博士論文（要約）

Studies on altered immune functions and their mechanisms during aging process

（加齢による免疫機能の変化及びそのメカニズムに関する研究）

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Contents

Abbreviations

APCs	antigen presenting cells
ATP	adenosine triphosphate
BEC	blood endothelial cells
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CD	cluster of differentiation
cDNA	complementary DNA
CRP	C-reactive protein
DC	dendritic cell
DHE	dihydroethidium
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothyocyanate
FRC	fibroblastic reticular cells
IFN-γ	interferon-γ
IL	interleukin
IL-1	interleukin-1
IL-6	interleukin-6
IL-17	interleukin-17
Ig	immunoglobulin
LEC	lymphatic endothelial cells
LPS	lipopolysaccharide
MACS	magnetic cell sorting
MAPK	mitogen-activated protein kinase
MLN	mesenteric lymph node
mtDNA	mitochondrial DNA
NF-kappaB	nuclear factor-kappa B

OVA	ovalbumin
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PP	Peyer's patch
RA	retinoic acid
RALDH	retinaldehyde dehydrogenase
RAR	retinoic acid receptor
RNA	ribonucleic acid
RXR	retinoid X receptor
SLO	secondary lymphoid organs
SPL	spleen
TCR	T cell receptor
TNF-α	tumor necrosis factor α
T reg	regulatory T cell

Background

Progress in medical and healthcare and invention of vaccination and antibiotics make an increased lifespan (1). Population aged over 60 is rapidly increasing and it is estimated that people older than 65 years would be 2 billion by 2050 (2, 3). It implies the necessity of the planning and social care for the elderly. Aging process is sometimes accompanied by age-related diseases and it also causes alterations of organ systems, response to antigen, and cellular maintenance pathways, which result in impinging on the individual's quality of life (4).

It has been assumed that lifelong accumulation of molecular and/or cellular damages causes aging but there is no clear answer why people age and when people start to age (5). Many theories exist which explain the aging process, but there is no theory fully explains aging process. Programmed theory and the damage or error theory have been considered as a basis theory of biological aging and immunological theory, which belong to the programmed theory categories, also explains aging process. It accounts that augmented vulnerability to infection results from immune system programmed to decline with aging induces aging process and eventually death (6, 7).

The ability to resist against to pathogens, diseases or injury decreases with aging and some of the noticeable features are alterations in the development and maintenance of immune response and cellular function (8-10). Pro-inflammatory cytokines show age-associated increased production and these productions could induce the onset of inflammatory diseases (11). Intestinal function, which is important in maintain of health, also changes with aging (12). Aging triggers suchlike changes of immune responses and as a result of unregulated immune functions against infection and inflammation, a number of complications and chronic medical conditions occur (10, 13, 14).

Systemic inflammatory response syndrome is induced by uncontrolled immune response to trauma or infection that normally protective responses became harmful provoking shock and organ dysfunctions (10). Concerning the adaptive immune system, the age-associated changes are well characterized. The apparent features are changes

in T cell subsets and thymic involution (11, 15). T cells show decreased CD28 expression on their surface, which is a crucial factor for stimulation needed for TCR-mediated activation (16) and changed chemokine receptor expression on the surface (17). Innate immunity, however, is assumed to be activated during aging, which called inflamm-aging. Inflamm-aging indicates the chronic inflammatory state resulting from increased secretion of pro-inflammatory cytokines and mediators in the elderly (18, 19). With increasing age, a decline in the function of immune system is accompanied, which results in augmented morbidity and mortality. Immune aging is believed as a loss of effectiveness and it also causes increased inflammation and makes easy to develop autoimmune diseases (20). With aging, increased inflammatory markers such as IL-6, TNF-α, and CRP (C-reactive protein) lead to chronic diseases and disability (21). Thymic involution, which induces change of naïve T and B cell compartments, and increased titers of autoantibodies generate impaired immune response (22). These age-associated alterations are referred as immunosenescence, which means the state of changed immune function with aging. Although age-associated change in immune system may have significant impact on health of individuals, little is known about the mechanisms of unregulated immune response with aging.

The aim of this study was to unveil the mechanisms of immunosenescence by examining the altered immune functions and responses with aging. I investigated the roles of aging on functional changes of immune response on cellular level and explored several substances that have an ability to modulate the age-associated altered immune functions.

Chapter 1. Splenic stromal cells from aged mice produced high level of IL-6 during aging

1.1 Introduction

The elderly are susceptible to autoimmune diseases, bacterial pathogens, and viral exposure and the occurrence of autoimmune diseases, infectious diseases, and cancer increases in the elderly (23, 24). Also a number of alterations in immune system, such as altered cytokine profile might facilitate allergic associated conditions in the elderly (25, 26).

Pro-inflammatory cytokines, such as TNF-α, IL-1, and IL-6 are significantly related to the age-associated diseases and disability, and these are important factors on inflammatory response in the elderly (27-29). Especially, aged IL-6 knockout mice show reduced mortality compared to wild type mice after injection with LPS and anti-IL-6 receptor antibodies are good formulation for rheumatoid arthritis (10). IL-6 produced in multiple tissues during inflammation stimulates immune process and the production of IL-6 is elevated in some tissues of aged mice compared to young adult mice such as lung, kidney, skeletal muscle, heart, fat, and spleen (10, 30). Under stressful or depressed conditions, IL-6 levels are increased (31). Moreover, IL-6 is detected in high levels in disease conditions including autoimmune disease, such as rheumatoid arthritis, atherosclerosis, diabetes, and inflammatory-bowel diseases (32, 33). It implies IL-6 might play roles that boost the risk and mortality in inflammation in the elderly.

