J. Ginseng Res.
Vol. 33, No. 3, 177-182 (2009)

Effects of Non-Saponin Red Ginseng Components (NSRG) on Functions of Macrophages Isolated from Young and Aged Mice

Kyung-Ho Kim[1], Seon-A Jang[2], Kyung-Suk Kim[2], Sulkyoung Park[2], Hye-Jin Park[1], Soo-Jin Lee[1], Suhkneung Pyo[1] and Eun-Hwa Sohn*[,2]

[1]*College of Pharmacy, Sungkyunkwan University, Suwon, 440-746, Republic of Korea*
[2]*Department of Herbal Medicine Resource, Kangwon National University, Samcheok, 245-711, Republic of Korea*
(Received June 8, 2009; Revised July 28, 2009; Accepted August 13, 2009)

Abstract : Macrophages play an important role in the first line of immunologic effects against tumor cells. The effects of nonsaponin red ginseng (NSRG) components on macrophage functions like tumoricidal activity, phagocytic activity, and NO production in young (8-weeks-old) and aged (82-weeks-old) male C57BL/6 mice were assessed in vitro, respectively. The treatment of tumor cells (melanoma B16 cells) with the supernatants of NSRG-treated macrophages resulted in an increase of cytotoxicity at 300 μg/ml in the aged mice, whereas the supernatants did not have a cytotoxic effect in the young mice. It was observed that the supernatants induced the increase of tumor cell proliferation at 150 μg/ml in the young mice, suggesting that the supernatants contain growth factors rather than cytotoxic molecules. In addition, NSRG alone had a direct cytotoxic effect on the B16 tumor cells. NSRG had no effect on the NO production by the macrophages in the young mice, while it significantly increased the level of NO release in the aged mice. There was no difference in the phagocytic activities of the macrophages by NSRG in both groups of mice. These results suggest that NSRG has differential effects on the macrophage functions in young and aged mice.

Key words : nonsaponin red ginseng, aging, macrophage, NO, cytotoxicity

INTRODUCTION

Red ginseng (*Panax ginseng* C.A. Meyer Radix Rubra) is prepared by sequential events of steaming and drying of the root of White ginseng (Ginseng Radix Alba). The heat process during the red ginseng preparation has been found to causes some changes in the composition of ginsenosides.[1)] Hiromichi *et al*. suggests that pharmacological actions of ginseng roots may be derived from its non-saponin fractions as well as from ginsenosides, which is more well-known as active ingredients.[2)] Moreover, several previous studies have also demonstrated the biological activities of NSRG.[2)] For example, acidic polysaccharides from non-saponin red ginseng components (NSRGs) has been shown to prevent obesity or hyperlipemia by enhancing immunity against cancer, inhibiting lipolysis and lowering intestinal absorption of cholesterol or fatty acids.[3,4)] Larina *et al*. also showed that saponin-free fraction from Ginseng significantly improves an oxygen-dependent antimicrobial function of neutrophils.[5)]

Macrophages have been known as an important component of host defense mechanism against bacterial infections and tumorigenesis tumor cells (lymphoma and mastocytoma).[6,7)] In vitro studies have shown that macrophages can be activated by non-specific extracellular signaling to kill tumor cells and this suggest that macrophages might be important in host defense against neoplastic cells in vivo.[8)] Peritoneal macrophages, one of tissue-specific macrophages, can be stimulated by a variety of agents, such as IFN-γ, lipopolysaccharides, and other microbial products.[9,10)] Some of these molecules have been also shown to trigger the release of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, and NO (nitric oxide), and to induce tumoricidal activity in macrophages. Activated macrophages also exhibit increases in their phagocytic activity and the release of various molecules such as cytokines and reactive oxygen intermediates, in order to carry out strong nonspecific immune responses.[11,12)]

Aging is a natural phenomenon, accompanied by physical and memory dysfunction, and is also believed to be

* Corresponding author. E-mail: ehson@kangwon.ac.kr
Phone: +82-33-570-6492, Fax: +82-33-570-6499

concomitant with immune dysfunction.[13,14] Even though there have been several reports on the age-related phenotype changes in immune cell, the effects of immunomodulators on aged cells have not been studied well. Therefore, it would be interesting to examine the differences in the macrophage function between young and aged mice. In this study, we determined whether the macrophage response to NSRG in aged animals is different from that of young ones.

