Age-related alteration of expression and function of TLRs and NK activity in oral candidiasis

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Running title: Toll-like receptors and NK cell in oral candidiasis

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Abstract

Objective: Roles of aging or immune responses mediated by Toll-like receptors and natural killer cell in the onset or progression of human candidiasis remain unclear. This study was designed to elucidate the roles by using peripheral blood mononuclear cells from healthy donors and patients with oral candidiasis.

Subjects and methods: Subjects tested were healthy volunteers and patients who visited Dental Clinical Division of Hokkaido University Hospital. The patients with oral candidiasis included 39 individuals (25-89 years of age) with major complaints on pain in oral mucosa and/or dysgeusia. Healthy volunteers include students (25-35 years of age) and teaching staffs (50-65 years of age) of Hokkaido University Graduate School of Dental Medicine.

Results: Functions of Toll-like receptor 2 and 4 were downregulated significantly and the natural killer activity was slightly, but not significantly downregulated in aged healthy volunteers compared with healthy young volunteers. Functions of Toll-like receptor 2 and 4 and the natural killer activity were significantly downregulated in patients with oral candidiasis compared with healthy volunteers.

Conclusion: Downregulation of functions of Toll-like 2 and 4 as well as natural killer activity are suggested to be associated with the onset or progression of oral candidiasis

in human.

Introduction

Recently, the number of compromised patients is increasing as a result of advance in medical care, such as treatment with anti-cancer drugs, anti-inflammatory steroids and immunosuppressive agents. Candidiasis is one of the opportunistic infection which can be a serious problem for compromised patients, because candidiasis can be systemic (invasive candidiasis) and associated with high mortality (Williams & Lewis, 2011). *Candida albicans* is one of the causative of human oral candidiasis, although it is a commensal resident of human oral cavity and has been reported to be detected from 1.9% to 62.3% of healthy donors (Scully et al., 1994). *C. albicans* possesses the ability to perform a dimorphic switch from a spherical yeast growth form to filamentous hyphal cells, which is crucial for its pathogenicity (Calderone & Fonzi, 2001).

Hyphal formation on mucosal epithelium is considered a major virulence determinant for oral candidiasis, which can be physically uncomfortable, and the patient may complain of burning mouth, dysgeusia, dysphagia, anorexia, and weight loss, leading to nutritional deficiency and impaired quality of life (Sharon & Fazel, 2010). In addition, oral candidiasis can be a symptom of an undiagnosed or poorly controlled systemic disease (Samaranayake et al., 2009). For example, it will occur in 50% to 95% of all HIV positive persons during their progression to AIDS (Fidel, 2011). Patients with

diabetes or in an immunosuppressive condition due to malignancies have been implicated in oral candidiasis (Akpan & Morgan, 2002). Drugs such as broad spectrum antibiotics have also been reported to alter the local oral flora creating a suitable environment for candida to proliferate (Epstein et al., 1984, Soysa et al., 2008). Recently it has been revealed that local factors such as using a denture or xerostomia are also responsible for oral candidiasis by causing change in oral flora (Akpan & Morgan, 2002). However, it is also known that colonization of *C. albicans* in oral cavity and oral candidiasis may develop to healthy individuals who are elderly or in malnutrition (Lockhart et al., 1999, Paillaud et al., 2004).

Toll-like receptors (TLRs) are representative pattern recognition receptors (PRRs) and play important roles in recognition of microbial invasion (Akira et al., 2006). To date, more than 10 members of the TLR family have been identified in mammals (Akira et al., 2006), and numerous lines of evidence have indicated that TLRs orchestrate not only the innate immune system but also adaptive immune responses to microbial infections (Parker et al., 2007). TLR2 and 4 are key PRRs in innate immune system that play important roles in immune response against many microbes including fungi by recognizing zymosan and mannan (Roeder et al., 2004, Tada et al., 2002). Natural killer (NK) cells are not only innate immune effector cells capable of

recognizing and destroying virally infected cells and neoplastic cells but also effectors of acquired immune responses (Horowitz et al., 2010) through their ability to mediate antibody-dependent cellular cytotoxicity (ADCC) (Sondel & Hank, 2001). In addition, the importance of NK cells in antifungal host response is also increasing (Schmidt et al., 2013). Therefore, we attempted to examine the relationship, because there are few studies on relationship among human candidiasis, innate immune system and aging.

In this study, expression and functions of TLRs and NK activity were examined in peripheral blood mononuclear cells (PBMCs) from healthy volunteers and patients with oral candidiasis who are free from diabetes, HIV-infected, auto immune disorder and tumour bearing.

