Phenotypic Correction of Age-associated Functional Decline in Murine Immune Cells by Thymax, A Thymic Extract

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Abstract. Background: We have recently demonstrated that Thymax, a gross thymic extract, induces the functional activity of human dendritic cells (DCs) in vitro. In this study, the role of Thymax in phenotypic correction of age-associated functional decline in immune cells in mice was evaluated. Materials and Methods: C57BL/6 mice (13 months old) were treated with Thymax orally (20% v/v) for 4 weeks. Different splenic cell types, dendritic cells (DCs), B-cells, T-cells and natural killer (NK) cells, were analyzed using flow cytometry. Results: Treatment with Thymax resulted in: i) a significant increase in the percentages of DCs (1.6-fold), B-cells (7-fold) and T-cells (5-fold) over the control (p<0.05); ii) an increase in the percentages of activation markers (CD25 and CD69) of CD4+ and CD8+ T-cells; and iii) an enhancement in NK activity. Thymax showed no adverse side-effects. Conclusion: Thymax might have a role in reversing immune dysfunction in the elderly.

Aging is an inevitable process that affects all cells, organs and organisms, diminishing homeostasis and increasing organism vulnerability (1). Immunosenescence refers to the decline in immune function associated with aging in humans and animals (2). The consequences of dysregulation of both acquired and innate immunity in the elderly may partially account for the increased susceptibility to infections, malignancies and autoimmunity (3, 4). Age-associated immunological alterations occur in many types of immune cells, affecting both the phenotype and function. These include dendritic cells (DCs), T-cells, B-cells, natural killer (NK) cells, macrophages, neutrophils and natural killer T cells (5, 6).

Hematopoietic precursor stem cells differentiate into immature dendritic cells (iDCs), which are recruited to the periphery where they continuously sample antigens. These iDCs then migrate to the regional lymph nodes where they phenotypically mature (7). Maturation of DCs results in the up-regulation of co-stimulatory and maturation markers, enhancing their capacity to present antigens to T-cells. Thus, DCs are essential for the induction of a primary cytotoxic T lymphocyte (CTL) response to tumor cells (8). In addition, these mature DCs interact with B-cells and NK cells to elicit their specific responses and differentiate into functionally mature DCs. Therefore, DCs play a major role in the induction of tolerance and the initiation and regulation of the immune response (9-13). Immunotherapy with DCs has been applied to various types of cancer (14, 15).

Recently, the role of DCs in immune senescence and in the chronic inflammatory state in aging has attracted the attention of many scientists. Aged individuals demonstrated a reduced number of circulating and plasmacytoid DCs (PDCs) and reduced IFN-α secretion (16). An impairment of migration of DCs is seen in the aged, in spite of the similarity of the cell surface receptors between young and aged individuals (17), suggesting that the defect is in downstream-signaling pathways. Phosphoinositide-3- (PI3) kinase has been demonstrated to play a critical role in both phagocytosis and migration of DCs (18, 19).

Studies over the last four decades on the immunological view of aging suggest that alterations in T- and B-cell populations and in both antibody- and cell-mediated immune responses lead to immune dysfunctions, which may affect disease incidence and life span (20). Age affects cell numbers and functions rather than the cellular milieu (21). Similarly, aging has been demonstrated to have deleterious effects upon NK cells. Previous studies by us and others showed aged mice exhibit a significant decline in NK cell activation, which was greatly associated with an increased incidence of neoplasia as the mice aged (22-24).

Since age is associated with a decline in immune function, it is of utmost interest to find agents that activate the immune cells during aging. Thymax, a gross thymic extract, exerts an apoptotic effect on human breast cancer cells in vitro (25). In addition, we have recently demonstrated that Thymax has

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the ability to activate human DCs in vitro (26). In the current study, we extend our research to evaluate the role of Thymax in phenotypic correction of age-associated functional decline in immune cells in mice.