MATERIALS AND METHODS

NSRG preparations

Six-year Korean red ginseng was obtained from the Korean Society of Ginseng. 100 g of 6-year red ginseng was extracted three times with 1000 ml of MeOH for 4 h under reflux. The resulting extract 13.5 g, was subjected to filtration and concentration. 10 g of this was dissolved in 100 ml of water and was extracted 3 times with 100 ml ether. The supernatant was dehydrated by Na_2SO_4, and was decompressed for concentration within 40°C yielding 830 mg of the powder (Fig. 1). The water saturated fraction was extracted three times with 100 ml ethylacetate and was separated with ethylacetate (EtOAc fr.) and water. The water fraction was extracted three more times with 100 ml n-BuOH, filtered and concentrated yielding a 1.4 g saponin fraction. Finally, 10.2 g of the H_2O fraction was obtained by the decompression followed by the condensation of the remaining supernatants (Fig. 1).

Aged mice housing and feeding

Male C57BL/6 mice (7 weeks old) were obtained from the Charles River Breeding Laboratories (Japan). The animals were randomly distributed into five per group. The aged (82 weeks) and young (8 weeks) C57BL/6 male mice were used in this study. The mice were provided with water and food, ad libitum and quarantined under 12 h light : 12 h dark photoperiod in the animal care facility of the Sungkyunkwan University, Suwon, Korea. Animal care was performed following the Institute for Laboratory Animal Research (ILAR) guideline.

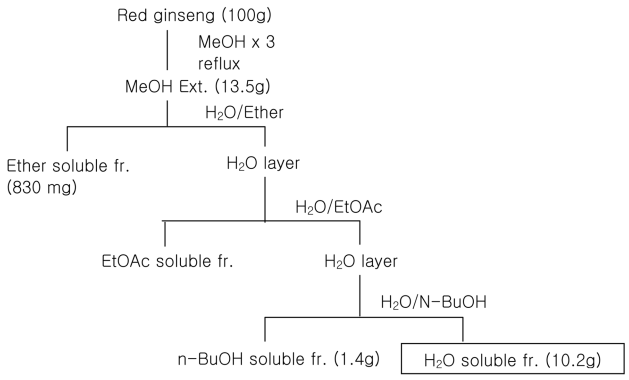

Fig. 1. Various solvent fraction of red ginseng. 10.2 g of the H_2O soluble fraction was obtained from six-year Korean red ginseng as described in Materials and Methods.

Chemicals

Unless stated otherwise, all chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO). The RPMI 1640 medium and fetal bovine serum (FBS) were purchased from GIBCO (Grand Island, NY). The XTT {2,3-Bis (2-methoxy-4-nitro-5-sulfophenyl) -2H-tetrazolium-5-carboxanilide inner salt} cell viability assay kit was purchased from WelGENE (Daegu, South Korea). All the tissue culture reagents and NSRG were assayed for any endotoxin contamination using the Limulus lysate test (E-Toxate, Sigma), and the endotoxin levels were found to be <10 pg/ml.

Macrophage-mediated tumoricidal activity and cell viability

The assay for macrophage-mediated tumoricidal activity was determined using a modification of the method reported by Mosmann *et al.*[15] Briefly, macrophages (1×10^5cells/well) incubated in either medium alone or in medium supplemented with various doses of NSRG for 24 h in 96-well plates. The culture supernatants of macrophages were treated with B16 melanoma cells (5×10^4 cells/well). After incubation of cells for 24 h, 20 μl of phenazine methosulphate (PMS; electron-coupling reagent) and 25 μl of XTT was added to each well. The cells were further incubated for 3 h to allow XTT formazan production. The absorbances were determined with a microplate reader (Menlo Park, CA, USA) at a test wavelength of 450 nm and a reference wavelength of 690 nm.

NBT assay for phagocytosis

Phagocytosis was measured by nitro blue tetrazolium (NBT) reduction assay.[16] Peritoneal macrophages were seeded in 96-well plates at a density 5×10^4 cells per well, treated with various concentration of NSRG and cultured for 24 h. The cultured media was then removed and 50 μl of 5×10^6 particles/mL zymosan and 0.6 mg/mL NBT was added into each well. After an additional incubation for 1 h, wells were washed with cold D-PBS two times and the optical density of reduction product of NBT, a purple

insoluble formazan, was determined at 540 nm using a microplate reader. It was not required to solubilize the formazan before taking the measurement of absorbance.