Materials and Methods

Subjects

Subjects tested were healthy volunteers and patients who visited Dental Clinical Division of Hokkaido University Hospital. The patients with oral candidiasis included 39 individuals (25-89 years of age) with major complaints on pain in oral mucosa and/or dysgeusia. Patients with underlying diseases caused by immunological disorders and patients under treatment with antimicrobial and immunosuppressive agents, such as patients with diabetes, HIV-infected, auto immune disorder and tumour bearing, were not included. Healthy volunteers as non-candidiasis group include students (25-35 years of age) and teaching staffs (50-65 years of age) of Hokkaido University Graduate School of Dental Medicine (Table). Regardless of the existence of coated tongue, the subjects diagnosed as oral candidiasis were patients with pain in oral mucosa and/or dysgeusia and with existence of *C. albicans* on their tongue dorsum by culture test.

All the subjects were informed the purpose of this study. The experiments were undertaken with the understanding and written consent by each subject according to ethical principles, including the World Medical Association Declaration of Helsinki. All experiments in this study were approved by the Medical Ethics Committees of Hokkaido University Graduate School of Dental Medicine.

C. albicans *culture test*

C. albicans culture test was performed as follows. Subject's tongue dorsum was scraped by a sterilized dental mirror for 10 times, and then the sample was inoculated directly on a CHROMagar ® plate (Kanto Chemical Co. Inc., Tokyo, Japan) using the dental mirror. The plate was incubated under aerobic condition at 35 °C, and the number of colony on the plate more than 10 was determined as *C. albicans* positive patients.

Antibodies and reagents

Phycoerythrin (PE)-conjugated monoclonal antibody (mAb) against TLR1 (clone GD2.G4) and its isotype control (clone P3. 6. 2. 1) were purchased from eBioscience (San Diego, CA). Alexa flour 488-conjugated anti TLR2 mAb (clone T2.5), PE-conjugated anti TLR4 mAb (clone HTA125), PE-conjugated anti TLR6 mAb (clone TLR6. 127) and their respective isotype controls (clone MOPC-21, MOPC-173 and MOPC-21) were purchased from BioLegend (San Diego, CA).

TLR2 ligand used was mycoplasmal lipopeptide, FSL-1 (Shibata et al., 2000) and TLR4 ligand used was *Esherichia coli* LPS purchased from Sigma (St Louis, MO).

All other reagents were purchased from commercial sources and were of analytical or reagent grade.

Determination of TLR1, 2, 4 and 6 expression levels

Human peripheral blood mononuclear cells (PBMCs) were separated from peripheral blood of subjects by gradient centrifugation on Ficoll-Paque (GE Healthcare Bioscience, Uppsala, Sweden) at room temperature. PBMCs collected were washed with RPMI 1640 base medium (Sigma), adjusted to be 1×10^6 /ml and then incubated with phosphate buffered saline (PBS) including 1% (w/v) bovine serum albumin (BSA). After washing the cells, they were incubated with anti TLR1, 2, 4, 6 mAbs, or their appropriate isotype control antibodies. Data for 30,000 cells falling within appropriate forward light scatter (FSC) and side light scatter (SSC) were analyzed with a FACS Calibur (BD Biosciences, San Diego, CA). Cell populations were gated in 7 areas (G1-G7) in SSC/FSC dot plots. Data were analyzed using FlowJo software (Tree Star, Ashland, OR). Results were expressed as relative mean fluorescence intensity (MFI) calculated as:

Relative MFI = (MFI by anti TLR ab) / (MFI by isotype control ab).

Assay for TLR2 and TLR4 functions

Suspension of PBMC (1×10^6 /ml) in RPMI 1640 base medium was stimulated with

FSL-1 (10 ng/ml, 100 ng/ml) or LPS (100 ng/ml, 1,000 ng/ml), and then incubated at 37°C in 5% CO_2 for 16 h. After centrifugation of the suspension at 400×g for 10 min, the supernatants were collected and examined for the amount of tumour necrosis factor (TNF)-α, which was determined by ELISA (BioLegend, San Diego, CA).

