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The increasing hematopoietic effect of the combined treatment of Korean Red Ginseng and *Colla corii asini* on cyclophosphamide-induced immunosuppression in mice

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ABSTRACT

Background: Hematopoiesis is the production of blood cells from hematopoietic stem cells (HSCs) that reside in the bone marrow. Cyclophosphamide (CTX) is a chemotherapy drug that suppresses the immune system. Korean Red Ginseng (KRG) and *Colla corii asini* (CCA) have been traditionally used for boosting the immune system.
Methods: HSCs in the bone marrow, and immune cell subtype in splenocytes, PBMCs, and thymocytes were investigated. Serum levels of hematopoietic-related markers were analyzed using ELISA. Protein expression in spleen tissue was analyzed using western blot analysis. Hematoxylin & eosin staining in the femurs of mice were also conducted.
Results: The combination of KRG and CCA with a ratio of 3:2 increased HSCs, CD3 and $CD8^+$ T cells in the circulation, and CD3 T cells in the spleen. A ratio of 2:3 (KRG:CCA) increased the thymic regulatory T cells and recovered the CD3 T cells in the spleen and circulation while recovering proteins in the JAK-STAT pathway in the spleen. Overall, blood cell population and differentiating factors vital for cell differentiation were also significantly recovered by all combinations especially in ratios of 3:2 and 2:3.
Conclusion: A ratio of 3:2 (KRG:CCA) is the most ideal combination as it recovered the HSC population in the bone marrow of mice.
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1. Introduction

Hematopoiesis is the process of the production of blood cells and hematopoietic stem cells (HSCs) that reside in the bone marrow. HSCs consist of pluripotent, long-term self-renewing HSCs (LT-HSC). They eventually differentiate into short-term HSCs (ST-HSC), common myeloid progenitors (CMPs), and common lymphoid progenitor (CLPs). The CMPs will differentiate into megakaryocyte-erythroid progenitors (MEPs) and granulocyte-macrophage progenitors (GMPs), where all the above stated are multipotent progenitors. Further, differentiation of the MEPs will yield RBCs and platelets; GMP will produce monocytes, neutrophils, and eosinophils, whereas CLP will produce lymphoid cells like B lymphocytes and T lymphocytes [1]. They constantly differentiate to ensure a sufficient amount of blood cells due to their short-lived life span. Cyclophosphamide (CTX) is a chemotherapy drug that suppresses the immune system [2]. As cyclophosphamide is non-specific to cancer cells, it also induces damage to the stem cell microenvironment, especially the bone marrow. Therefore, it is important for us to discover herbal formulations to supplement chemotherapy and prevent immunosuppression with cases of suppressed white blood cells (WBC).

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Herbal formulations have been commonly used in traditional medicine and accepted well as compared to over-the-counter medicines. In a study comparing the preferences of the participants for herbal medicines or commonly prescribed drugs, most of them would opt for a natural alternative due to concerns of side effects and the lack of knowledge of over-the-counter prescriptions [3]. In traditional Chinese medicine, formulations were made based on the "emperor-minister-assistant-courier" principle or also known as the "Jun-Chen-Zuo-Shi" principle: the emperor acts as the main herb; the minister acts as the adjuvant herb; the assistant reduces the toxicity or side effects of the herb; and the courier will assist or guide the active ingredients of the main herbs to the respective target organs [4]. Therefore, herb combinations and their ratio of usage should be determined.

Korean Red Ginseng (KRG) is known to boost immunity [5,6], and *Colla corii asini* (CCA) is donkey-hide gelatin that is commonly used in traditional Chinese medicine, which has been known to increase hematopoiesis [7], improve anemia [8], enhance immunity and has been traditionally reported for its anti-coagulatory and vasodilatory properties [9]. As CTX is known to decrease the number of HSCs [10], the present study focused on HSCs in order to determine whether the combined treatment of KRG and CCA is capable of restoring the number of HSCs in the bone marrow of mice. We aim to investigate the best combination ratio of KRG: CCA having optimal effects against CTX-induced immunosuppression in mice.

2. Materials and methods

2.1. Materials

CTX was purchased from Sigma–Aldrich (St. Louis, MO, USA) and antibodies for CD4, CD8, CD3, CD19, sca-1, Flk-2, c-kit, CD34, and IL-7R were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Lineage antibody were purchased from BioLegend (San Diego, CA, USA). ACK lysing buffer was purchased from Gibco (Waltham, MA, USA). Methocult™ GF M3434 used for culturing bone marrow cells was purchased from STEMCELL Technologies Inc. (VN, Canada). Recombinant human granulocyte colony-stimulating factors (rhG-CSF) were purchased from R&D Systems (Minneapolis, MN, USA). Fetal bovine serum (FBS) was acquired from Welgene (Gyeongsan-si, Gyeongsangbuk-do, Republic of Korea). Antibodies for Janus kinase 2 (JAK-2), p-JAK2, signal transducer and activator transcription 3 (STAT3), p-STAT3, suppressor of cytokines signaling 1 (SOCS1), suppressor of cytokines signaling 3 (SOCS3), c-Myc, β-actin, total c-Jun N-terminal kinase (T-JNK), phospho-c-Jun N-terminal kinase (p-JNK), total extracellular signal-regulated kinase (T-ERK), phospho-extracellular signal-regulated kinase (p-ERK), total-p38 (T-p38), and phospho-p38 (p-p38) were purchased from Cell Signaling Technology (Danver, MA, USA).

2.2. Preparation of KRG and CCA

KRG was obtained from the Korean Ginseng Corporation (Daedeok-gu, Daejeon, Republic of Korea) (batch number: H1312-9016). CCA (batch number: 1901063) was obtained from Dong E E Jiao Corporation (Liaocheng, Shandong, China). CCA was then size reduced, weighed, and dissolved in ultrapure water (Welgene, Gyeongsangbuk-do, Republic of Korea). KRG was weighed accordingly and dissolved in water.