Nitrite determination

The cells were treated with various doses of NSRG for 24 h and the accumulation of nitrite in culture supernatants was measured using the assay system described by Ding *et al*.[17] 100 μL aliquots of culture supernatants were mixed with an equal volume of Griess reagent (mixture at 1:1 of naphthylethylenediamine dihydrochloride and 1% sulphanilamide in 5% H_3PO_4) and incubated at room temperature for 10 min. Nitrite concentration was calculated from a $NaNO_2$ standard curve.

Statistical analysis

Each experiment was repeated at least two times, and the results of one representative experiment are shown. The results were expressed as means±S.E.M. and analyzed via ANOVA. The significant values are represented by an asterisk (*p<0.05 and **p<0.01).

RESULTS AND DISCUSSION

Effects of NSRG on macrophage-mediated tumoricidal activity and tumor cell cytotoxicity

The immune system may play an important role in aging and the changes in the immune status are associated with treatment of various immunomodulators.[18] This study examined the immunomodulatory effects of NSRG on the functions of macrophage in young and aged mice. We previously reported that NSRG increased NK cell tumoricidal activity at 100 and 300 μg/ml.[19] Therefore we choose these concentrations to determine the immunomodulatory effects of NSRG on peritoneal macrophages from the old and young mice.

To examine whether NSRG enhances the production of tumoricidal components of macrophages against target tumor cells, macrophages from the old and young mice were treated with NSRG for 24 h, and the culture supernatant was added to B16 tumor cells for 24 h. B16 cells were used as target tumor cells since they are either TNF-α or NO sensitive. As shown in Fig. 2, NSRG-treated supernatant enhanced the production of tumoricidal components from macrophages in aged mice at 300 μg/ml. However, it increased the cell proliferation at 150 μg/ml, suggesting that growth factors were produced rather than cytotoxic molecules in macrophages of young mice. Though NSRG-treated macrophages in young mice promotes cell proliferation in B16 tumor cells, this effect should be confirmed in both normal cells and various tumor cell lines to examine whether this effect is cell type specific or not. These results suggest that NSRG induced the production of different molecules from activated macrophages in young and old mice. The present results also showed that NSRG has a direct cytostatic or cytotoxicity of B16 tumor cells (Fig. 3).

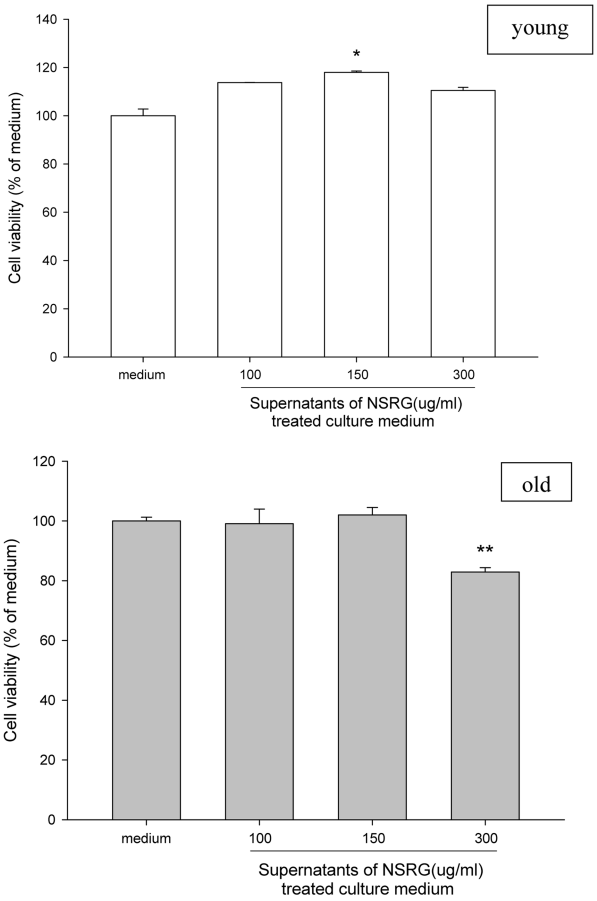

Fig. 2. Effects of NSRG on macrophage-mediated tumoricidal activity in the young and aged mice. The peritoneal macrophages (1×10^5cells/well) from the young and old mice were treated with NSRG and the culture supernatants were added in B16 tumor cells (1×10^4cells/well) for 24 h. Macrophage-mediated tumoricidal activity was determined by XTT assay. The data represents the means±S.E.M. of quadruplicate experiments. *p<0.05, **p<0.01; significantly different from the control supernatant treatment.