IL-6 is mainly produced by cells such as macrophages and dendritic cells (DCs). In secondary lymphoid organs (SLOs), where immune responses are initiated, a number of hematopoietic cells, including macrophages and DCs that were mentioned above, interact with each other (34). In addition to these immune cells, stromal cells are also present in SLOs. Stromal cells have been considered as cells that do not significantly affect the immune response but give a structural support to the lymphoid organs. However, the functions of stromal cells in immunehomeostasis and immune responses have begun to emerge (35). Stromal cell population can be distinguished into four groups depending on the expression of CD31 and Gp38 on the cell surface, BECs (blood endothelial cells, $CD31^{+}Gp38^{-}$), FRCs (fibroblastic reticular cells, $CD31^{-}Gp38^{+}$), LEC (lymphatic endothelial cells, $CD31^{+}Gp38^{+}$), and the double negative populations.

In this study, the principle objective was to identify cell types that were affected by

aging concerning secretion of IL-6 in the murine model. To answer these questions, IL-6 production in splenocytes was compared from both young and aged mice and a number of cell types were isolated from spleen and investigated IL-6 mRNA or protein expression. It was found that stromal cells express IL-6 highly with aging both in noninflammatory and inflammatory conditions.

1.2 Materials and Methods

1.2.1 Animals

C57BL/6 female mice were obtained from CLEA Japan, Inc. The mice were used in the experiments at 2-4 mo for young mice, and at 11-18 mo for aged mice, respectively. All procedures were performed in accordance with guidelines for animal use and care of the University of Tokyo.

1.2.2 Culture medium

Cells were cultured in RPMI 1640 medium supplemented with 5 % FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 3 mM L-glutamine, and 50 μM 2-ME.

1.2.3 Isolation of cells and cell culture

Spleens were removed from mice and digested with collagenase type1. After red blood cell lysis, white blood cells were counted. Five×10^5 whole splenocytes were seeded in 96-well plates in 200 μl RPMI medium and incubated for 24 hours at 37 °C and 5 % CO_2. Macrophages, $CD11c^+$ DCs and $CD45^-$ stromal cells were isolated by MACS (Miltenyi Biotech). In short, cells were incubated with anti-mouse F4/80, CD11b, CD11c, and CD45-coated magnetic beads (Miltenyi Biotech), respectively, and selected on MACS separation columns (Miltenyi Biotech). Macrophages were prepared by F4/80 positive selection, or CD11c negative selection and CD11b positive selection. DCs were isolated as $CD11c^+$ cells, and stromal cells as $CD45^-$ cells, respectively. In certain experiments, $CD45^+$ cells were also used. MACS purified macrophages, DCs or $CD45^-$ cells were seeded in 96-well plates (5 × 10^5 cells/well) in 200 μl RPMI medium with or without LPS and cultured for 24h. Five×10^4 cells of $CD45^-$ stromal cells were cultured in the same manner as above. Since the splenic $CD45^-$ stromal cells form a dense network and FRCs, a kind of stromal cells, are connected to each other in spleen forming a

three-dimensional reticulum, lower concentration of cells were used for measurement of IL-6 protein production compared to other cell types (36, 37).

1.2.4 Exposure to LPS

Acute inflammation was induced by i.p. injection with LPS derived from E. coli (Sigma). LPS was dissolved in phosphate-buffered saline (PBS) and administered by i.p. with a dose of 3.3 μg/g body weight in 100 μl in in vivo experiments. In control mice, PBS was injected. Ninety minutes after LPS injection, mice were sacrificed and spleens were aseptically removed. For in vitro experiments, LPS dissolved in distilled water was diluted to 1 μg/ml and 10 μg/ml with RPMI medium. Cells were cultured with LPS for 24 h and culture supernatants were subjected to ELISA.

1.2.5 Measurement of IL-6

IL-6 level in the culture supernatant was assayed by Enzyme-linked immunosorbent Assay (ELISA). Cells were cultured with medium containing 0, 1, or 10 μg/ml LPS. Supernatants were collected after 24 h and analyzed by ELISA.

1.2.6 Quantitative PCR

Total RNA was isolated from purified cells by QIA shredder and RNeasy Mini kit (QIAGEN) and by SV total RNA isolation system (Promega) from organs. Quantitative PCR reaction was performed with SYBR green PCR mastermix (QIAGEN). Gene expression levels for each sample were normalized to GapDH serving as internal standard. Sequences of the forward and reverse primers were as follows: GAPDH (*Gapdh*), 5' – TGTCCGTCGTGGATCTGAC – 3' and 5' – CCTGCTTCACCACCTTCTTG – 3'; IL-6 *(Il6),* 5' – TGGAGTCACAGAAGGAGTGGCTAAG – 3' and 5' – TCTGACCACAGTGAGGAATGTCAA – 3'; IL-17A *(Il17a)*, 5' – GAAGCTCAGTGCCGCCA – 3' and 5' – TTCATGTGGTGGTCCAGCTTT – 3'; IL-1β

(Il1b), 5' – CAGGATGAGGACATGAGCAC – 3' and 5' – CAGTTGTCTAATGGGAACGTCA – 3'.

1.2.7 Cell population with flow cytometry analysis

Spleens were recovered from young and aged mice and spleen cells were used for flow cytometry analysis after red blood cell lysis. Cell staining for flow cytometry was performed at 4 °C after Fc-block step for 15min with the following monoclonal antibodies: FITC-conjugated anti-CD11b, FITC-conjugated anti-CD4, FITC-conjugated anti-CD45, PE-conjugated anti-F4/80, and PE-conjugated anti-B220 for 20 min. For stromal cell analyses, FITC-conjugated anti-CD45, PE-conjugated anti-gp38, biotinylated anti-CD31, streptavidin-APC, and propidium iodide were used. All data were analyzed with FlowJo.