2.3. Cyclophosphamide-induced immunosuppression in mice

Six-week-old C57/Bl6 mice were purchased from Orient Bio (Gyeonggi-do, Republic of Korea). The mice were housed in a pathogen-free facility with a temperature maintained at 22 °C ± 2 °C and a relative humidity of 50% ± 10%, with a 12/12 h light/dark cycle. Feed and water were provided *ad libitum*. Animal experiments were conducted and approved by the Institutional Animal Care Committee of Kyungpook National University (approval number: KNU2019-131). After 1 week of acclimatization, the mice were divided into 10 groups randomly (n = 8 per group): control, CTX (100 mg/kg *i.p.*), KRG (300 mg/kg), CCA (300 mg/kg), 1:1 (KRG 150 mg/kg: CCA 150 mg/kg), 1:2 (KRG 100 mg/kg: CCA 200 mg/kg), 2:1 (KRG 200 mg/kg: CCA 100 mg/kg), 3:2 (KRG 180 mg/kg: CCA 120 mg/kg), 2:3 (KRG 120 mg/kg: CCA 180 mg/kg) and rhG-CSF (22.5 μg/kg). All of the mice were administered with CTX except the control group for 3 consecutive days, followed by the oral administration of samples accordingly for 10 consecutive days. rhG-CSF was administered *i.p.* on Days 1, 3, 5, 7, and 9 after the CTX administration. On Day 11, the mice were euthanized with an overdose of isoflurane. The spleen and thymus were collected and weighed immediately. The right femur was collected for fluorescence-activated cell sorting (FACS) analysis and the left femur was directly fixed in 10% neutral buffered formalin for hematoxylin & eosin (H&E) analysis. Moreover, blood was collected for peripheral blood mononuclear cell (PBMC) isolation.

2.4. Peripheral hemogram of mice

Blood was harvested and stored in a microvette (BD, Franklin Lakes, NJ, USA) for blood analysis. The WBC, lymphocytes, monocytes (MID), granulocytes, hemoglobin (HGB), platelet count (PLT), and red blood cell count (RBC) were evaluated using a URIT-3000 Vet Plus hematology analyzer (Diamond Diagnostics Inc., Holliston, MA, USA).

2.5. Fluorescence-activated cell sorting (FACS) analysis of splenocytes, thymocytes, PBMC, and bone marrow nucleated cells of mice

Blood was harvested through cardiac puncture and stored in EDTA tubes (BD, Franklin Lakes, NJ, USA). RBC was lysed using an ACK lysing buffer to obtain the blood cells. To obtain the bone marrow cells, the femur was harvested and the marrow cells were flushed using a 23G needle with phosphate buffered saline (PBS). Cells were strained through a 70 μm cell strainer (SPL Life Sciences, Gyeonggi-do, Republic of Korea). For splenocyte and thymocyte preparation, the spleen and thymus were homogenized using the plunger of a syringe and passed through a 70 μm cell strainer. Collected cells were centrifuged at 1800 rpm for 5 min. The cells were resuspended in an ACK lysing buffer to lyse the RBCs, quenched with FACS buffer (PBS supplemented with 2% FBS), and centrifuged at 1800 rpm for 5 min to remove the lysed RBCs. Collected bone marrow cells, thymocytes, and splenocytes were counted and adjusted to 2×10^5 cells/mL in 5 mL round bottom tubes (SPL Life Sciences, Gyeonggi-do, Republic of Korea). The tubes were then centrifuged, and the supernatant was discarded and stained with respective surface markers for FACS analysis. The bone marrow cells were investigated for the hematopoietic stem cell markers sca-1, c-kit, CD34, Lin, Flk-2, and IL-7R, whereas the splenocytes and PBMC were checked for CD3 naïve T cells, CD19 B cells, CD4 Th cells, and CD8 cytotoxic T cells (CTL). Moreover, thymocytes were checked for CD4 Th cells, CD8 CTL, and $CD4^+CD25^+$ regulatory T cells (Tregs). FACS analysis was conducted using the BD FACSAria™III (BD Biosciences, Franklin Lakes, NJ, USA). Data were post-

analyzed using the Flowlogic version 7 software (Miltenyi Biotec, Bergisch Gladbach, Germany).

2.6. Spleen and thymus indices

The weight of the mice was measured before euthanization. The spleen and thymus were weighed directly after harvesting. The index was taken as the weight of the spleen and thymus per weight of mice (mg/g). The spleens were then snap-frozen for western blot analysis.

2.7. Western blot analysis of spleen tissue

The spleens were homogenized using a hand grinder with a protein extraction solution (Pro-Prep, iNtRON, Daejeon, Republic of Korea). Protein concentration was measured using the Bradford method (Pro-Measure, iNtRON, Daejeon, Republic of Korea). The proteins were then separated using 10% SDS-PAGE gels and transferred onto PVDF membranes (Milipore, Burlington, MA, USA). The membranes were then blocked with 5% skim milk and washed with Tris-Buffered Saline with 0.1% TWEEN® 20 (TBS-T). After that, they were incubated with primary antibodies at a 1:1,000 dilution overnight on a roller at 4 °C. After washing with TBS-T, the membranes were incubated at a dilution of 1:3,000 for 75 min. The membranes were developed with enhanced chemiluminescence (WesternBright ECL, Advansta, San Jose, CA, USA) and detected using an image developer (General Electrics, Boston, MA, USA). Experiments were conducted in triplicates and blots were quantified using ImageJ (NIH, USA).

2.8. Hematoxylin & eosin (H&E) staining

After euthanization, the femurs were harvested and fixed in formaldehyde immediately. They were decalcified with formic acid after 2 days in formaldehyde fixation, embedded in paraffin, and sectioned into 5 μm slides before undergoing H&E staining. Histological images were acquired using the Aperio ImageScope x64 software (Leica Biosystems, Buffalo Grove, IL, USA). The images were obtained by a different researcher uninformed of the grouping of slides to ensure blinding. Moreover, the images were acquired at a magnification of 200×.

2.9. ELISA assay for serum

Hematopoietic-related cytokines were investigated in the serum of mice. The blood was left to separate in a vacutainer for 2 h and then centrifuged at 3000 rpm for 5 min. The serum was then separated and stored at −70 °C until analysis. Serum IL-4, IL-10, IFN-γ, GM-CSF, TPO, and EPO were investigated using ELISA kits (R&D Systems, Minneapolis, MN, USA) in accordance with the manufacturer's instructions.

2.10. Colony-forming cell assays

Murine bone marrow cells at a density of 2×10^4 cells/well were seeded and incubated for 7 days in methylcellulose-based medium with recombinant cytokines (GF M3434; Methocult™, STEMCELL Technologies Inc., VN, Canada). Colony-forming unit-granulocyte-macrophage (CFU-GM), burst-forming unit (BFU-E), and colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) were observed under an inverted microscope and counted.

