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ORIGINAL

Relationship between expression and function of Toll-like receptor 2 and 4 with aging in humans and in mice

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ABSTRACT : Splenocytes prepared from 8-week old and 20-month old C57BL/6 mice were stimulated with various concentrations of the TLR2 ligand FSL-1 or the TLR4 ligand *E. coli* LPS and the growth of the splenocytes was measured. Both FSL-1 and *E. coli* LPS stimulated the growth of the cells from the young mice more strongly than that from the aged mice in a dose-dependent manner. The splenocytes from the young and aged mice expressed both TLR2 and TLR4 on the cell surface and the expression levels of both TLRs were higher in the young mice than in the old mice. The expression and functions of TLR2 and TLR4 were then examined with nine each of 60-week and 8-week old mice. The expression levels of TLR4 were significantly higher in the young mice than in the aged mice, whereas the expression level of TLR2 was significantly lower in the young mice than in the aged mice. However, the amounts of TNF-α and IL-6 produced by splenocytes from the young mice were significantly larger than those from the aged mice, suggesting that the function of TLRs is impaired in the aged mice. The expression and functions of TLR2 and TLR4 in human PBMC were also examined. The expression levels of both TLR2 and TLR4 in the young individuals were slightly but not statistically significantly higher than those in the aged individuals. FSL-1 but not the LPS stimulation induced significantly larger amounts of IL-6 in young human adults when compared with aged adults. In addition, both FSL-1 and LPS stimulation induced significantly larger amounts of TNF-α in young volunteers than in those from the aged ones.

Thus, the present study suggests that the functions of TLR2 and TLR4 become defective with aging in humans and mice.

Key Words : Toll-like receptor 2, Toll-like receptor 4, Aging

Introduction

Human aging is concerned with increased morbidity and mortality from infectious diseases and impaired responses to immunization, which results in part from immunosenescence (1). Immunosenescence is defined as the state of dysregulated immune function that contributed to the increased susceptibility of the elderly to infection and, possibly, to autoimmune disease and cancer (1). Numerous lines of evidence have been described that there are alterations in adaptive immunity associated with aging which include a shift from naïve to memory phenotype T cells, as well as decreased CD28 expression and decrease in number of clone in the T cell repertoire (2). In addition, the effects of aging on the innate immune system in human and mice have been reviewed recently (3, 4). However, most of the studies focus on expression and function of Toll-like receptors (TLRs), cell numbers and functions of innate immune cells isolated such as neutrophils, monocytes/macrophages and dendritic cells (3, 4). Therefore, in this study, we focused on expression and function of TLRs of the whole cells in lymphoid tissue such as mouse splenocytes or human peripheral blood mononuclear cells (PBMC) which include various types of immune cells such as lymphocytes,

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monocytes, polymorphonuclear cells and so on.

TLRs are pattern recognition receptors that recognize structural components of many bacteria, viruses, and fungi (5). TLR activation induces production of pro- and anti-inflammatory events, including cytokine production and costimulatory molecule up-regulation, which are mediated in part by the translocation of NF-κB and signal transduction via mitogen-activated protein kinases (6). TLRs play important roles in the link between innate and adaptive immunity by thus activating antigen-presenting cells (APCs) (7). In human, aging has been implicated in atherosclerosis and autoimmune diseases as well as in altered susceptibility to infections (8). Furthermore, it has been reported that TLR function and expression were generally impaired in macrophages with aging (9). However, it has also been reported that LPS and zymosan stimulation did not change murine TLR2 or TLR4 surface expression but noted a decrease in proinflammatory cytokine production (10, 11). In humans, several reports have examined LPS-induced cytokine production in older adults, mostly using ELISA-based assays following stimulation of bulk PBMC or whole blood and yielding conflicting results (12-15). For example, Bruunsgaad *et al.* (13) have reported that TNF-α and IL-1β produced by whole blood after stimulation with LPS were significantly lower in elderly humans, but no differences are detected with regard to IL-6. Duin *et al.* (15) have reported that TLR1 surface expression was decreased by 36% in older adults, whereas TLR2 surface expression was unaffected by aging. Thus, relationship of aging with changes in the innate immune system is not fully understood.