1.2.8 Western Blotting

Spleens were removed after 1.5 h i.p. LPS stimulation from young and aged mice. Nuclear extracts were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL). One µg of protein were separated by sodium dodecyl sulfate polyacrylamide (10 %) gel electrophoresis and transferred to polyvinylidene fluoride membrane by electroblotting. Membranes were incubated for 1 hour with primary antibody p65 (Santa Cruz Biotechnology, sc-8008). Detection was done with horseradish peroxidase-conjugated anti-mouse IgG (1:1000) for 1 hour and visualization was done with Super signal west femto maximum sensitivity substrate (Thermo).

1.2.9 Statistical analysis

Results comparisons were performed by student's t-test. A value of $p < 0.05$ was considered to be significant.

1.3 Results

1.3.1 IL-6 production of spleen cells is higher in aged mice compared to young mice.

To examine the aging effect on IL-6 protein production levels in the spleen of young and aged mice in vitro, splenocytes of young and aged mice were incubated with or without LPS for 24 h. After incubation, I analyzed IL-6 protein concentrations in supernatant by ELISA. As shown in previous studies, IL-6 protein concentrations increased in the supernatant dependent on LPS concentration in both of young and aged mice splenocytes. In the spleen cells of aged mice, stimulation of LPS led to IL-6 protein up-regulation in the supernatant, while in splenocytes of young mice, although the concentration of IL-6 protein increased when stimulated with LPS, this was only a modest increase. The difference of IL-6 protein levels between young and aged mice became significantly greater in LPS-concentration dependent manner. Macrophages are well known for important cells that produce the major pro-inflammatory cytokines such as IL-6 and TNF-α (38). To analyze the effect of the aging process on the IL-6 secretion of splenic macrophages, macrophages were isolated from spleen using two different cell markers. $F4/80^+$ cells and $CD11b^+CD11c^-$ cells were prepared by using MACS. In either case macrophages produced higher levels of IL-6 protein in young mice group than aged mice group after LPS stimulation. DCs are also known as a source of IL-6 (39). DCs from young and aged mice were isolated and IL-6 protein levels were investigated. DCs from the aged mice group produced slightly higher amounts of IL-6 proteins compared to young mice DCs. In case of the expression of IL-6 mRNA, however, the differences between young and aged mice were negligible. These results show that aging process does not greatly affect the function of macrophages and DCs in IL-6 secretion.

1.3.2 Stromal cells from aged mice produce higher levels of IL-6 compared to those from young mice.

To identify cells highly produce IL-6 protein with aging, I next focused on stromal cells. $CD45^-$ stromal cells were isolated from young and aged mice spleen by MACS.

FACS analysis was performed and the cell population was characterized as > 98% for $CD45^-$ stromal cells. The IL-6 protein production levels were high in aged mice compared to young mice in all tested LPS concentrations (0, 1, and 10 μM). $CD45^-$ stromal cells from young mice produced lower amounts of IL-6 protein than from aged mice and they showed only modest changes in IL-6 protein production when stimulated by LPS. Contrary to young $CD45^-$ stromal cells, aged $CD45^-$ stromal cells yielded higher levels of IL-6 protein even in noninflammatory state, and by stimulating with LPS, IL-6 production further increased and the production was significantly high compared to those of young mice. Then RNA was extracted from freshly isolated $CD45^-$ stromal cells and mRNA levels were investigated. Expression of IL-6 mRNA was significantly increased in the aged $CD45^-$ stromal cells.

1.3.3 Cell populations of spleen from both young and aged mice do not show age-dependent differences but those of splenic $CD45^-$ stromal cells show different profile.

Cell populations might change with aging in spleen, and the difference in cell population may influence on the overall production of IL-6 in the spleen of young and aged mice. To analyze the effect of population, the proportion of cells was characterized that expressing $F4/80^+$, $CD11b^+$, $CD11c^+$, $CD4^+$, $B220^+$, and $CD45^-$ in spleen of young and aged mice by performing flow cytometric analysis. Aging process did not significantly affect cell populations defined by these surface markers in the spleen and it was suggested that the difference in IL-6 production of splenocytes in between young and aged mice was not due to the difference of the proportion of each cell population. To test the effect of age on altered distribution of stromal cells, flow cytometry analyses were performed with $CD45^-$ stromal cells. Distribution of population within $CD45^-$ stromal cells determined and $CD45^-$ stromal cells from aged mice displayed different populations compared to young mice. Difference between young and aged mice was observed in BEC population in $CD45^-$ stromal cells. Aged mice $CD45^-$ stromal cells show high population of BEC and low population of DNC.

1.3.4 IL-6 secretion of $CD45^-$ stromal cells but not $CD45^+$ cells is enhanced by aging.

To investigate further whether the enhanced IL-6 secretion of splenocytes from aged mice results from IL-6 production of aged $CD45^-$ stromal cells, I cultured $CD45^-$ stromal cells and $CD45^+$ cells from young and aged mice and these cells were activated with LPS for 24 h. Five$\times10^4$ of $CD45^-$ stromal cells and 5×10^5 of $CD45^+$ cells from young and aged mice were incubated respectively and I confirmed that $CD45^-$ stromal cells of aged mice secreted almost the same amount of IL-6 protein as ten-times number of $CD45^+$ cells. When $CD45^+$ cells were incubated, young mice $CD45^+$ cells secreted significantly higher IL-6 protein than those of aged mice. These results suggested that higher IL-6 production from splenocytes of aged mice compared to young mice was due to the enhanced production of IL-6 in stromal cells of aged mice.