2.11. Statistical analysis

Statistical analyses were done using the GraphPad Prism version 7.00 software (San Diego, CA, USA) and one-way ANOVA was performed with Dunnett's post-test. Data were presented as the mean ± SD. $^{\#}$ indicates $P < 0.05$, $^{\#\#}$ indicates $P < 0.01$, and $^{\#\#\#}$ indicates $P < 0.001$ as compared to the control group, whereas * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$ as compared to the model group.

3. Results

3.1. The efficacy of the combined treatment of KRG and CCA in improving CTX-suppressed hematopoietic stem cell differentiation

3.1.1. The combined treatment of KRG and CCA improves blood cell count suppressed by CTX treatment

From our results, WBC, lymphocytes, and MID have been significantly suppressed by the CTX group. Ratios of 3:2 and 2:3 have significantly recovered the WBC count. In the case of lymphocytes, the KRG 300 and 2:3 groups have shown a significant increase in lymphocytes. The CCA 300 and 3:2 groups have also shown signs of increase but were not significant. Furthermore, no change was observed in hemoglobin, RBC levels, and platelet count in all groups (Fig. 1A–G).

3.1.2. The combined treatment of KRG and CCA increased the hematopoietic stem cells in the bone marrow of CTX-treated mice

The combination of the markers for LT-HSC, ST-HSC, MPP, CLP, and CMP is shown in Fig. 2E [11]. LKS^+ includes LT-HSC, ST-HSC, and MPP. MPP is capable of differentiating into CLPs and CMPs, which are both capable of proliferation and differentiation. Comparatively, the LKS^+ cells were significantly suppressed in the CTX-treated mice and were significantly increased in the 3:2 and rhG-CSF groups (Fig. S1A & B). In segregating the LKS^+ into LT-HSC, ST-HSC, and MPP, it can be observed that CTX has suppressed LT-HSC, ST-HSC, and MPP (Fig. 2A–D). A ratio of 3:2 and rhG-CSF have significantly increased MPP and ST-HSC. CTX significantly suppressed the CLPs. However, not much recovery was observed in all treatment groups except for the 3:2 and rhG-CSF groups (Fig. 3A and B). CTX has also significantly suppressed the CMPs. Moreover, no significant increase in CMP was observed in all groups (Fig. S2A &B), but a slight increase of CMP was observed in the 3:2 and 2:3 groups. CFU-GM, CFU-GEMM, and BFU-E assays are essential in evaluating the differentiating abilities of HSCs. Based on our findings, CTX has prevented the differentiation of CFU-GM, CFU-GEMM, and BFU-E. Moreover, CFU-GM was increased significantly in the KRG 300, 3:2, and 2:3 groups, the CFU-GEMM colonies were increased in all groups, and BFU-E has been only significantly increased in the CCA 300 group (Fig. 4A–C).

3.1.3. The combined treatment of KRG and CCA recovered the hematopoietic growth factors in the serum of mice induced with CTX

All treatment groups did not alter TPO levels (Fig. 4E). EPO was significantly increased with CTX treatment (Fig. 4D), whereas GM-CSF was significantly decreased (Fig. 4F). The increase of EPO may be due to a recovery mechanism to increase the amount of erythrocytes that has been reduced with CTX treatment. The expressions of GM-CSF were significantly increased in the CCA 300, 1:1, 2:1, 3:2 2:3, and rhG-CSF groups. Based on the results of the present study, the expressions of IFN-γ, IL-10, and IL-4 were suppressed by CTX treatment (Fig. 4G–I). The combination of ratios 3:2 and 2:3 has significantly increased IFN-γ and IL-10 expression. Overall, the combination ratios of 3:2 and 2:3 are capable of increasing the

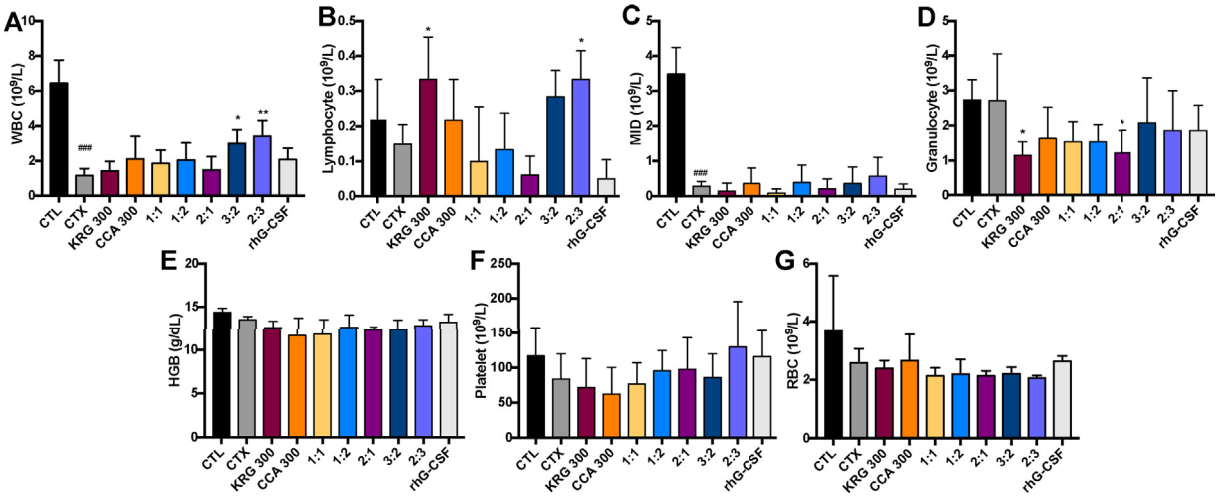

Fig. 1. The combined treatment of KRG and CCA attenuated change in the peripheral blood cell count. Blood was collected from mice after 10 days of sample treatment and analyzed. (A) White blood cells (WBC), (B) lymphocytes, (C) MID, (D) granulocytes, (E) hemoglobin, (F) platelet count, and (G) red blood cell (RBC) levels in mice with or without cyclophosphamide and treated with or without KRG or CCA and their combination. Statistical significance was taken as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ against CTX. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ against the control group.