In this study, therefore, we also tried to evaluate the relationship of function and expression of TLR2 and TLR4 with aging in mouse splenocytes and human PBMC.

Materials and Methods

1. Reagents and antibodies

Phycoerythrin (PE) conjugated anti-mouse TLR2 antibody (Ab) (clone 6C2) and its isotype control (PE-conjugated rat IgG2b, κ), PE-conjugated anti-mouse TLR4 Ab (clone Vt41) and its isotype control (PE-conjugated mouse IgG1, κ) were purchased from eBioscience (San Diego, CA, USA). Anti-human TLR2 Ab and its isotype control (mouse IgG1, κ) were purchased from IMGENEX (San Diego, CA, USA) and FITC-conjugated anti-mouse IgG Ab from Jackson ImmunoResearch (West Grove, PA, USA). Biotinylated Anti-human TLR4 Ab and biotinylated -anti-mouse IgG2a were from BD Pharmingen (San Diego, CA, USA). Streptoavidin-PE was purchased from Beckman Coulter (Brea, CA, USA). The TLR2 ligand FSL-1 was synthesized according to the method described previously (16). Highly purified *E. coli* LPS was purchased from Sigma (St. Louis, MO, USA).

2. Human volunteers and isolation of PBMC

Volunteers include five undergraduate students in Hokkaido University Graduate School of Dental Medicine (20-30 years) and teaching staffs (50-60 years) in the same university for this study. They are all healthy male adults with no self-reported symptoms of recent infection. This study was approved by the Human Investigation Committee at Hokkaido University Graduate School of Dental Medicine. Informed consent was obtained from all volunteers.

Blood were drawn into heparinized tubes and centrifuged on Ficoll-Paque gradients (GE Healthcare Bioscience, Tokyo). PBMC were recovered, washed with RPMI1640 complete medium and adjusted to 2 x 10^6 cells/ml of the medium.

3. Mice

Female 8-week-old (8w) C57BL/6 (B6) mice and 60w mice were purchased from Japan Clea (Tokyo). All mice were maintained in specific pathogen-free conditions at our animal facility at Hokkaido University, and all experiments were carried out in accordance with the regulations of the Hokkaido University Animal Care and Use Committee. A 20 month-old (20m) mouse was raised in our animal facility.

4. Proliferation of splenocytes in response to TLR ligands

Splenocytes (1.0 x 10^6 cells) isolated from 8w and 20m mice were incubated for 48 h in a 96-well flat-bottomed plate (0.2 ml) with various amounts (0, 0.01, 0.1, 1, 10, 100, 1000 ng/ml) of the TLR2 ligand FSL-1, or *E. coli* LPS. Cultures were pulsed with 0.2 μCi/well of [^{3}H]-thymidine for the last 16 h of culture. The cultures were harvested with a Labo Mash cell harvester. The radioactivity of the cells deposited on filters was measured in a liquid scintillation counter.

5. Determination of cytokines

Splenocytes (1 x 10^6 cells) derived from 10 each of 8w and 60w mice were stimulated for 24 h with 100 ng/ml of FSL-1 or *E. coli* LPS, the cultures were centrifuged and supernatants were collected. The cell culture supernatants

were assayed for IL-6 and TNF-α, by using ELISA kits purchased from R&D Systems (Minneapolis, MN, USA). Briefly, flat-bottomed 96-well Nunc-Immuno MaxSorp assay plates were coated overnight with the appropriate anti-cytokine antibodies. After blocking the plates with bovine serum albumin, the plates were incubated for 2 h with the culture supernatants and then incubated for 1 h with each of biotin-conjugated anti-cytokine antibodies. Then horseradish peroxidase-conjugated streptavidin was added and developed with TMB peroxidase substrate. The optical densities were measured at 405 nm using a microplate reader.

6. Flow cytometry

Splenocytes (1 x 10^6 cells) derived from young and aged mice were incubated on ice (10^6 cells in 200 μl of PBS with 1% BSA) with 1 μg each of PE conjugated anti-mouse TLR2 or TLR4 Ab or its isotype control. After 30 min, aliquots were washed three times with PBS containing 1% BSA, resuspended in the same solvent and examined for surface expression of TLR2 or TLR4 using a FACS Calibur (BD Biosciences, Sunnyvale, CA, USA). Data for 50000 cells falling within appropriate forward and side light scatter gates were collected from each sample. Data were analyzed using CellQuest software (BD Biosciences). The expression levels of TLRs are expressed as the ratios of mean fluorescence intensity (MFI) obtained by anti-TLRs Abs vs the MFI by isotype controls.