Assay for NK cell activity

The use of the leukemic cancer cell line K562 for NK cell-mediated lysis has been described before (Pross et al., 1981, West et al., 1977). K562 cells in RPMI 1640 medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) (Gibco BRL, Rockville, MD), 100 units/ml penicillin (Sigma), and 100 μg/ml streptomycin (Sigma) were adjusted to 2×10^4 / 100 μl and used as a target. PBMCs including NK cells were used as effector cells and incubated for 4 h together with K562 cells in a 96 well V-bottomed plate at effector/target (E/T) cell ratios of 5:1, 10:1, and 20:1. After centrifugation at 400×g for 5 min, 100 μl of the supernatant was removed from each well. Lactate dehydrogenase (LDH) released in the supernatants was measured using a CytoTox 96 non-radioactive assay kit (Madison, WI, Promega) according to manufacturer' instructions. The formula used to calculate the specific lysis (% cytotoxicity) is:

% cytotoxicity = (Aexp - Asp)/(Amax - Asp) × 100,

where Aexp or Asp represents the absorbance at 490 nm of supernatants from mixtures consisting of PBMCs and K562 cells or K562 cells alone, respectively, and Amax is the absorbance of supernatant from K562 cells lysed with 1% TritonX-100. The NK activity of whole PBMCs was evaluated by the slope of the linear lines of best fit calculated on the basis of data obtained from three different E/T ratio (5:1, 10:1, and 20:1).

Statistical analysis

All statistical calculations were performed using statistical software from Social Survey Research Information Co. ltd (Tokyo, Japan). Student's T-test for multiple observations was used for statistical analysis. Statistical significance was set at a P-value of less than 0.05.

Results

Age-related alteration of expression and functions of TLRs in healthy volunteers

TLRs play crucial roles in innate immune system. TLR1, 2, 4 and 6 are expressed on cell surface, and it has been reported that TLR2 recognizes bacterial lipoprotein/lipopeptide with TLR1 or 6 as a co-receptor, and TLR4 recognizes Gram-negative bacterial LPS (Akira et al., 2006). Recently, it is also accepted that TLR2 and 4 are involved in the host interaction with *C. albicans* and play an important role in the development of host immune responses during candidiasis (Gil & Gozalbo, 2009). We thought that decline of immune responses in elderly may be explained by age-related alteration of expression and function of TLRs. Therefore PBMCs were isolated from healthy volunteers (25-89 years) and divided into 6 groups by age (25-34, 35-44, 45-54, 55-64, 65-74 and 75-) (Table), and the expression levels of TLRs on PBMCs were analyzed by using a flow cytometer. It was found that there were no significant differences in expression levels of TLR1, 2, 4 and 6 among these groups in any 7 gated cell populations (G1-G7). G4 data as a representative are shown in Figure 1a and b. Therefore, attempts were made to elucidate age-related alteration in functions of these TLRs.

Since recognition of appropriate ligands by TLR2 and 4 mainly leads to NF-κB

activation which induces the production of proinflammatory cytokines (Akira & Takeda, 2004), TNF-α production by PBMCs was measured after stimulation with the TLR2 ligand FSL-1 alone or the TLR4 ligand LPS alone to elucidate the function of these TLRs. The amounts of TNF-α in the age groups 25-34 and 35-44 in response to two different doses of FSL-1 (10 ng/ml, 100 ng/ml) were significantly larger than those in the age-groups older than 45 years (Figure 2a), although there was no significant difference between the age-group 25-34 and 35-44. No significant differences were observed among the 4 age groups older than 45 (Figure 2a). Similar results were also obtained in the case of LPS stimulation (Figure 2b).

These results suggest that the functions of both TLR2 and TLR4 do not vary from 25 to 44 years, and were kept downregulated significantly after 45 years.

Age-related alteration of NK activity in healthy volunteers

NK cells are not only innate immune effector cells capable of recognizing and destroying virally infected cells and neoplastic cells but also effectors of acquired immune responses (Horowitz et al., 2010) through their ability to mediate ADCC (Sondel & Hank, 2001). To date, there are a number of reports on relationship between aging and NK activity. For example, it has been reported that there was no age-related

decline in the NK cell activity (Ogata et al., 1997, Solana & Mariani, 2000, Sansoni et al., 1993), whereas it has been reported that functional activity of NK cells in aged subjects was impaired (Mariani et al., 1990, Panda et al., 2009). Thus, age-related alteration of NK activity is still controversial. We also examined the age-related alteration of NK activity in healthy volunteers. NK activity tended to be downregulated with aging, although no significant differences among any age-group were observed (Figure 3). Thus our result suggest that there is no significant age-related decline in NK activity of healthy volunteers.

Difference in expression and functions of TLRs between healthy volunteers and patients with oral candidiasis

In this analysis, subjects who use a denture and/or have xerostomia were excluded because local factors such as using a denture or xerostomia are also responsible for oral candidiasis by causing change in oral flora (Akpan & Morgan, 2002). Since the patients with oral candidiasis had xerostomia in age younger than 44 years (Table), we compared the expression and functions of TLRs in PBMCs between healthy volunteers and patients with oral candidiasis in age older than 45 years. The same subjects were analyzed in the experiments hereafter.