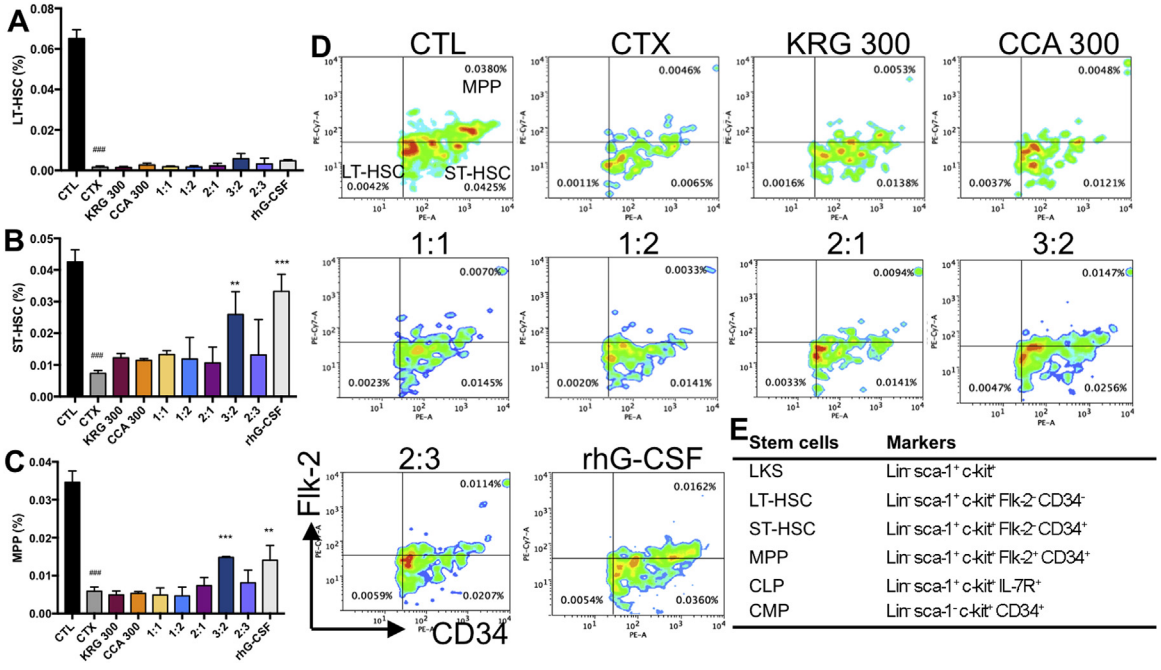

Fig. 2. The combined treatment of KRG and CCA improved the hematopoietic stem cell markers in the bone marrow of mice. The percentage of bone marrow cells that were (A) LT-HSC, (B) ST-HSC, and (C) MPP positive in the bone marrow of mice. Representative analyses of flow cytometry analysis for each treatment group are as shown in (D). Surface markers for the respective populations are stated in (E). Statistical significance was taken as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ against CTX. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ against the control group.

proliferation of immune cells that has been suppressed with CTX treatment.

3.2. Immunostimulatory effects of the combined treatment of KRG and CCA suppressed by CTX treatment

3.2.1. Combined treatment of KRG and CCA suppressed the enlargement of spleen induced by CTX

Increased spleen weight in mice in the CTX-treated group was due to the disruption of hematopoiesis function in the bone marrow. Hence, extramedullary hematopoiesis has to take place in the spleen. Based on the findings of the present study, CTX has significantly increased the size of the spleen (Fig. 4J), but no significant change was observed in the thymus (Fig. 4K). Treatment ratios of 1:2, 2:1 3:2, and 2:3 have significantly reversed the increase in spleen index induced by CTX.

3.2.2. KRG and CCA combination recovered PBMC T cells suppressed by CTX treatment

The CD3 T cells and CD19 B cells in PBMC have been significantly suppressed with CTX treatment (Fig. S3). No significant recovery was observed in all treatment groups. However, signs of increase in