Effects of NSRG on nitric oxide production

Activated macrophages have been known to produce various cytokines such as TNF-α, IL-1, IL-6 and nitrite

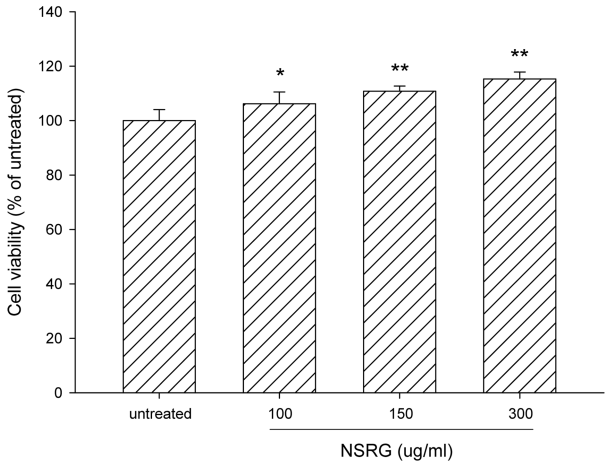

Fig. 3. Effects of NSRG on the cell viability of B16, a mouse melanoma cells. The B16 tumor cells (4×10^4cells/well) were treated with NSRG for 24 h. Cell viability was determined by XTT assay. The data represents the means±S.E.M. of quadruplicate experiments. *p<0.05, **p<0.01; significantly different from the control supernatant treatment.

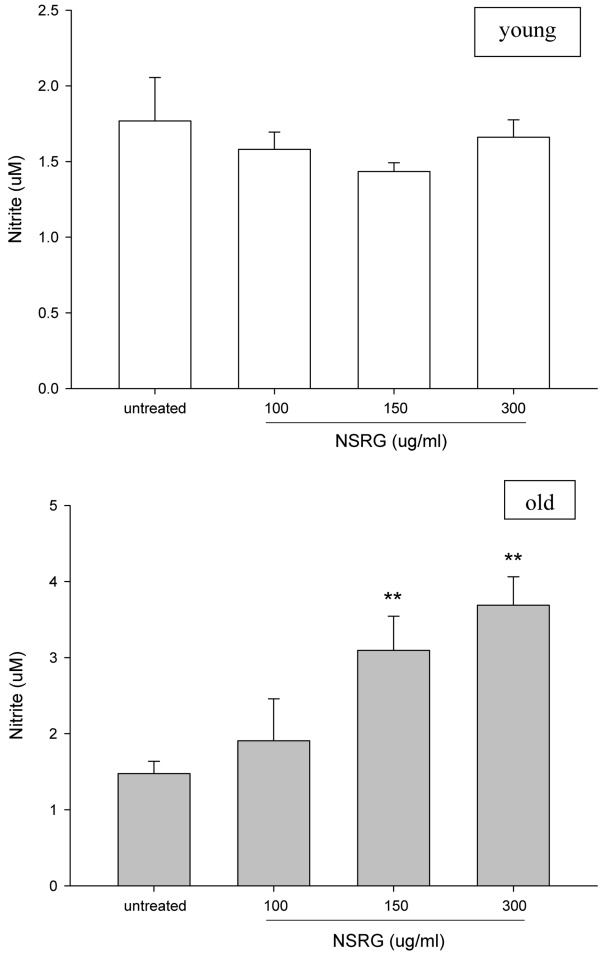

Fig. 4. The effects of NSRG on production of nitrite in peritoneal mcarophages from the young and aged mice. Macrophages (1×10^4cells/well) were treated with various concentration of in NSRG for 24 h. Culture supernatants were collected and the levels of nitrite were measured as described in Materials and Methods. The data represents the mean± S.E.M. of quadruplicate experiments. **p<0.01; significantly different from the control (no treatment).

that are involved in tumoricidal activity. Since NO is related to cytostatic or cytotoxic function of macrophages against a variety of tumors,[12,20] we examined the effects of NSRG on NO production from macrophages in the old and young mice, respectively. As shown in Fig. 4, NSRG significantly increased NO production in old mice in dose-dependant manners. With increasing amount of NO released, the supernatant from NSRG-treated macrophages slightly increased tumoricidal activity at 300 μg/ml in aged mice, while NSRG had no effect on both NO production and tumoricidal activity in young mice. These data indicate that NO might be involved in tumoricidal activity of NSRG-treated macrophage in old mice.