The cell surface expression levels of TLRs on PMBCs were analyzed by using a flow cytometer (Figure 4a and b). No significant differences in expression levels of TLR1, 2, 4 and 6 were observed among these groups in any 7 gated cell populations, suggesting no significant differences of these TLRs expression between healthy volunteers and patients with oral candidiasis. Therefore, attempts were made to elucidate the differences in functions of these TLRs. The amounts of TNF-α produced in response to both FSL-1 and LPS were significantly lower in patients with oral candidiasis than in healthy volunteers (Figure 5). This result suggests that the functions of both TLR2 and 4 are downregulated in patients with oral candidiasis, compared with healthy volunteers.

Difference in NK activity between healthy volunteers and patients with oral candidiasis

NK activity in PBMCs was compared between healthy volunteers and patients with oral candidiasis also in age older than 45 years as described. It was found that NK activity in PBMCs from patients with oral candidiasis was significantly downregulated than those from healthy volunteers (Figure 6).

Taken together, these results suggest that the functions of TLR2 and TLR4 as well as NK activity are downregulated significantly in PBMCs from patients with oral

candidiasis, compared with those from healthy volunteers, although it is still unclear whether onset of oral candidiasis is due to these donwregulation or not.

Discussion

In this study, subjects tested were healthy volunteers and patients who visited Dental Clinical Division of Hokkaido University Hospital as described. The patients who are supposed to have immunological disorders were not included in subjects, because the purpose of this study is to elucidate the differences in innate immune responses between patients with oral candidiasis without immunological disorders and healthy volunteers in wide range of age groups. This study elucidated the age related alteration in the expression and function of TLR2, 4 and NK activity, and the etiological roles of the alteration in oral candidiasis.

Oral candidiasis can be a symptom of systemic diseases with immunological disorders (Samaranayake et al., 2009). However, as shown in this study, there are patients with oral candidiasis who are free from diabetes, HIV-infection, auto immune disorder and tumour bearing. The number of patients with oral candidiasis younger than 54 years were very small, and most of the patients were older than 55 years (Table). Therefore, we speculated that age-related decline in immune responses is associated with the onset of oral candidiasis. In this study, significant age-related decrease was observed in functions of TLR1, 2, 4 and 6 (Figure 2), but not in their expressions (Figure 1). These results were supported by our previous study (Donen et al., 2010),

whereas van Duin *et al*. reported that decreased surface expression of TLR1 but not TLR2 was observed in human monocytes from aged individuals compared with those from young adults, and the decrease results in less TNF and IL-6 production in response to TLR1/2 stimulation (van Duin et al., 2007b). They also reported that expression of co-stimulatory molecule CD80 in monocytes stimulated with TLR1/2, 2/6, 4, 5, 7 or 8 ligands was reduced in older (> 65 years of age) compared with young adults (van Duin et al., 2007a). These reports suggest that there are decline in both innate and acquired immune system in elderly. The discrepancy on TLR1 expression is speculated to be due to the difference of cells used. That is, the cells we used were whole PBMCs whereas the cells van Duin *et al*. used were peripheral blood monocytes.

TLRs are known to play important roles in recognition of *C. albicans* by host cells. There are many components of *C. albicans* to activate host cells through TLRs. For example, it has been reported that TLR2 recognizes β-glucans (Netea et al., 2006), phospholipomannan and linear beta-1,2-oligomannoside structures (Jouault et al., 2003), TLR4 is activated by O-linked mannans (Netea et al., 2006), TLR7 is activated single-stranded RNA (Biondo et al., 2011), and TLR9 recognizes genomic DNA (Miyazato et al., 2009) derived from *C. albicans*. TLR2 is one of the most important PRRs in recognition of *C. albicans* (Netea et al., 2002, Villamon et al., 2004). However,

the roles of TLR2 in candidiasis have been controversial. In this study, we demonstrated that significant decrease was observed in the function but not in expression of TLR2 in patients with oral candidiasis compared with healthy volunteers (Figure 5). This result is supported by the study by Yanez *et al* (Yanez et al., 2011). They reported that *C. albicans* can directly stimulate progenitor cells through TLR2 and Dectin-1 during the infection to generate inflammatory macrophages and monocyte-derived dendritic cells that may play an important role in defense mechanisms against *C. albicans*. Therefore, downregulation in function of TLR2 in elderly (Figure 2) and in patient with candidiasis (Figure 5) may result in attenuation of host defense against *C. albicans*.