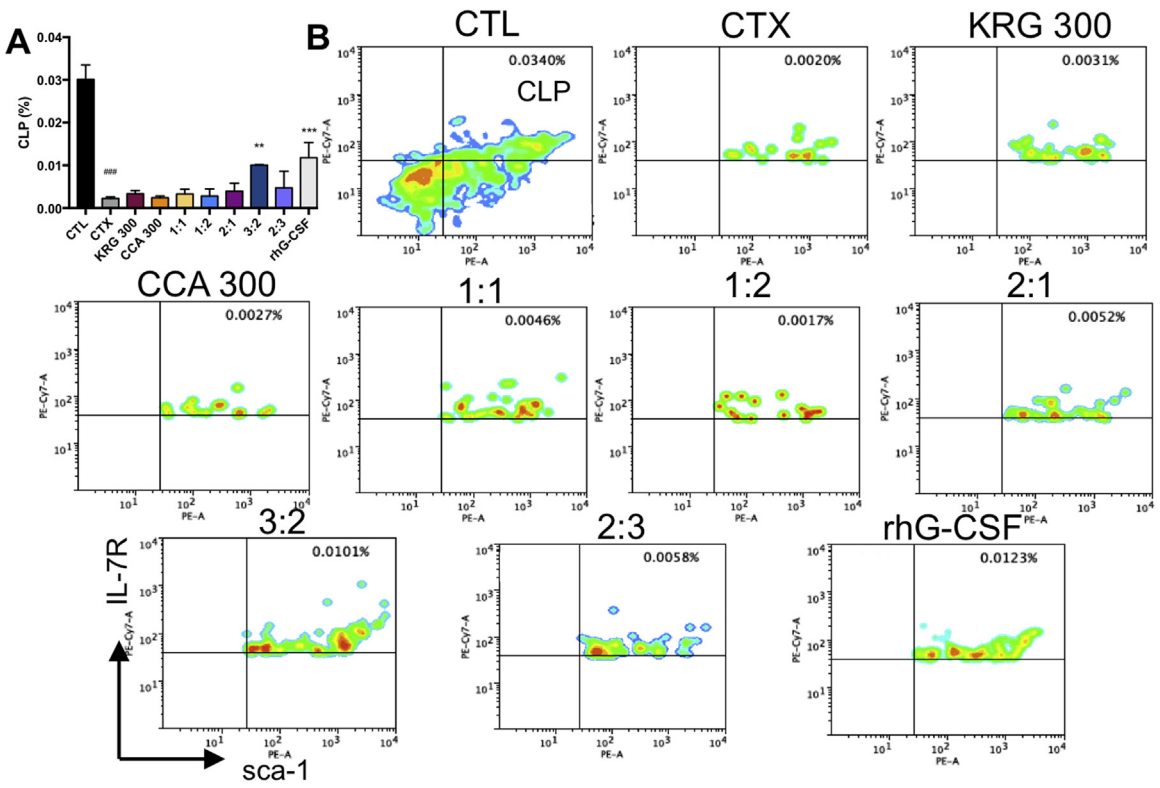

Fig. 3. A combined treatment of KRG and CCA improved the CLP population in the bone marrow of immunosuppressed mice. (A) The percentage of stem cells that are CLPs in the bone marrow of mice. (B) Representative flow cytometry analysis for each treatment group. Statistical significance was taken as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ against CTX. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ against the control group.

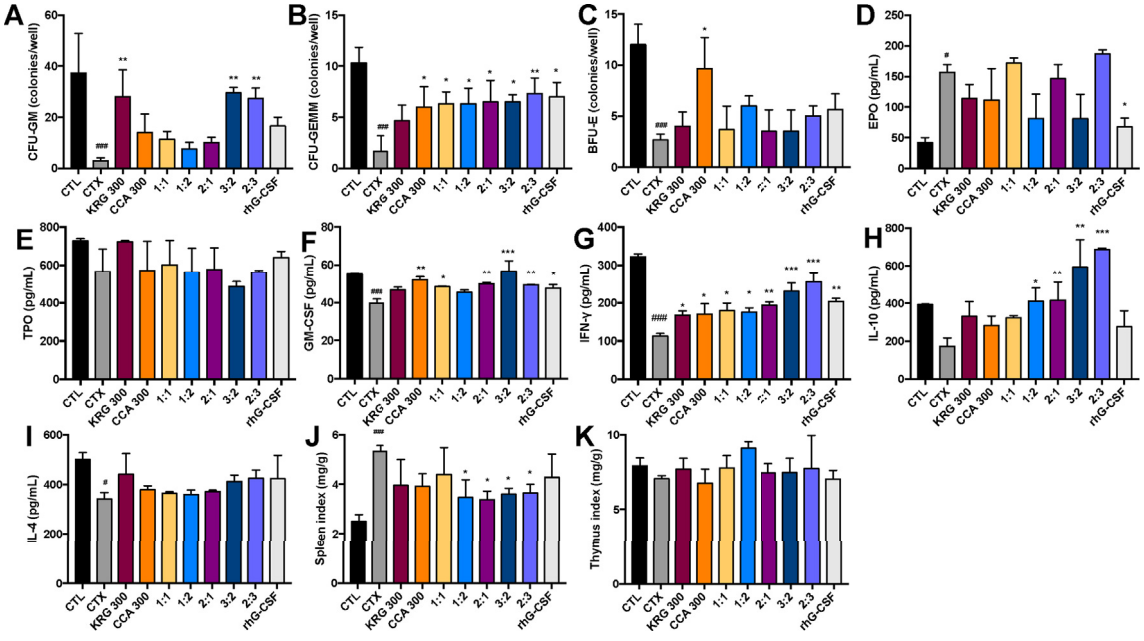

Fig. 4. The combined treatment of KRG and CCA showed immunostimulatory effects. It also improved the growth of CFU-GM, CFU-GEMM, and BFU-E colonies in the bone marrow of mice. Bone marrow cells were cultured in Methocult™ media and left to differentiate before the colonies were counted under an inverted microscope (A-C). Combined treatment of KRG and CCA on the EPO, TPO, GM-CSF, IFN-γ, IL-10, and IL-4 levels in the serum of mice were investigated using ELISA kits (D-I). The spleen and thymus indices of mice in different groups with various combination ratios of KRG and CCA were measured directly after harvest (J-K). The index was calculated by comparing the weight of the organ with the weight of each mice. Statistical significance was taken as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ against CTX. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ against the control group.

PBMC CD3 were observed in the 1:1 and 2:1 groups. Based on the findings of the present study, the CTX group has shown signs of suppressed numbers of CD4 T cells and CD8 CTL, but not significant. For the CD4 T cells, KRG 300 and a ratio of 1:1 have shown a

significant increase in the CTX-treated group (Fig. S4A & C). For the CD8 CTL, the positive control, rhG-CSF, and 3:2 groups have shown a significant increase. Furthermore, the KRG 300, CCA 300, 1:1, 1:2, and 2:3 groups have also shown increments, but not significant (Fig. S4B & C).

3.2.3. The combined treatment of KRG and CCA increased the CD3 T cells in the spleen of CTX-induced mice

The levels of CD3 T cells in the spleen have been significantly suppressed by CTX treatment but have been recovered in all groups, except the CCA 300 group. CD19 B cells show signs of decrease in the CTX-treated group, but not significant (Fig. S5). Moreover, the CTX treatment significantly suppressed the CD4 T cells and CD8 CTL cells in the spleen. However, no significant changes were observed in all the other treatment groups (Fig. S6).

3.2.4. Combined treatment of KRG and CCA increased the regulatory T cells in the thymus of CTX-induced mice

In the thymus, no significant change was observed in the CD4 T cells and CD8 CTL cells in all groups (Fig. S7). $CD4^{+}CD25^{+}$ Tregs were suppressed in the CTX group but not significant. Signs of recovery in Tregs were observed in the KRG 300, CCA 300, 1:2, 2:1, 3:2, 2:3, and rhG-CSF groups, but significant results were observed only in the 2:3 group (Fig. S8).

3.2.5. Protein expressions of hematopoietic factors in the spleen of mice

Protein expressions of p-JAK2, p-STAT3, c-Myc, p-JNK, and p-ERK were suppressed by the CTX treatment. However, the reduction in p-STAT3 was not significant. Expressions of SOCS1 was increased by CTX but not significant. The ratio of 2:3 significantly increased the expressions of p-JAK2, p-STAT3, c-Myc, p-ERK, and p-JNK that had been suppressed by the CTX treatment, while significantly suppressing the SOCS1 expression that had been increased by the CTX treatment. A ratio of 3:2 had increased the expressions of c-Myc, p-ERK, and p-JNK, while decreasing the expression of SOCS1 as a recovery from the CTX treatment. No significant change was observed in the protein expressions of SOCS3 and p-p38 in all the treatment groups (Fig. 5A and B). This indicates that only the treatment ratio of 2:3 had an effect on the JAK-STAT pathway in the spleen.