Materials and Methods

Animals. Male C57BL/6 mice (13 months old) weighing 19 to 21 g were purchased from Harlan Laboratories (Chicago, IL, USA). Mice were housed 5 per cage at constant temperature (24˚C ± 2˚C) with alternating 12-h light and dark cycles. Animals were provided with standard cube pellets and water ad libitum. The water intake (water alone and water mixed with Thymax) was monitored during the course of the experiment (4 weeks). This study was approved by the Institutional Animal Care and Use Committee (IACUC) at Drew University of Medicine and Science.

Tumor cell line. A yeast artificial chromosome-1 (YAC-1) cell line (a Moloney leukemia virus-induced mouse T-cell lymphoma of A/Sn mice origin) obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) was acquired for use in NK activity assays.

Complete medium (CM). RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum, 2 mM glutamine, and 100 μg/ml streptomycin and penicillin was used to maintain cell cultures.

Thymax. Thymax is a gross thymic extract with heat-resistant peptides contained in acidic pH (through treatment with NaCl and L-ascorbic acid) as described elsewhere (25). Thymax was offered by YS Nature Company, Tokyo, Japan. Thymax differs from other thymus extracts in that it is a complex of thymosin, thymomodulin, and many other peptides. In addition, unlike other thymic factors, Thymax is introduced into the body by mouth. Attempts are currently being made to identify its active components.

Experimental design. Mice were randomly divided into 2 groups: i) mice receiving Thymax (330 μl/day/mouse [20% v/v]; n=5) and ii) a control group of mice (n=5) given only water. Animals received Thymax orally by mixing Thymax with drinking water. Mice received freshly prepared Thymax daily and treatment was continued daily for 28 days. At the end of the treatment period, mice were euthanized, and spleens were removed and examined for: i) Animal behavior. Thymax-treated mice were examined daily for adverse side-effects by assessing the changes in the normal feeding/drinking cycles and life activity patterns for the entire treatment period. (ii) Histopathological analysis. Thymax-treated mice were killed after the 28-day treatment period. Twelve different organs were excised and tissues from bone marrow, brain, colon, esophagus, heart, kidneys, liver, lungs, pancreas, small intestine, skeletal muscle, and stomach were fixed in 10% formalin and processed overnight. Paraffin embedded sections of 3-5 μm were stained with hematoxylin and eosin (H&E) and examined using a light microscope. Histopathological changes were compared with those of control mice.

Statistical analysis. Statistical significance was determined by the Student’s t-test. Differences were considered significant at the p<0.05 level.

Results

Percentage of dendritic cells (DCs). Treatment with Thymax caused a significant increase in the percentage of DCs. Data in Figure 1 show that in Thymax-treated mice, DCs reached
8%, as compared to 5% for control mice, representing a 1.6-fold increase over control mice ($p<0.05$).

**Proliferation of B-cells and T-cells.** Treatment with Thymax caused a significant increase in proliferation of B-cells by LPS. Data in Figure 2 show 36% B-cell proliferation in Thymax-treated mice as compared with 5% for control mice. This represents about a 7-fold increase over the control value ($p<0.01$). Data also show a 5-fold increase in proliferation of T-cells post-treatment with Thymax as compared with the controls.

**Percentages of CD25 and CD69 cells.** Data in Figure 3 show that treatment with Thymax caused up-regulation of CD25 and CD69, the activation markers of CD4+ and CD8+ T-cells.

**NK cell activity.** Thymax-treated mice showed elevated NK cell activity as compared to control untreated mice. Thymax-induced enhancement in NK activity was dependent on various effector:target (E:T) ratios. Data depicted in Figure 4 show a 2.5-, 1.9-, and 1.5-fold increase in NK activity for Thymax-treated mice over the control values at E:T ratios of 12.5:1, 25:1, and 100:1 respectively. When data were calculated in terms of LUs, statistical analysis showed a significant increase ($p<0.05$) in NK cell activity in Thymax-treated mice (17.9 LUs) as compared to control mice (9.1 LUs).