7. Statistical analysis

Statistical analysis was done by Student's T test and $p<0.05$ was considered to indicate statistical significance.

Results

1. Effects of *E. coli* LPS or FSL-1 on the growth of mouse splenocytes

Splenocytes prepared from 8w and 20m old B6 mice were stimulated with various concentrations of the TLR2/6 ligand FSL-1 or the TLR4 ligand *E. coli* LPS and the growth of the splenocytes was assessed by being pulsed with [^{3}H]-thymidine uptake. It was found that both FSL-1 and LPS stimulated the growth of the cells from the 8w mouse more strongly than that from the 20m old mouse in a dose-dependent manner (Fig. 1A). We thought that the expression level and function of TLR4 or TLR2 in splenocytes from the young mouse are higher than those from the

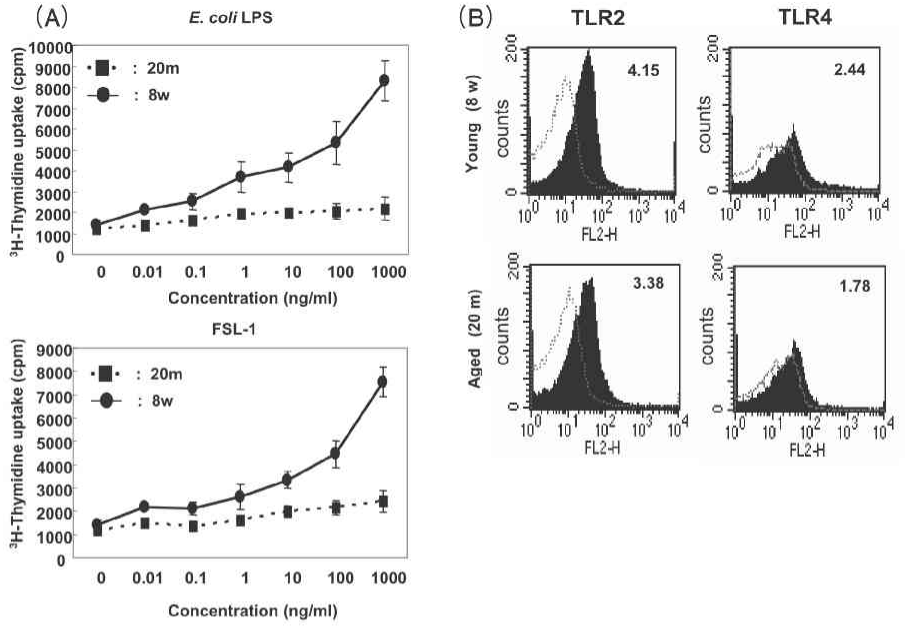

Fig.1. Proliferation of mouse splenocytes in response to the TLR4 ligand *E. coli* LPS and the TLR2 ligand FSL-1 and the expression of TLR2 and TLR4.

(A): Splenocytes (1.0 x 10^6 cells) isolated from 8w and 20 m B6 mice were incubated for 48 h in a 96-well flat-bottomed plate (0.2ml) with various amounts (0, 0.01, 0.1, 1, 10, 100, 1000 ng/ml) of the TLR2 ligand FSL-1, or *E. coli* LPS. Cultures were pulsed with 0.2 μCi/well of [^{3}H]-thymidine for the last 16h of culture. Results are expressed as means ± SD of three determinations. See text for details.