1.3.5 IL-6 expression was elevated in splenic stromal cells of aged mice after LPS-stimulation in vivo.

To investigate age-related overexpression of IL-6 after LPS stimulation in vivo, mice were injected i.p. with LPS, and IL-6 mRNA expression of splenocytes was assessed. It was found that after LPS-stimulation in vivo, aged mice spleen expressed significantly higher levels of IL-6 mRNA. Since NF-κB p65 mainly regulates IL-6 gene expression, p65 expression was compared by western blot after separation of nuclear extracts from young and aged mice spleen (40, 41). A difference in translocation of NF-κB p65 into the nuclei between young and aged mice was found. In nuclear extract from aged mice, more NF-κB p65 was found than young mice. To further elucidate whether $CD45^-$ stromal cells of spleen exhibited age-related differences in IL-6 mRNA levels after LPS administration in vivo, spleens were obtained from young and aged mice after LPS injection, and stromal cells were isolated. As expected, $CD45^-$ stromal cells of aged mice expressed significantly higher IL-6 mRNA after LPS-stimulation in vivo. Since IL-17 and IL-1β can enhance IL-6 production in concert with IL-6 in non-immune cells, the expression of IL-17 and IL-1β mRNA was also examined (31).

The results demonstrated that IL-17 and IL-1ß mRNA expression was also significantly higher in splenocytes of aged mice compared to young mice.

1.4. Discussion

Aging induces various changes in immune system. In this study, I examined the effect of aging-process on IL-6 secretion, one of the pro-inflammatory cytokines, in several types of cells from spleen of young and aged mice. In autoimmune diseases, unbalance and dysregulation of proinflammatory cytokines are considered to play a pivotal role to develop autoimmunity (42). Patients with elevated IL-6 show higher mortality and organ failure and anti-IL-6 receptor antibody is supposed to be effective medication for rheumatoid arthritis and Castleman's diseases (43). It implies that IL-6 is a critical factor in autoimmune diseases, especially in rheumatoid arthritis (44-46). There are a number of studies concerning cytokine secretions in aged mice and analyzing the age-related defects by showing changes of proinflammatory cytokine profile (19). Aged mice have been shown to express increased levels of IL-6, and the results of our present study are consistent with these reports (10, 47-49). In rheumatoid arthritis, it has been proposed that bacterial infections might play important roles, so the expression of pro-inflammatory cytokines in response to bacterial stimulation may also be important (50). In this study, I observed that upon LPS stimulation, splenocytes of aged mice secreted more IL-6 than those of young mice. Translocation of NF-κB to the nucleus activates the transcription of a number of genes, including IL-6 (51). NF-κB p65 protein content of aged mice nuclear extracts was significantly high which is in line with the higher production of IL-6 between young and aged mice, which supports previous studies. It implies that via activated NF-κB, a number of inflammatory genes, including IL-6, are induced and results in a characteristic proinflammatory profile in aged mice.

There are many cell types in the spleen including macrophages, DCs, and other immune and non-immune cells. Several types of cells were separated from spleen and these cells were cultured with or without LPS to identify cells that play roles in production of IL-6 with aging in inflammatory and noninflammatory environment with young and aged mice. Although macrophages produce IL-6 in the early phase of infectious inflammatory response (52), the present results suggest that the aging process does not affect the IL-6 production of these cells. Similarly, DCs from both young

and aged mice had almost similar ability to secrete IL-6. Rather, it was newly found that IL-6 production of $CD45^-$ stromal cells was greatly increased in aged mice. $CD45^-$ stromal cells were separated from young and aged mice spleen and cultured these cells with or without LPS for 24 h. The results showed that $CD45^-$ stromal cells of aged mice strongly produced IL-6 even under the noninflammatory condition and the IL-6 protein production increased upon LPS stimulation. In addition, IL-6 mRNA was expressed highly in freshly isolated $CD45^-$ stromal cells from aged mice compared to young mice, which alludes to the function of $CD45^-$ stromal cells in secreting IL-6 changes with aging. To further investigate the effect of $CD45^-$ stromal cells on IL-6 protein production of spleen upon LPS stimulation, $CD45^+$ cells and $CD45^-$ stromal cells were cultured under LPS stimulated condition for 24 h, respectively. $CD45^+$ cells from young mice secreted higher IL-6 protein compared to aged mice, suggesting $CD45^+$ cells do not play a role in increased production of IL-6 with aging. This demonstrates that although hemopoietic cells other than macrophages and DCs may produce IL-6, the level of production was not higher in aged mice, and these cells did not contribute to the elevation of IL-6 secretion in aged mice. $CD45^-$ stromal cells from aged mice secreted almost the same amount of IL-6 protein with $CD45^+$ cells with one tenth number of $CD45^+$ cells and $CD45^-$ stromal cells from aged mice showed three times higher IL-6 protein yields compared to young mice. These results suggest that $CD45^-$ stromal cells are crucial to increase the IL-6 protein production after LPS stimulation in aging process. The distribution of subsets of these $CD45^-$ stromal cells changed with aging. The BEC population increased population in $CD45^-$ stromal cells of aged mice. Although the ratio of $CD45^-$ stromal cells did not differ, the composition of $CD45^-$ stromal cells changed after aging. It implies that altered distribution of $CD45^-$ stromal cells, especially increased population of BECs might contribute to augment IL-6 production. It is still not clear, however, how many cell subsets of stromal cells exist within SLOs and further study is necessary on the function of these subsets. Also, clarification of the stromal cell subset(s) that produce increased levels of IL-6 upon aging will be subject of future studies.