**Adverse effects of Thymax (toxicity).** We examined adverse effects of Thymax treatments from two different aspects: animal behavior and histopathological examination of different organs extracted from mice treated with Thymax.

(i) **Animal behavior.** Daily examinations of mice showed that the 28-day treatment period with Thymax resulted in no adverse side-effects as indicated by normal feeding/drinking and life activity patterns during the treatment period.

(ii) **Histopathology.** Thymax-treated animals were sacrificed and 12 different organs were examined for pathology. Data in Figure 5 show that histopathological examination of the liver, lung and brain obtained from Thymax-treated animals were within the normal limits as compared to control mice. Similar results were seen with other organs.

**Water/Thymax consumption.** We did notice that the control, untreated mice consumed only 3 ml/day/mouse drinking water (Figure 6). On the other hand, Thymax-treated mice consumed significantly more, 5ml/day/mouse of drinking water mixed with Thymax ($p<0.001$).

**Discussion**

Different cell types in the immune system exhibit a decline in their activity with advancing age, which contributes to an increased incidence of cancer and infectious diseases (28, 29). As mice age, the immune system displays an impairment of DC activation (30, 31), decline in T-cell number, proliferation and activation (32-35), a qualitative deficiency of B-cell antibody-mediated immune responses (36-38) and decline in NK cell cytotoxicity (39-41). Recent attention has focused on the ability of biological response modifiers (BRMs) to augment immune responses in the aged. We have established the fact that Thymax is a potent immunomodulator in the aged based on the findings that Thymax treatment resulted in an increase in the percentages of DCs, proliferation B-cells, and T-cells, an increase in the percentages of activation markers of T-cells, and an enhancement in NK activity. These data complement our recent study on the role of Thymax in induction of the

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Figure 1. Effect of Thymax on the percentage of splenic DCs in vivo. Mice were treated with Thymax for 4 weeks. Spleens were used to examine DC numbers by surface CD11c antibody staining. Data represent the mean±SD of 5 mice in each group (Thymax-treated group and control group). *$p<0.05$, as compared to control untreated mice.

Figure 2. Effect of Thymax on the percentages of splenic B-cell and T-cell proliferation in vivo. Mice were treated with Thymax for 4 weeks. Spleens were used to examine B-cell and T-cell proliferation stimulated with LPS using CFSE dye dilution assay. Data represent the mean±SD of 5 mice in each group (Thymax-treated group and control group). *$p<0.01$, as compared to control untreated mice.
functional activity of human DCs, in the increase in CD4+ T-cells and in the secretion of interferon (IFN)-γ in vitro (26). Some thymic extracts have been shown to enhance immune function, such as TP-5 (42, 43), while others such as TP-1 did not affect the immune system (44) or had a differential effect within a group of patients (45). Thymax is a gross thymic extract with heat-resistant peptides contained in acidic pH (25). Thymax differs from other thymus extracts in that it is a complex of thymosin, thymomodulin and many other peptides, and is introduced into the body by mouth. The mechanism(s) by which Thymax exerts its effect on splenic immune cells is not known, but could be attributed to up-regulation of IFN-γ and interleukin (IL)-12 production. Our recent study showed that Thymax enhances the production of both cytokines from human peripheral blood mononuclear cells (PBMC) (26). These cytokines play an important role in the activation and differentiation of NK cells and T-cells (46-49). Alternatively, the effect could be due to the suppressive effect of Thymax on IL-10 cytokine secretion. A recent study demonstrated that impaired NK cell activation and IFN-γ-producing CD4+ T-cell differentiation in aged mice could be related to IL-10-mediated suppression of the innate IL-12/ IFN-γ axis (50). The authors confirmed this finding by blocking IL-10 signaling, which resulted in the restoration of IFN-γ-producing NK cells in aged mice. In addition, the increase of T-cell proliferation post-treatment with Thymax in vitro may be due to up-regulation of CD80 and CD86. We
recently demonstrated that Thymax-treated human DCs caused up-regulation of these two molecules, which provide co-stimulatory signals for optimal T-cell activation (51). This cell signaling is mediated by CD28/CTLA4-CD80/CD86 (CD28/B7) (52) and plays an essential role in the proliferation and survival of CD4+ T-cells (53-56). In the current study, Thymax demonstrated the ability to reverse age-related functional decline in B-cells. A significant increase in the percentage of splenic B-cells responding to LPS occurred after treatment with Thymax (36%), as compared to 5% for the aged control mice (*p<0.01), representing about a 7-fold increase over the control mice. The mechanism by which Thymax exerts its effect on B-cells remains to be investigated.