(B): Splenocytes (1.0 x 10^6 cells) isolated from 8w and 20 m B6 mice were stained with anti-mouse TLR2 or anti-mouse TLR4 and their isotype controls. Data for 50000 cells falling within appropriate forward and side light scatter gates were collected from each sample. Data were analyzed using CellQuest software. The expression levels of TLRs written in the right upper corner of the histogram are expressed as the ratios of MFI obtained by anti-TLRs Abs vs the MFI by their isotype controls. See text for details.

aged mouse, because several lines of evidence are accumulated that the expression levels and functions of TLRs decrease with aging (9). Therefore, the surface expression levels of TLR2 and TLR4 in splenocytes from these mice were examined. The splenocytes from the young and aged mice expressed both TLR2 and TLR4 on the cell surface, although the expression level of TLR2 was significantly higher than that of TLR4 (Fig. 1B). Expectedly, the expression levels of both TLRs were higher in the young mice than in the old mice (Fig. 1B).

2. Differences in the expression and function of TLRs in between young and aged mice

The results described above were obtained from just two mice, one young mouse and one aged mouse. Therefore, we tried to investigate the relationship between expression and function of TLRs and aging by using numbers of young and age of mice. However, it was very hard to obtain 20m mice. Therefore we used 9 mice with the age of 60 w as the aged mice and the same number of young (8w) mice in the following experiments. First of all, we examined whether there are differences in expression level of TLR2 and TLR4 in these young and aged mice. Expectedly, the expression level of TLR4 was significantly higher in the young mice than in the aged mice (Fig. 2). However, unexpectedly, the expression level of TLR2 was significantly lower in the young mice than in the aged mice (Fig. 2). Next experiment therefore was carried out to examine whether there are dif-