Recent data showed that IL-17 stimulates stromal cells to induce production of inflammatory mediators such as IL-6 in concert with IL-6, which indicates an

IL-17-mediated IL-6 amplifying system (32, 53, 54). Under the presence of IL-17, non-immune cells including fibroblasts can secrete increased amounts of cytokines in response to infection (53, 54). IL-17 treatment alone, however, is a poor stimulus for gene expression in these cells. Nevertheless, if IL-17 cooperates with other cytokines, especially TNF-α and IL-6, synergic response would occur, and therefore it might be considered as a potential mechanism that in aged mice IL-6 production can increase significantly by IL-17A-triggered positive-feedback loop mediated by IL-17 and IL-6 (55). IL-6 and IL-17A mRNA of young and aged mice spleen were examined after 1.5 h LPS injection in vivo and the results show that splenocytes of aged mice highly expressed IL-6 and IL-17A mRNA than splenocytes of young. Also, $CD45^-$ stromal cells from aged mice expressed 5-fold higher IL-6 mRNA after 1.5 h LPS injection than from young mice. These results imply that highly produced IL-6 by stromal cells and IL-17A produced by some cell population in aged mice trigger the IL-6 amplifier, and result in increasing IL-6 production in aged mice spleen. It has been reported that IL-1β produced in LPS-injected mice stimulates cardiac cells to produce IL-6 (56). The expression of IL-1β, a pro-inflammatory cytokine, was higher in splenocytes of aged mice compared to young mice after LPS injection. Yet another possibility maybe that IL-1β produced upon LPS stimulation enhances IL-6 secretion of stromal cells in aged mice.

Present results that increased IL-6 expression in splenic $CD45^-$ stromal cells of aged mice suggest general etiological mechanisms of the chronic inflammatory condition found in the elderly, referred to inflamm-aging. Such altered immune functions might have influence on developing diseases, susceptibility to infections, and conditions related to allergy in the elderly. In both of our in vivo and in vitro results, stromal cells play a role of causing difference in IL-6 production between young and aged mice. These results propose that increased risk with age of autoimmune diseases, especially induced by IL-6 is due partly to elevated IL-6 production by stromal cells. Additional studies need to make clear the mechanisms related to elevated secretion of IL-6 in stromal cells with age. And to better understand age-related IL-6 production in stromal cells, further studies concerning secretion of IL-6 in each subset of stromal cells might be necessary.

Chapter 2.

（第2章の内容は、学術雑誌に掲載される予定であり、公表できない。）

Chapter 3.

（第2章の内容は、学術雑誌に掲載される予定であり、公表できない。）

Chapter 4. Attenuation of migration properties of $CD4^+$ T cells from aged mice correlated with decreased chemokine receptor expression.

4.1 Introduction

Immunosenescence characterizes a state of significant age-related changes in the immune system that are associated with decline of antigen specific immunity (57). In the elderly, the immunological functions alter and these result in the decreased ability to respond to vaccinations and infections (58, 59).

The immune system can be divided into two parts, innate and adaptive immunity, and aging affects both arms of these immune systems, but in the adaptive immune system, more detrimental changes occur with aging (60). In particular, T cell functions are affected by aging. One of the most outstanding age-related events is thymus involution both functionally and structurally (1, 58). T cell functions such as T cell proliferation, receptor signaling, IL-2 response, and expansion of memory T cells are impaired upon aging in both aged human and murine models (61). Moreover, the number of naïve T cells present in the thymus significantly reduces with aging (62), while programmed cell death-1 (PD-1) expressing T cells are present in higher percentages in aged mice compared to young mice (63). Because of drastic decrease of newly produced T cells and a lifelong exposure to antigens, the proportion of naïve T cells decrease, and memory and effector T cells increase in the elderly, and these relate with declining immune response (64).

T cells circulate tissue, blood, and lymph, and thus migration of T cells in a proper way is important in immune homeostasis (65). T cells can respond to pathogens only when they meet pathogen derived antigen, and therefore T cells should migrate to sites where the antigen locates (66). Such migration of T cells is regulated by chemokines and their receptors (67). Chemokines, small proteins important for determination of immune function and inflammatory response, induce lymphocytes migration in response to chemo-attractant gradients, and chemokine-chemokine receptor interactions play critical roles in a number of diseases (68, 69). Mice lack of CCL19, the ligand of CCR7, show incompetence of T cell migration (70). Also, CCL25, the ligand of CCR9, an important chemokine for gut specific migration, has several important functions in immune response and inflammation (71). Thus, for the efficacy of immune response, chemokine-chemokine receptor directed migration ability of T cells is an important

factor.

Previous studies suggest that the chemokine receptor expressions of T cells are affected by aging (17). Aged murine $CD4^+$ T cells expressed higher levels of the chemokine receptors CCR1, 2, 4, 5, 6 and 8 and lower levels of CCR7 and 9 compared to young mice under non-stimulated conditions. However, the actual changes in migration abilities by aging have not been well studied. Moreover, chemokine receptor expression and migration activities of lymphocytes are regulated by various factors. Retinoic acid (RA), a metabolite of vitamin A, is one of such factors which induces gut homing receptor expression, and as a result, regulates T cell migration function to the intestine, such as to Peyer's patch and mesenteric lymph nodes (69, 72, 73).

In this chapter, I examined CCL19-CCR7 and CCL25-CCR9 interactions and the influence of aging process on $CD4^+$ T cell migration ability between young and aged mice. Attenuated migration abilities of $CD4^+$ T cells were observed in aged mice, which correlated with decreased chemokine receptor expression. Furthermore, the results propose that reduced production and response to RA by aging in the intestinal immune system may be one of the causes of such attenuated migration abilities.

4.2 Materials and Methods

4.2.1 Animals

Young (2-4 mo) and aged (12-17 mo) DO11.10 female mice (74) were obtained from Institute for Animal Reproduction, Japan and young (2-3 mo) and aged (12-13 mo) Balb/c female mice from Charles River, Japan. All procedures were performed in accordance with guidelines for animal use and care of the University of Tokyo.