3.2.6. Ratios 3:2 and 2:3 recovered the hematopoietic microenvironment in the bone marrow

Based on the findings of the present study, the opacity of the bone marrow had been severely reduced by CTX. Compared to the CTX group, all treatment groups showed visible improvement in the recovery of the bone marrow nucleated cells. CCA 300, 1:2, 3:2, and 2:3 groups had a better recovery rate as compared to the other groups (Fig. 6). This further confirms the efficacy of the treatment ratios of 3:2 and 2:3.

4. Discussion

External factors like radiation and chemotherapy cause hematopoietic stem cell senescence, which poses a threat to the wellbeing of recovering cancer patients as they develop hypoplastic anemia due to long-term bone marrow injury [12]. Existing treatments such as rhG-CSF treatment have been used to boost the mobilization of hematopoietic progenitors [13] and reported to improve neutropenia [14]. However, G-CSF treatment has been reported to show signs of myelodysplasia and acute myeloid leukemia in stem cell donors [15]. This raises concern on the safety of the routine use of G-CSF.

A majority of HSCs reside in the bone marrow to maintain blood cell production. They differentiate into different types of cells and are able to mobilize into the periphery. The cells will mobilize into the circulation to maintain a bodily function. The T cells migrate to the thymus where they differentiate to express both CD4 and CD8. Cells would differentiate into $CD4^{+}$ T cells if they were to be in contact with MHC II proteins, while they differentiate into $CD8^{+}$ cytotoxic T cells when in contact with MHC I proteins [16]. $CD4^{+}$ T cells can further differentiate into T helper cells or Tregs. IFN-γ, IL-10, and IL-4 are important in the regulation of T cell differentiation.

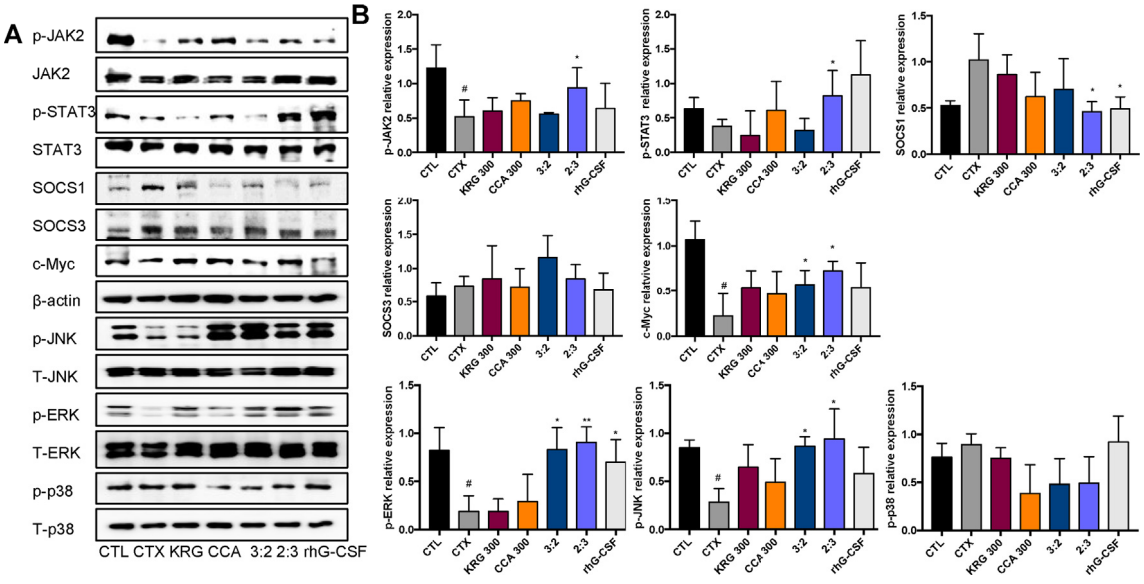

Fig. 5. Combined treatment of KRG and CCA recovered the proteins in the JAK-STAT pathway in the spleen of mice. Protein was extracted from the spleen of mice, separated using 10% SDS-PAGE, transferred onto PVDF membranes, blocked with skim milk, and incubated with the target primary antibodies overnight before incubation with HRP-conjugated secondary antibodies for visualization. The proteins in the JAK-STAT and MAPK pathways expressed in the spleen of mice (A). Western blot was performed in triplicate and the relative expressions were compared with their total forms or the housekeeping gene, β-actin (B). Statistical significance was taken as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ against CTX. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ against the control group.