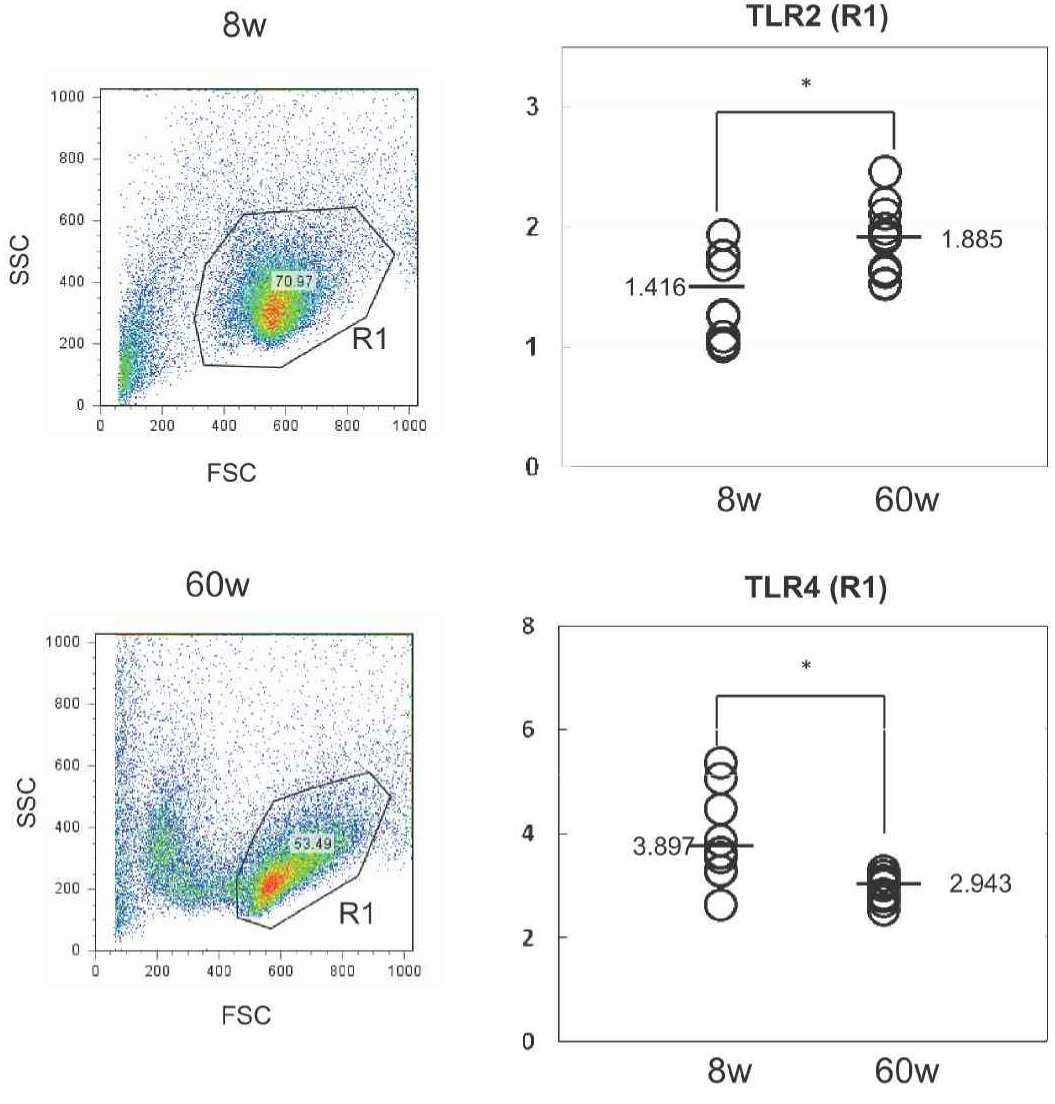

Fig. 2. Expression of TLR2 and TLR4 on the surface of splenocytes from young and aged mice.

Splenocytes (1.0×10^6 cells) isolated from 9 each of 8w and 60 w B6 mice were stained with anti-mouse TLR2 or anti-mouse TLR4 and their isotype controls. Data for 50000 cells falling within f appropriate forward and side scatter gates were collected from each sample. Data within the gate R1 in left figures were analyzed using CellQuest software. See text for details. *: $P < 0.05$

ferences in function of TLRs in splenocytes from between young and aged mice, which are assessed by cytokine production after stimulation with FSL-1 or *E. coli* LPS. As a result, it was found that the amounts of TNF-α and IL-6 produced by splenocytes from the young mice were significantly higher than those from the aged mice (Fig. 3).

3. Expression levels of TLR2 and TLR4 in human PBMC from young and aged adults

The findings obtained by mouse splenocytes that the expression level of TLR4 but not TLR2 and their function decreased with aging encouraged us to examine expression level and function of TLR2 and TLR4 in human PBMC from young and aged adults, although several lines of evidence have been reported on the effects of aging on expression and function of TLRs (12-15). PBMC prepared from young healthy individuals (5 volunteers with ages of 20 to 30-year old) and aged healthy individuals (5 volunteers with ages of 50 to 60-year old) were examined for surface expression of TLR2 and TLR4. As a result, it was found that expression level of both TLR2 and TLR4 in the young individuals was slightly but not statisically significantly higher than those in the aged individuals (Fig. 4).

Next experiment therefore was carried out to examine whether there are differences in function of TLRs in PBMC from young and aged volunteers, which are assessed by cytokine production after stimulation with FSL-1 or *E. coli* LPS. We found that FSL-1 induced significantly larger amount of IL-6 in young adults when compared with the aged ones (Fig. 5). No significant difference between them was observed in the case of LPS stimulation (Fig. 5). However, both FSL-1 and LPS stimulation induced significantly larger amount of TNF-α in young volunteers than in those from the aged ones (Fig. 5).

Thus, our results also support the previous findings that the expression and function of TLR2 and TLR4 are defective in human aging (12-15).

Discussion

Several lines of evidence have been accumulated that TLR function is impaired with aging in human and mouse (9-15). Most of these studies have described the effects of aging on the functions of a specific cell type such as monocytes/macrophages, neutrophils and dendritic cells. Therefore, this study was designed to investigate the effects of advanced age on functions of murine splenocytes and human PBMC that include many types of immune cells such as monocytes/macrophages, dendritic cells, NK cells, T and B lymphocytes, because we thought that defects in the immune systems should be assessed by outcome of the interaction of many types of immune cell but not by a specific type of cell.