4.2.2 Culture medium

Cells were cultured in RPMI 1640 medium supplemented with 5 % FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 3 mM L-glutamine, and 50 μM 2-ME.

4.2.3 Isolation of $CD4^+$ T cells from spleen, Peyer's patch (PP), and mesenteric lymph nodes (MLN) and cell culture with OVA

Spleen, PP, and MLN were removed from young and aged DO11.10 mice and $CD4^+$ T cells were depleted using Mini MACS columns (Miltenyi Biotech). Five × 10^5 cells of $CD4^+$ T cells were incubated with 2 × 10^6 cells of antigen presenting cells (APC) and 1mg/ml of ovalbumin (OVA) in 96 well plates for 3 days at 37 °C. After incubation, cells were harvested and washed twice with RPMI medium for chemotactic assay. For APC, splenocytes from Balb/c mice were treated with mytomycin C for 30 min at 37 °C.

4.2.4 Cell culture with retinoic acid

Five × 10^5 cells of purified splenic $CD4^+$ T cells were incubated with 2 × 10^6 cells of APC and 1mg/ml of OVA in 96 well plates for 2 days at 37 °C. Two days after, the cells were resuspended in three times as much volume of fresh RPMI medium containing recombinant murine IL-2 and were transferred into new wells and cultured for 2 days. At the start of each culture, 1 μM of RA was added to the $CD4^+$ T cells containing wells.

4.2.5 Chemotactic migration assay

Chemotactic migration assay was performed using 24-well chemotaxis chambers (5 μm pore, Costar) and 5×10^5 cells were placed into upper chamber of a trans-well migration plate for an assay toward CCL19 and 1×10^5 cells for an assay toward CCL25. For splenic $CD4^+$ T cells migration, 600 μl of 0, 50, 200, and 400 ng/ml of CCL19 was added in the lower chamber respectively and cells were allowed to migrate for 90 min at 37 °C. For the migration of $CD4^+$ T cells from PP, MLN, and spleen that cultured with RA, 600 μl of 500 ng/ml of CCL25 was added to the lower chamber. The number of migrated cells into the lower chamber was counted by Trypan Blue staining and their percentages of the input cell number were calculated.

4.2.6 Quantitative PCR

Total RNA was isolated from purified cells by QIA shredder and RNeasy Mini kit (QIAGEN). Quantitative PCR reaction was performed with SYBR green PCR mastermix (QIAGEN). Gene expression levels for each sample were normalized to GAPDH serving as internal standard. Sequences of the forward and reverse primers were as follows: GAPDH (*Gapdh*), 5'- TGTCCGTCGTGGATCTGAC - 3' and 5' - CCTGCTTCACCACCTTCTTG -3'; CCR7 (*CCR7*), 5'- GTGTGCTTCTGCCAAGATGA -3' and 5' - CCACGAAGCAGATGACAGAA -3'; CCR9 (*Ccr9*), 5' - TGCAGGCTGTTGACGCTTATG -3' and 5' - CATCCCAGGTTCTTCAGGGTC - 3'; α4 (*Itga4*), 5' - GCTTGTGAACCCAACTTCAT - 3' and 5' - CATTTGGAGCCATGCTAATC - 3'; β7 (*Itgb7*), 5' - CAAGTCACCATGTGAGCAG - 3' and 5' - GTCAAGGTCACATTCACGTC - 3'; RALDH1 (*Aldh1a1*), 5' – ATGGTTTAGCAGCAGGACTCTTC – 3' and 5' – CCAGACATCTTGAATCCACCGAA – 3'; RALDH2 (*Aldh1a2*), 5' – GACTTGTAGCAGCTGTCTTCACT – 3' and 5' – TCACCCATTTCTCTCCCATTTCC – 3'.

4.2.7 Statistical analysis

Results comparisons were performed by student's t-test. A value of $p < 0.05$ was considered to be significant.

4.3 Results

4.3.1 The intrinsic ability of splenic $CD4^+$ T cells of young versus aged mice to migrate toward CCL19 and CCL25 chemokine.

Here the possibility that splenic $CD4^+$ T cells from aged mice have intrinsic defects in migration toward chemokine, CCL19, was investigated using chmotaxis assay. If the chemokine and chemokine receptor of aged mice $CD4^+$ T cells work in appropriate way, there would be no difference in the number of migrated $CD4^+$ T cells between young and aged mice. Chemotactic response toward CCL19 increased in chemokine concentration dependent manner in both young and aged $CD4^+$ T cells. As the chemokine concentration rose, the number of migrated cells increased in both young and aged mice $CD4^+$ T cells. In aged mice cells, however, it showed only modest increase. $CD4^+$ T cells from aged mice were less competent in migration than the young mice in all tested chemokine concentrations. $CD4^+$ T cells from young mice responded sensitively to even low concentration of CCL19. In the migration ability of $CD4^+$ T cells toward 50 ng/ml CCL19, only about 10 % of total input aged mice $CD4^+$ T cells migrated to lower chamber and over 25 % of young mice input cells migrated. About 40 % of the young adult $CD4^+$ T cells migrated toward 400 ng/ml of CCL19 in contrast to 20 % of the aged mice cells did. Additionally, expression levels of CCR7, the CCL19 receptor, were decreased in aged mice $CD4^+$ T cell cells compared with young mice. In migration assay toward CCL25 chemokine, no migrated cells were observed for both young and aged mice.

4.3.2 Influence of RA on CCR9 and $\alpha4\beta7$ expression level and migration ability toward CCL25 of young and aged mice splenic $CD4^+$ T cells.