Effects of NSRG on Phagocytosis

Phagocytosis is the primary role of macrophage, which leads macrophage to enhance a diverse range of antimicrobial and cytotoxic responses including generation of respiratory bust, secretion of inflammatory mediators and antigen presentation. In this study, we examined the effects of NSRG on phagocytosis of macrophages from the old and young mice. Our data demonstrate that treatment with NSRG resulted in no effect on phagocytosis of macrophage from the both mice (data not shown).

Age-related involution brings about many alterations in immune system. Despite the fact that thymic atrophy and diminished output of T lymphocytes are the most recognized changes, it is increasingly evident that the effects of aging on altering immune system are widespread, extending from hematopoietic stem cells and earlier lymphoid progenitors to mature lymphocytes in secondary lymphoid organs and immune cells.[21-25]

It has been reported that aging-associated immunological alterations can be found in lymphoid organs as well as in cellular components of immune system, and T cells are especially more susceptible to the effects of aging.[26] Hiromoto *et al*. has been reported that there is a significant difference of gene expression in murine thymic aged-related cell cycle genes.[27] These data indicated that expres-

sion of some genes is exclusive in thymus from 4 to 40 weeks old mice, while other genes are expressed at different ages. In addition, it has been recently known that total antibody production, frequency of antibody-producing cells, and proliferative response of spleen T cells were enhanced in aged mice, whereas some antigen-specific antibody responses were decreased.[18] Our results also showed that NSRG has several different effects on functions of macrophage because of age-related alterations in immune system.

In summary, NSRG has cytotoxic effects on B16 tumor cells directly. The supernatant of NSRG-treated macrophages increased tumoricidal activity and NO production in aged mice, whereas it had no effect in young mice at same concentration. However, the culture supernatant of NSRG-stimulated macrophage increased B16 cell proliferation in young mice. This suggests that NSRG might induce the production of different type of molecules from macrophages in young and old mice. In addition, it appears that NSRG mediates cellular activities such as cell growth or cell death at different ages.

Many researchers have believed that nutritional effects of natural products are similar in young and old age. If one have good immunological effects in young, it might be good for the old. However, because our data suggest that NSRG has differential immunomodulating effects on macrophages in young and aged mice, nutritional intake for elder people might need to be considered to select proper immunomodulators and NSRG is more useful for old people to treat cancer.