The spleen contains CD8α+CD205+ DCs in its periarteriolar lymphatic sheaths and CD8α− DCs in its marginal zones (57, 58). As mice age, DCs display an impairment of CD40 and CD86 co-stimulatory molecule expression. This expression could be restored to levels of young mice by inactivating T regulatory cells with anti-CD25 monoclonal antibodies (30). In our study, Thymax treatment also increased the percentages of DCs. The

Figure 5. Histopathology of Thymax-treated mice. Mice were treated with Thymax for 4 weeks. Animals were euthanized and different organs were harvested for histopathological examination, stained with H&E. Results indicate that pathology induced by Thymax is within normal limits.

Figure 6. Water/Thymax consumption. Mice received drinking water alone (control) or drinking water mixed with Thymax. Water consumption was recorded daily for the 4 week treatment period. Data represent the means±SD of 5 mice in each group. *p<0.001.
The mechanism underlying the effect of Thymax is not known, but it may involve interference in the maturation/activation state of DCs. The DC system contains conventional DCs and PDCs. Both types of cells are produced from bone marrow (59). It has been proposed that tolerance induction by DCs requires maturation signals different from microbial or inflammatory stimuli. Thymax may induce maturation signals driving the differentiation of tolerogenic DCs. Our recent study showed the role of Thymax in induction of the functional activity of human DCs with respect to co-stimulatory molecule expression and cytokine secretion in vitro. Thymax activated DCs to secrete IL-12p40 and IL-6 cytokines and inhibited IL-10 production. Additionally, Thymax caused up-regulation of DC CD80 and CD86 (26).

The immune system can be modified by pharmacological agents and naturally occurring biological response modifiers (BRMs). A few polynucleotides such as Poly I:C and double-stranded RNAs, the bacterial immunopotentiators, bacille Calmette-Guérin (BCG), killed streptococcal preparations (OK432) and Corynebacterium parvum (CP), and IL-2 are capable of augmentation of natural immunity or interfering directly with cancer cell proliferation (39, 60–63). However, the clinical use of these BRMs and lymphokine-activated killer (LAK) cell therapy has been limited because of their severe side effects (64–66). The use of natural BRMs to modulate the immune system in aged mice and in humans was the focus of research in our laboratory. These BRMs include a modified arabinoxylan from rice bran (MGN-3) (67, 68), and marina crystal minerals (MCM) (69). Enhancement of NK activity was associated with an increase in tumor necrosis factor (TNF)-α and IFN-γ production and in the granular content of NK cells. In addition, an increase in T- and B-cell mitogen response was noted, and an inhibition in tumor growth was detected using an experimental animal model system (70, 71) and in cancer patients (72, 73).

Data of the immune modulatory function by Thymax in aged mice in vivo and in human PBMC in vitro suggest that such interest should be given to this newly developed BRM, which does not appear to have side-effects. Thymax-treated animals were monitored to observe potential toxic side-effects of Thymax treatment. Results demonstrated normal animal behavior and different organs of these mice had no pathology detected up to 28 days post-treatment with Thymax. Further studies are needed to optimize the immune modulatory activities of Thymax with respect to duration of treatment and dose responsiveness.

Conclusion

Thymax induces phenotypic correction of age-associated functional decline in immune cells in mice in vivo, which might have a potential role in reversing immune dysfunction in the elderly.

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References


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