CCR9 is largely detected in PP and in the small intestine and it binds with CCL25 and forms chemokine-receptor pair in gut specific migration of leukocytes (66). The effect of RA addition was investigated on the expression of CCR9 and $\alpha4\beta7$ on $CD4^+$ T cells after activation with OVA. Naïve $CD4^+$ T cells were stimulated with OVA, APC and

with RA for 4 days. Addition of 1 μM RA induced higher expression of gut-homing receptor, CCR9, in $CD4^+$ T cells from young mice compared to those from aged mice but not the expression of α4β7. To determine if the changed CCR9 expression levels by addition of RA have influence on the migration ability of splenic $CD4^+$ T cells, the same amount of splenic $CD4^+$ T cells were input in the upper chamber of transwell and migration assay was performed. In migration assay, about 25 % of RA treated $CD4^+$ T cells of young mice migrated toward CCL25, whereas few aged mice $CD4^+$ T cells detected in lower chamber of transwell.

4.3.3 RALDH expression in MLN and PP $CD11c^+$ DCs of young and aged mice.

MLN-DCs and PP-DCs can induce RA production by expressing RALDH (68). It was tested if there was difference in production ability of RA between young and aged mice with DCs from MLN and PP. Naïve $CD11c^+$ DCs were isolated from Balb/c mice, which are background of DO11.10, and RALDH1 and RALDH2 mRNA levels were investigated. PP-DC expressed higher level of RALDH1 compared to MLN-DCs and MLN-DCs expressed higher level of RALDH2 compared to PP- DCs in both young and aged mice. The expressions of RALDH1 of PP-DCs and RALDH2 of MLN-DCs were decreased in aged mice compared to young mice.

4.3.4 Migration ability toward CCL25 of $CD4^+$ T cells from young and aged mice PP and MLN.

Small intestinal epithelium mainly expresses CCL25 and interaction between CCL25 and CCR9 is related to migration of leukocytes, especially gut-specific migration. The effect of aging process on migration of $CD4^+$ T cells originated from PP and MLN both of young and aged mice was investigated. Young mice PP and MLN $CD4^+$ T cells migrated to lower chamber were approximately 13 % and 35 %, respectively, of their input in upper chamber. Compared to $CD4^+$ T cells from young mice, PP and MLN $CD4^+$ T cells from aged mice moving to lower chamber were seldom detected.

4.4 Discussion

Aging is related to the changes of immune functions and altered immune function induces higher risks of infectious diseases and diminished efficacy of vaccines. Most remarkably, in the elderly, the changes of T cell functions are apparent (64). To respond to pathogens, T cells should migrate to the sites where antigens are found. Chemokine and chemokine receptors expressing on T cell surface are of important factor in leukocytes traffic (75).

In this study, it was investigated if aging process affects expressions of chemokine receptors and altered chemokine receptor expression levels induce changed migration ability of $CD4^+$ T cells. The results presented in this chapter proved attenuation of aged mice $CD4^+$ T cells in migration abilities and suggested these might result from the decreased chemokine receptor expression levels. It was reported that $CD4^+$ T cells from young mice highly expressed CCR7 and CCR9 compared with aged mice under unstimulated condition and under the stimulated condition, the expression levels change (71). In this study, the migration ability of splenic $CD4^+$ T cells from young and aged mice toward CCL19 was examined. CCL19 is one of the important chemokine for lymphocytes to home to lymph nodes and PP (65), and it was found the declined function of migration ability in splenic $CD4^+$ T cells of aged mice toward the same concentration of CCL19 compared with young mice. The expression of CCR7, the chemokine receptor for CCL19, may have an impact on the migration ability of $CD4^+$ T cells, and decreased expression level of CCR7 on aged mice $CD4^+$ T cells may lead to decline of migration ability. As expected, aged mice $CD4^+$ T cells showed decreased CCR7 mRNA expression levels than young mice after OVA stimulation. These results extended previous observations in unstimulated $CD4^+$ T cells showing that CCR7 expression and migration towards CCL19 were decreased in antigen stimulated $CD4^+$ T cells of aged mice compared to young mice. The mechanism for expression of CCR7 is still not fully understood. With aging, TCR sensitivity and signaling strength decrease in naïve $CD4^+$ T cells (76) and these TCR-mediated signaling changes might affect CCR7 expression level in aged mice after antigen stimulation. Moreover, there still may be other factors that affect CCR7 expression level, and aging might influence such factors. It was

investigated the migration ability of splenic $CD4^+$ T cells from young and aged mice using CCL25 by the same manner, however, few cells both young and aged $CD4^+$ T cells moved toward CCL25.

To examine the influence of RA on the expression of cell surface chemokine receptor CCR9 and α4ß7, splenic $CD4^+$ T cells were cultured with OVA with RA. RA, derived from dietary vitamin A, is a main oxidative metabolite of vitamin A. From early 20^{th} century, vitamin A is considered as important for immunity, called vitamin A anti-infective (77). Iwata et al. examined the change of migration ability with vitamin A deficient mice and found that migrated T cells were remarkably decreased in secondary lymphoid organs. Moreover, the addition of RA induced CCR9 expression in naïve $CD4^+$ T cells under the stimulated condition (73). It was found that by addition of RA, although α4ß7 expression was not altered both in young and aged mice, CCR9 expression of splenic $CD4^+$ T cells from young mice was remarkably high upon OVA stimulation. In contrary, aged mice splenic $CD4^+$ T cells showed decreased CCR9 expression level under the same condition. Since CCR9 expression tends to be more RA-dependent than the expressions of α4 and ß7, (73, 78) the expressions of α4 and ß7 may not be as susceptible to age-associated RA response. $CD4^+$ T cell migration ability decreased with aging under the presence of RA and it might result from decreased CCR9 expression in these cells. Furthermore, it was suggested that the ability of producing RA was affected by aging process. RALDH1 and RALDH2 expression levels of PP DC and MLN DC, respectively, were significantly decreased with aging and it implies attenuated RA production in aged mice.