REFERENCES

1. Nam KY. The comparative understanding between red ginseng and white ginsengs, processed ginseng (*Panax ginseng* C.A. Mayer). Korea J Ginseng Sci. 29: 1-18 (2005)
2. Okuda H, Lee SD, Matsuura Y, Zheng Y, Sekiya K, Takaku T, Kameda K, Hirose K, Ohtani K, Tanaka O, Sakata T. Biological activities of non-saponin compounds isolated from Korea Red Ginseng. Korea J Ginseng Sci. 14: 157-161 (1990)
3. Lee SD, Lee KS, Okuda H, Hwang WI. Inhibitory effect of crude acidic polysaccharide of Korean ginseng on lipolutic action of toxohormone-L from cancerous ascites fluid. Korea J Ginseng Sci. 14: 10-13 (1990)
4. Okuda H. Biological activities of non-saponin compounds isolated from Korean red ginseng. Proc. Int'l Ginseng Symp. on Korean Ginseng. P. 15-19 (1990)
5. Larina L, Cho BG, Ten L, Park H. Isolation of saponin-free fraction from ginseng (*Panax ginseng* C.A. Meyer) and its effects on the function of neutrophils. soluble non saponin constituents from Korean red ginseng (*Panax ginseng* C.A. Meyer): Immunomodulating activities of acidic polysaccharide. Proc of 99 Korea-Japan Ginseng Symp. P. 191 (1999)
6. Hahn H, Kaufmann SH. The role of cellmediated immunity in bacterial infections. Rev Infect Dis. 3: 1221–1250 (1981)
7. Verstovsek S, Maccubbin DL, Ehrke MJ, Mihich E. Tumoricidal activation of murine resident peritoneal macrophages by interleukin-2 and tumor necrosis factor. Cancer Res. 52: 3880-3885 (1992)
8. Klimp AH, de Vries EG, Scherphof GL, Daemen T. A potential role of macrophage activation in the treatment of cancer. Crit Rev Oncol Hematol. 44: 143-161 (2002)
9. Gautam S, Deodhar S. Generation of tumoricidal effector cells by human C-reactive protein and muramyl tripeptide: a comparative study. J Biol Response Mod. 8: 560-569 (1989)
10. Paulnock DM, Lambert LE. Identification and characterization of monoclonal antibodies specific for macrophages at intermediate stages in the tumoricidal activation pathway. J Immunol. 144: 765-773 (1988)
11. Choriki M, Freudenberg M, Calanos C, Poindron P, Bartholeyns J. Antitumoral effects of lipopolisacchride, tumor necrosis factor, interferon and activated macrophages: synergism and tissue distribution. Anticancer Res. 9: 1185-1190 (1989)
12. Keller R, Keist R, Frei K. Lymphokines and bacteria that induce tumoricidal activity, trigger a different secretory response in macrophages. Eur J Immunol. 20: 695-698 (1990)
13. Takeoka Y, Chen SY, Yago H, Boyd R, Suehiro S, Shultz LD, Ansari AA, Gershwin ME. The murine thymic microenvironment: changes with age. Int Arch Allergy Immunol. 111: 5-12 (1996)
14. Boehmer ED, Goral J, Faunce DE, Kovacs EJ. Age-dependent decrease in Toll-like receptor 4-mediated proinflammatory cytokine production and mitogen-activated protein kinase expression. J Leukoc Biol. 75: 342-9 (2004)
15. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55-63 (1983)
16. Okimura T, Ogawa M, Yamauchi T. Stress and immune responses. III. Effect of resistant stress on delayed type hypersensitivity (DTH) response, natural killer (NK) activity and phagocytosis in mice. Jpn J Pharmacol. 41: 229-235 (1986)
17. Ding AH, Nathan CF, Stuer DJ. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. J Immunol. 141: 2407-2412 (1988)
18. Speziali E, Santiago AF, Fernandes RM, Vaz NM, Menezes JS, Faria AM. Specific immune responses but not basal functions of B and T cells are impaired in aged mice. Cell

Immunol. 256: 1-5 (2009)
19. Sohn EH, Kim J, Choi H, Park HJ, Kim BO, Rhlee DK, Pyo SN. Immunomodulatory effects of non-saponin red ginseng components on innate immune cells. J Ginseng Res. 32: 67-72 (2008)
20. Hibbs JB Jr, Taintor RR, Vavrin Z. Macrophage cytotoxicity: role for L-arginine deaminase and imino nitrogen oxidation to nitrite. Science 235: 473-476 (1987)
21. Taub DD, Longo DL. Insights into thymic aging and regeneration. Immunol Rev. 205: 72-93 (2005)
22. Haynes L, Eaton SM, Burns EM, Randall TD, Swain SL. CD4T cell memory derived from young na cells functions well into old age, while memory generated from aged na cells functions poorly. Proc Natl Acad Sci USA 100: 15053-15058 (2003)
23. Miller JP, Allman D. The decline in B lymphopoiesis in aged mice reflects loss of very early B-lineage precursors. J Immunol. 171: 2326-2330 (2003)
24. Benner R, Hijmans W, Haaijman JJ. The bone marrow: the major source of serum immunoglobulins, but still a neglected site of antibody formation. Clin exp Immunol. 46: 1-8 (1981)
25. Miller RA. The aging immune system: primer and prospectus. Science 273: 70-79 (1996)
26. Taub DD, Longo DL. Insights into thymic aging and regeneration. Immunol Rev. 205: 72-93 (2005)
27. Li YH, Su H, He W, Cui LX, Hiromoto O. Differential expression analysis and cloning of murine thymic aged-related genes. Zhongguo Yi Xue Ke Xue Yuan Xue Bao. 23: 158-62 (2001)