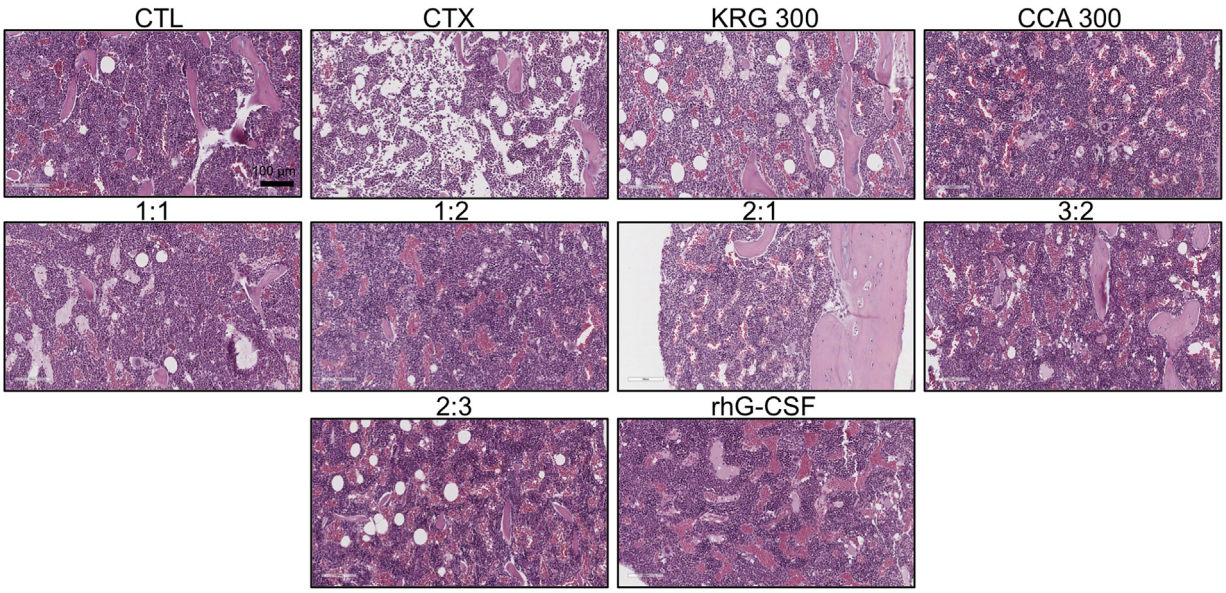

Fig. 6. The combined treatment of KRG and CCA recovered the bone marrow cell population in CTX-immunosuppressed mice. The femur was collected from mice and stained with H&E for visualization. Sections were observed at 200×. Scale bar indicated in control group is 100 μm.

Th cells differentiate into Th1 with the help of IFN-γ [17], whereas IL-4 induces the differentiation of Th2 cells [18]. IL-10 was reported to induce the differentiation of Tregs [19]. In the 2:3 group, the increase in thymic Tregs may be associated with the increase in the IL-10 levels in the blood (Fig. 4 & S8). TPO is produced by the liver and kidney and induces the differentiation of platelets, and EPO induces the differentiation of erythrocytes [20]. GM-CSF is secreted by activated T cells and also helps regulate the Th1/Th2 cytokine balance [21]. IL-4 has also been shown to induce the differentiation of B cells from CLP, whereas GM-CSF is vital for the differentiation of GMPs into dendritic cells [11]. Therefore, the expressions of these cytokines were investigated in this study.

Extramedullary hematopoiesis occurs in other organs like the liver and spleen. It is vital for immune response to pathogens. It also occurs when hematopoiesis is impaired in the bone marrow. A previous study also reported the existence of extramedullary hematopoiesis in mice administered with CTX [22]. Another study also showed that splenomegaly is a sign of extramedullary hematopoiesis in the spleen [23]. Moreover, this study showed that CTX induced a disruption of hematopoietic function in the bone marrow of mice, which forces extramedullary hematopoiesis to take place in the spleen as observed in Fig. 4J. The JAK-STAT pathway is known to play an important role in the homeostasis in humans and animals, with the activation of cell proliferation, cell differentiation, cell migration, cell apoptosis, and hematopoiesis [30]. The differentiation of T helper cells is also important. It can be activated by its ligand-like interleukins and interferons (e.g., IL-6, IFN-γ, and IL-4) which cause a conformational change in the receptor. The MAPK pathway is also a downstream pathway of the JAK-STAT pathway, which eventually gives rise to proteins like SOCS, B cell lymphoma (Bcl), and Myc [24]. In the JAK-STAT pathway, SOCS acts as a negative regulator which directly prevents JAK from further activating the JAK-STAT pathway. The human JAK family consists of JAK1, JAK2, JAK3, and TYK2. Resting STATs reside in the cytoplasm and translocate into the nucleus when activated, and the STAT family consists of STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. STATs are also vital in determining the fate of T cell differentiation. For example, STAT1 positively regulates Th1 differentiation, whereas STAT3 positively regulates Th2 and Th17 differentiation. Moreover, the SOCS family is required for T cell differentiation; SOCS1 positively regulates Th17 while negatively regulating Th1 and Th2, whereas SOCS3 positively regulates Th2 while negatively regulating Th1 and Th17 differentiation [25]. Hence, the expressions of SOCS1 and SOCS3 were investigated in this study. JAK2 can be activated by most ligands as compared to the other members in the JAK family [26]. STAT3 is vital for hematopoiesis according to previous studies [27,28]. Hence, the expressions of JAK2 and STAT3 were targeted in the present study. Based on our findings, suppressed p-JAK2 and p-STAT3 expressions by CTX treatment were recovered in the CCA and 2:3 groups (not significant in the CCA group). The 2:3 group had a higher recovery rate than the CCA group suggesting an increased potency in combination with KRG. A treatment ratio of 3:2 was more potent in recovering the proteins in the MAPK pathway. This indicates that a ratio of 2:3 may target the spleen to recover from immunosuppression instead of the HSCs. The increase in SOCS1 and 3 may indicate that in the early stages of immunosuppression, phosphorylation of JAK and STAT has increased due to the need of extramedullary hematopoiesis. This activated SOCS expression. In return, SOCS suppressed p-JAK2 and p-STAT3 expression. Only a ratio of 2:3 have significantly suppressed SOCS1 expression. Activation of SOCS3 was not altered by all treatment groups (Fig. 5).

This reveals an interesting finding that the ratios of 3:2 and 2:3 may be acting on different sites; a ratio of 3:2 targets the HSCs in the bone marrow, whereas a ratio of 2:3 targets the recovery in the spleen. Moreover, the findings of this study indicate that a treatment ratio of 3:2 increases the HSC population in the bone marrow of mice. Both combinations managed to increase the splenic CD3 T cells. A ratio of 2:3 increases the thymic Tregs possibly through increasing the thymic dendritic cells and activates extramedullary hematopoiesis via the activation of JAK-STAT pathway aside from increasing the splenic T cells. The positioning of T cells and B cells in the spleen allows them to identify antigen-presenting cells more effectively [29]. This indicates that all treatment groups (non-significant in CCA 300) are also capable of boosting the immune system by positioning and increasing T cells in the spleen. We also found that the combined treatment of KRG and CCA is more effective than any of the treatments alone. Comparatively, a ratio of

3:2 effectively targets hematopoiesis, hence making it a more potent combination as compared to a ratio of 2:3.

In conclusion, all of the ratios had increasing effects as compared to the KRG or CCA treatment alone. Based on the results, the potency ratios were 3:2 > 2:3 > 2:1 > 1:1 > 1:2. Considering the trend of the ratios, ginseng may be deduced as the "emperor" since a ratio of 3:2 is more dominant than a ratio of 2:3 comparatively. CCA may be the "assistant" in this combination which increased the potency of the combination. However, more studies on the immune cell subtypes in other lymphoid organs like the lymph nodes should be conducted. Furthermore, this is the first study to compare various combination ratios of KRG and CCA. The findings of this study suggest that a ratio of 3:2 is a more potent combination to be developed as a therapeutic supplement for patients undergoing chemotherapy.

