colon cancer. The results also showed that chronic sound stress induced increased expression of IL-4 and decreased expression of INF-γ in tumor-infiltrated lymphocytes, with a round, hematoxylin-stained nucleus. Taken together, these data suggest that chronic scream sound stress promoted colon cancer progression and induced an immune shift from the Th1 response to the Th2 response both in the circulation and in the tumor microenvironment.

4. Discussion

Clinical studies indicate that stress, chronic depression, and other psychological factors might influence cancer onset and progression. Chronic scream sound exposure in our experiment induced freezing behavior in the mice and decreased the bodyweight gain. Changes in the adrenal gland and serum corticosterone and norepinephrine levels indicate that chronic exposure to scream sound led to the HPA response and caused stress in Balb/c mice. Chronic scream sound stress caused a shift from the Th1 to the Th2 response and promoted colon cancer progression.

Stress is a state of threatened homeostasis that produces different physiological and pathological changes depending on the severity, type, and duration of exposure to the stressors [17]. Among a variety of stress models, chronic stress models are considered more naturalistic and are suggested to more closely resemble the human situation [12]. Restraint, social isolation, noise, exposure to cat odor, crowding, and the communication box method are frequently used in chronic stress research. Chronic unpredictable stress and chronic mild stress models have also been developed and frequently used in research on stress-induced diseases, with a paradigm of exposure to multiple different stressors for variable periods. Many of these stressors, such as restraint, electric foot shock, forced swimming, crowding, and cage inclination, involve physical factors and psychological factors simultaneously and are not suitable to observe the influence of psychological factors alone. The cat odor model is tedious and difficult to control. The communication box is inhumane as the rats repeatedly receive electric foot shocks. Therefore, we chose noise as a chronic psychological stressor [18]. Compared with white noise, we recorded the scream sound from inbred Balb/c mice stimulated with electric foot shock, lowered the sound decibel intensity to avoid audiogenic stress [19], and mimicked the psychological stress associated with environmental factors. In addition, we changed the starting time of the scream sound exposure everyday to prevent the adaptation of the HPA axis in the mice. In addition to the behavioral, hormonal, and immune changes shown in Figs. 1 and 2, chronic scream sound stress induced changes in learning and memory, as well as the levels of monoamine neurotransmitters in the brain (data not shown). These data demonstrated that chronic exposure to scream sound stress induced an allostasis load [15] in Balb/c mice and can be conveniently used as a novel chronic stress model.

Chronic scream sound stress led to a cytokine imbalance with a shift from Th1 cytokines, such as IFN-γ, to Th2 cytokines, such as IL-4 and IL-10, which has been shown to be induced by glucocorticoids [20]. Life stress is reported to influence tumor progression through many pathways: swim stress in rodents resulted in induction of chromosomal aberrations and sister chromatid exchange in bone marrow cells [21]; immobilization stress enhanced angiogenesis within tumors and increased the ovarian tumor burden [22]; catecholamines released by restraint stress activated β-adrenergic receptors on the tumor cell membrane and accelerated breast, ovarian, and pancreatic cancer growth [13,23,24]; stress hormones have been found to activate various human tumor viruses, such as the Epstein-Barr virus (EBV) and hepatitis B virus, to facilitate tumor growth [25,26]. Additionally, stress hormones have been shown to suppress different aspects of immune function, such as antigen presentation, T-cell proliferation, and humoral and cell-mediated immunity [16]. Our results are consistent with the above immune changes. Chronic stress could alter the HPA axis, induce a shift from Th1 cellular immunity to Th2 mediated humoral immunity, and suppress circulating type 1 cytokines and type 1 tumor infiltration by lymphocytes, which are known to be critical for the innate immune response and significantly increase the susceptibility to colon cancer.

Stress has become synonymous with modern life. Chronic exposure to scream sound stress mimics environmental stress and can be conveniently used as a novel chronic stress model. Chronic stress contributes to colon cancer progression. Chronic stress induces a Th1/Th2 imbalance both in circulation and in tumor-infiltrated lymphocytes. Additional studies should be carried out on the psychoneuroimmunological details of the influence of chronic stress on colon cancer progression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.08.101.

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