RA shows their biological effects by binding RA receptors (RARs) and retinoid receptors (RXRs) (79). Aging process might affect RA receptor expression and it influences on RA function compared with young mice. It is possible that the RA receptor expressions decrease and as a result, RA addition does not have an enhancing effect on the expression of CCR9. In addition, increased percentage of PD-1 in aged mice T cells might play roles in defects of RA-RAR interactions. Further investigation would be necessary on RA receptor expression in young and aged mice. In addition, decreased RALDH levels might influence expression of chemokine receptor, CCR9 and α4ß7 of MLN and PP $CD4^+$ T cells and resulting in attenuated migration toward CCL25. The increased expression of CCR9 in splenic $CD4^+$ T cells of young mice by addition of RA

and the interaction between CCR9 and CCL25 may provide clues to recover the T cell migration abilities in aged mice. In addition to CCR9 expression, the age-dependent difference of chemokine production should also be investigated.

In summary, in this chapter, it was elucidated that the migration ability of $CD4^+$ T cells declines with aging and the addition of RA induces $CD4^+$ T cells to augment CCR9 expression in young mice but not in aged mice. By understanding the mechanism of augmented CCR9 expression in young mice upon RA, aged mice migration ability might increase. Improving the understanding of the mechanism of migration ability of $CD4^+$ T cells may result in a better understanding of immune response and immune functions against antigen in the aged.

Chapter 5.

（第2章の内容は、学術雑誌に掲載される予定であり、公表できない。）

Overall discussion

The aim of present study is to clarify the age-associated altered immune functions and the mechanisms of those changes. Functions of the immune system declines with aging, which is referred as immunosenescence and it has been studied for decades. Advances in medical and nutritional conditions lead to increased life expectancy. The increased challenge of susceptibility to cancers and infections is seen in the elderly and it might be linked to deficiencies in the immune system.

Aging is a complicate process that has influence on immune system on various aspects. Immune system consists of different cells and these cells interact each other, directly or by numerous mediators to protect body from pathogens. Immune responses can be divided into two mechanisms, innate immunity and adaptive immunity. Aging triggers changes in these two mechanisms and in this study both innate immunity and adaptive immunity were investigated.

1. The effect of stromal cells on immunosenescence.

Within SLOs, there are not only hematopoietic cells but also stromal cells and these cells interact each other. So far, immune responses have been studied focusing on hematopoietic cells, apart from stromal cells. Stromal cells, however, have emerged recently to have abilities to affect immune system. In chapter 1, age-associated altered immune functions were investigated in the aspect of IL-6 production. IL-6 production ability of macrophage and DCs, which are known as IL-6 producing cells, was examined using young and aged mice spleen originated cells. Although these cells mainly secrete IL-6, there was no remarkable change in producing IL-6 between young and aged mice. Next, IL-6 production level was investigated with stromal cells. Stromal cells from aged mice showed increased IL-6 level after LPS-stimulation and expressed higher level of IL-6 mRNA even in naïve condition. IL-6 mRNA expression was investigated also in vivo after LPS injection and the result was consistent with those obtained in vitro. Stromal cells from aged mice expressed significantly higher IL-6 mRNA level in vivo and in vitro not only under LPS stimulated condition but also non-stimulated condition. This is the first study that investigated the ability of stromal cells to secrete

pro-inflammatory cytokine, particularly, IL-6 production. Remarkable differences of frequency of stromal cells were not found in this study. The subsets of stromal cells, however, were markedly different between young and aged mice. BEC increased in stromal cells from aged mice, and it implies that augmented BEC may contribute to induce increased IL-6 production. So far, the functions of each subset of stromal cells were not elucidated. Further studies would be necessary on the IL-6 production level of each subset of stromal cells. This study shed light on the age-associated alterations of stromal cells.

2. Age-related changes in adaptive immunity in the aspect of T cells

Aging affects not only innate immunity but also adaptive immunity. Particularly T cells change with aging and it affects wide range of immune responses. In this study, migration ability of $CD4^+$ T cells was investigated and $CD4^+$ T cells from aged mice showed attenuated migration ability. In migration, chemokine and chemokine receptor are important factors. The expression level of chemokine receptors such as CCR7 and CCR9 was also examined, and $CD4^+$ T cells from aged mice expressed lower level on chemokine receptors. RA can induce the expression of CCR9 and integrin, $\alpha4\beta7$ and RALDH catalyses retinal to RA. $CD4^+$ T cells from aged mice showed decreased level of CCR9 expression even when RA was added and RALDH level of MLN DCs were also decreased compared to young mice. RA binds to RA receptor, RARs, and plays biological roles. The RAR might change with aging which may be the cause of decreased CCR9 expression even when RA is added. Moreover, decreased RALDH level may also influence on migration ability of $CD4^+$ T cells.

3. Future prospects

With enhancement of medical and environmental condition, life expectancy has been increasing. Improving the quality of life for the elderly is important rather than simple longevity and immune system is deeply related to health of elderly. Immune responses changes with aging and it affects health of individuals. Both innate and

adaptive immunity showed attenuated ability to mount appropriate responses. More detailed mechanisms should be studied to improve immune responses in the elderly. Recently, a number of studies have been performed on the immune and aging but much still remains unclear. In this study, several age-associated changes of immune functions were investigated. It was the first time to examine the function of stromal cells in immunosenescence. Further studies are necessary to clarify the role of stromal cells in the elderly. It was shown that attenuation of migration properties of $CD4^+$ T cells from aged mice correlated with decreased chemokine receptor expression. Since age-associated decreased T cell migration ability might be one of causes of immunosenescence, effective medications to modulate such migration activity may help improve immune functions. To elucidate the mechanisms of immunosenescence would be effective for improving the quality of life for the elderly.

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