Data availability

Data are available upon request to the authors.

Declaration of competing interest

All authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2021.02.004.

References

[1] Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. Cell 2008;132(4):631–44.
[2] Ahlmann M, Hempel G. The effect of cyclophosphamide on the immune system: implications for clinical cancer therapy. Cancer Chemother Pharmacol 2016;78(4):661–71.
[3] Lynch N, Berry D. Differences in perceived risks and benefits of herbal, over-the-counter conventional, and prescribed conventional, medicines, and the implications of this for the safe and effective use of herbal products. Complement Ther Medicine 2007;15(2):84–91.
[4] Qiu J. Traditional medicine: a culture in the balance. Nature 2007;448(7150):126–8.
[5] Hyun SH, Ahn H-Y, Kim H-J, Kim SW, So S-H, In G, et al. Immuno-enhancement effects of Korean Red Ginseng in healthy adults: a randomized, double-blind, placebo-controlled trial. J Ginseng Res 2021;45(1):191–8.
[6] Ratan ZA, Youn SH, Kwak Y-S, Han C-K, Haidere MF, Kim JK, et al. Adaptogenic effects of Panax ginseng on modulation of immune functions. J Ginseng Res 2021;45(1):32–40.
[7] Wu H, Ren C, Yang F, Qin Y, Zhang Y, Liu J. Extraction and identification of collagen-derived peptides with hematopoietic activity from Colla Corii Asini. J Ethnopharmacol 2016;182:129–36.
[8] Li Y, He H, Yang L, Li X, Li D, Luo S. Therapeutic effect of Colla corii asini on improving anemia and hemoglobin compositions in pregnant women with thalassemia. Int J Hematol 2016;104(5):559–65.
[9] Luo X, Li S. Compendium of materia medica: bencao gangmu. Beijing: Foreign Languages Press; 2003.
[10] Passegué E, Jamieson CH, Ailles LE, Weissman IL. Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a reacquisition of stem cell characteristics? Proc Natl Acad Sci 2003;100(suppl 1):11842–9.
[11] Cheng H, Zheng Z, Cheng T. New paradigms on hematopoietic stem cell differentiation. Protein Cell 2020;11(1):34–44.
[12] Shao L, Wang Y, Chang J, Luo Y, Meng A, Zhou D. Hematopoietic stem cell senescence and cancer therapy-induced long-term bone marrow injury. Trans Cancer Res 2013;2(5):397–411.
[13] Thomas J, Liu F, Link DC. Mechanisms of mobilization of hematopoietic progenitors with granulocyte colony-stimulating factor. Curr Opin Hematol 2002;9(3):183–9.
[14] Freund M, Link H, Welte K. Cytokines in hemopoiesis, oncology, and AIDS. Heidelberg: Springer-Verlag; 2012.
[15] Avalos BR, Lazaryan A, Copelan EA. Can G-CSF cause leukemia in hematopoietic stem cell donors? Biol Blood Marrow Transplant 2011;17(12):1739–46.
[16] Kisielow P. How does the immune system learn to distinguish between good and evil? The first definitive studies of T cell central tolerance and positive selection. Immunogenetics 2019;71:513–8.
[17] Chang S, Collins PL, Aune TM. T-bet dependent removal of Sin3A-histone deacetylase complexes at the Ifng locus drives Th1 differentiation. J Immunol 2008;181(12):8372–81.
[18] Ansel KM, Djuretic I, Tanasa B, Rao A. Regulation of Th2 differentiation and Il4 locus accessibility. Annu Rev Immunol 2006;24:607–56.
[19] Groux H, Powrie F. Regulatory T cells and inflammatory bowel disease. Immunol Today 1999;20(10):442–5.
[20] Socolovsky M, Lodish HF, Daley GQ. Control of hematopoietic differentiation: lack of specificity in signaling by cytokine receptors. Proc Natl Acad Sci 1998;95(12):6573–5.
[21] Shi Y, Liu CH, Roberts AI, Das J, Xu G, Ren G, et al. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and T-cell responses: what we do and don't know. Cell Res 2006;16(2):126–33.
[22] Wang Y, Meng Q, Qiao H, Jiang H, Sun X. Role of the spleen in cyclophosphamide-induced hematosuppression and extramedullary hematopoiesis in mice. Arch Med Res 2009;40(4):249–55.
[23] Oda A, Tezuka T, Ueno Y, Hosoda S, Amemiya Y, Notsu C, Kasahara T, Nishiyama C, Goitsuka R. Niche-induced extramedullary hematopoiesis in the spleen is regulated by the transcription factor Tlx1. Sci Rep 2018;8(1):8308.
[24] Rawlings JS, Rosler KM, Harrison DA. The JAK/STAT signaling pathway. J Cell Sci 2004;117(8):1281–3.
[25] Seif F, Khoshmirsafa M, Aazami H, Mohsenzadegan M, Sedighi G, Bahar M. The role of JAK-STAT signaling pathway and its regulators in the fate of T helper cells. Cell Commun Signal 2017;15(1):23.
[26] Herrington J, Smit LS, Schwartz J, Carter-Su C. The role of STAT proteins in growth hormone signaling. Oncogene 2000;19(21):2585–97.
[27] Kortylewski M, Kujawski M, Wang T, Wei S, Zhang S, Pilon-Thomas S, Niu G, Kay H, Mulé J, Kerr WG, et al. Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. Nat Med 2005;11(12):1314–21.
[28] Welte T, Zhang SS, Wang T, Zhang Z, Hesslein DG, Yin Z, Kano A, Iwamoto Y, Li E, Craft JE, et al. STAT3 deletion during hematopoiesis causes Crohn's disease-like pathogenesis and lethality: a critical role of STAT3 in innate immunity. Proc Natl Acad Sci 2003;100(4):1879–84.
[29] Bronte V, Pittet MJ. The spleen in local and systemic regulation of immunity. Immunity 2013;39:806–18.
[30] Morris R, Kershaw NJ, Babon JJ. The molecular details of cytokine signaling via the JAK/STAT pathway. Protein Sci 2018;27:1984–2004.