This study demonstrated that aged mice have impaired responses to TLR2- and TLR4-specific stimulation with decreased TNF-α and IL-6 production (Fig. 3), which supports the previous findings that functions of TLRs assessed by the cytokine production decreased with aging (9, 17). However, the expression level of TLR4 in splenocytes decreased with aging, whereas that of TLR2 increased with aging (Fig. 2). This result suggests that the expression level of TLR2 is not always correlated with the TLR2 function. In addition, this is contradictory to the previous finding by Renshaw *et al.* (9) that the expression of TLR2 decreased with aging. They used macrophages isolated from

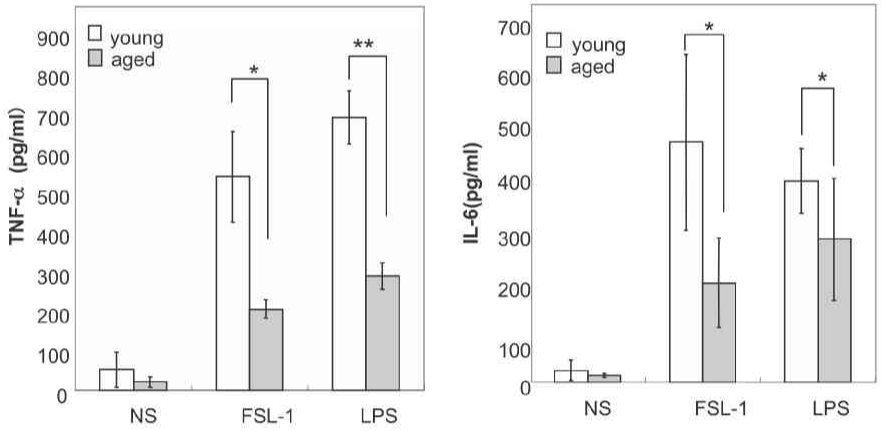

Fig. 3. Production of IL-6 and TNF-α by splenocytes from 8w and 60 w B6 mice in response to FSL-1 and *E. coli* LPS.
Splenocytes (1×10^6 cells) derived from 9 each of 8w and 60w mice were stimulated for 24 h with 100 ng/ml of FSL-1 or *E. coli* LPS, the cultures were centrifuged and supernatants were collected. The cell culture supernatants were assayed for IL-6 and TNF-α, by using ELISA kits. See text for details. *: $P < 0.05$, **: $P < 0.001$

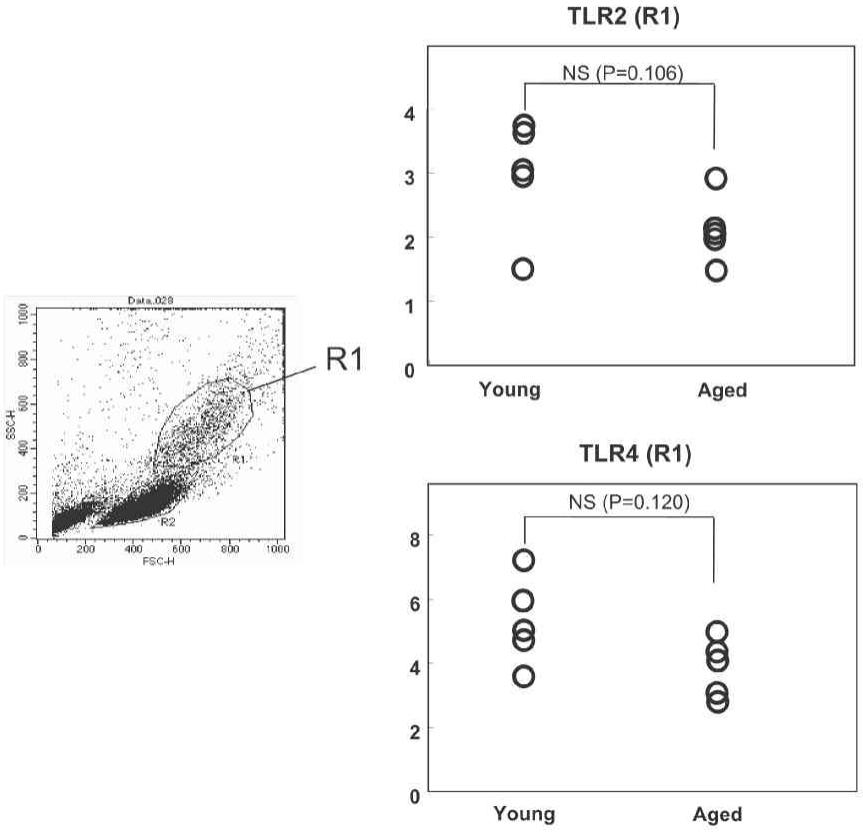

Fig. 4. Expression of TLR2 and TLR4 on the surface of PBMC from young and aged adults.

PBMC (1.0×10^6 cells) isolated f from 5 each of young and aged male adults were stained with anti- human TLR2 or anti-human TLR4 and their isotype controls. Data for 50000 cells falling within appropriate forward and side scatter gate were collected from each sample. Data falling within the gate R1 in left figure were analyzed using CellQuest software. See text for details. NS: No significant.

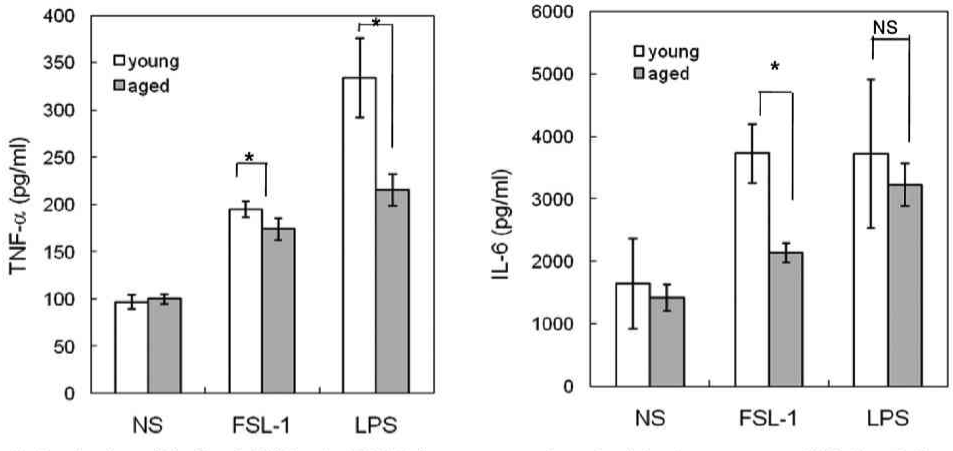

Fig. 5. Production of IL-6 and TNF-α by PBMC from young and aged adults in response to FSL-1 and *E. coli* LPS.

PBMC (1×10^6 cells) derived from 5 each of young (20-30 years) and aged adults (50-60 years) were stimulated for 24 h with 100 ng/ml of FSL-1 or *E. coli* LPS, the cultures were centrifuged and supernatants were collected. The cell culture supernatants were assayed for IL-6 and TNF-α, by using ELISA kits. See text for details. *: $P < 0.05$

spleen for expression of function of TLRs, whereas we used total splenocytes. This difference may cause the contradictory result.

In human PBMC, aged adults have impaired to TLR2- and TLR4-specific stimulation with decreased TNF-α and IL-6 production when compared with young adults (Fig. 5). However, there were no significant differences in the expression level of TLR2 and TLR4, although the expression level of both TLRs tended to decrease with aging (Fig. 4). This result also suggests that the expression level of

TLR2 is not always correlated with the TLR2 function. Bruunsgaad *et al.* (13) have reported that TNF-α and IL-1β produced by whole blood after stimulation with LPS were significantly smaller in elderly humans, but no differences are detected with regard to IL-6. However, this study demonstrated that production of IL-6 as well as TNF-α by PBMC after stimulation with LPS was smaller in aged adults (Fig. 5). It is speculated that this contradictory result is attributed to the difference in cell types used. That is, they use whole blood including polymorphonuclear leukocytes such as neutrophils expressing TLRs (3) which are not included in PBMC that we used.

Taken together, our findings suggest that TLR function decreased with aging in human and mouse. TLRs are expressed on a wide range of cells in the immune system, especially APCs, such as macrophages and dendritic cells, and the TLR activation induce production of proinflammatory cytokines or type I interferons via NF-κB- or IRF (interferon-regulatory factor) -dependent pathways. In addition, TLRs are responsible for the expression of costimulatory molecules such as CD80 and CD86 on the cell surface of APCs which are indispensable for activation of naïve T cells (18). Thus, TLRs play important roles in bridging the innate immunity and the acquired immunity. Therefore, the defect in TLR function is one of alterations in the immune system that occur with aging, which increases the risk of infection and malignancy.

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