On the contrary, it has also been reported that downregulation in function of TLR2 may potentiate host defense against *C. albicans* infection. TLR2 is also known to be important in controlling regulatory T (Treg) cells in *C. albicans* infection (Netea et al., 2004). Tregs play an essential role in sustaining self-tolerance and immune homeostasis by suppressing a wide variety of physiological and pathological immune responses against self and nonself (Sakaguchi, 2004). It has been reported that *C. albicans* induces immunosuppression through TLR2-derived signals that mediate increased IL-10 production and survival of Treg cells, which may facilitate the progress of the fungal infections (Netea et al., 2004). Therefore, it still remains to be elucidated

how TLR2-mediated innate immune response is involved in onset of oral candidiasis.

Age-related alteration on function of TLR4 has been studied by Bruunsgaard *et al.* (Bruunsgaard et al., 1999). They have reported that stimulation of whole blood cells with LPS induced the significantly lower amounts of TNF-α and IL-1β but not IL-6 in elderly humans compared with young controls. Downregulation of TLR4 function in elderly may be associated with onset of candidiasis, because it has been shown that TLR4-defective C3H/HeJ mice are more susceptible to *C. albicans* infection (Netea et al., 2002).

Age-related alteration on expression of TLRs was reported by Renshaw *et al.* (Renshaw et al., 2002). They elucidated that there are substantial age-associated decreases in *Tlr* gene including TLR2 and 4 in both splenic and activated peritoneal macrophages from mice. However, the pattern of decrease is less clear in human than in mice (Shaw et al., 2013). The age-related alteration on expression of TLRs in human is still to be elucidated.

There are many reports on age-related alteration of NK activity. As reviewed by Mocchegiani *et al*. (Mocchegiani et al., 2009), there are discrepancies at the levels of NK cell number and function in aged human among the previous studies. That is, contradictory data exist due mainly to the different selection of criteria of the elderly

population studied (Mocchegiani et al., 2009). Interestingly, this study demonstrated that there was a significant decline in patients with oral candidiasis compared with healthy volunteers in NK activity (Figure 6), whereas there was no significant age-related decline in NK activity of healthy volunteers (Figure 3). It remains unclear why there is a decline in NK activity in patients with oral candidiasis, although it was reported that the decline may be due to the ability of *C. albicans* to block NK activity (Zunino & Hudig, 1988).

Taken together, further studies are needed to elucidate the association between expression and functions of TLRs and onsets of candidiasis.

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Conflicts of interest

There is no conflict of interest.

Author contributions

MO, HH, YY, YK and KS designed the study. MO, HH, YY and YK collected blood from subjects. MO, TS and AH collaborated with different stages of various assays. MO, AH and KS drafted the manuscript. All authors participated in the discussion of the data and read and approved the final manuscript.

Table. Number of Subjects

age	25-34	35-44	45-54	55-64	65-74	74-	total
healthy volunteers	21 (21)	9 (9)	11 (11)	15 (12)	11 (3)	8 (2)	75 (58)
candidiasis patients	1 (0)	1 (0)	3 (3)	8 (4)	13 (3)	13 (4)	39 (14)
total	22	10	14	23	24	21	114

Shown in parentheses is the number of subjects who does not use a denture and/or have xerostomia.

Figure Legends

Figure 1. (a) Representative SSC/FSC dot plots of cell populations in PBMCs from healthy volunteers. Gate 4 (G4) is shown as one of the representative gates in the dot plots. (b) TLR1, 2, 4 and 6 expression on PBMCs in 6 groups devided by age. Their expression levels are shown as relative MFIs. Thick black line shows average level in each group.

Figure 2. Amounts of TNF-α in culture supernatants of PBMCs stimulated with FSL-1 or LPS. Cell suspensions of PBMCs (1×10^6) from healthy volunteers were stimulated with FSL-1 (10 ng/ml, 100 ng/ml) or LPS (100 ng/ml, 1000 ng/ml) (b) for 16 h at 37 °C. Thick black line shows average amount in each group. *$p<0.05$.

Figure 3. NK activity of PBMCs from healthy volunteers. PBMCs (2×10^4 / 100 μl) were incubated for 4 h with K562 cells at E/T cell ratios of 5:1, 10:1, and 20:1, and then LDH released in the supernatants was measured. The activity was evaluated by calculating slope of the lines on the basis of data obtained from three different E/T ratio. Thick black line shows average amount in each group.

Figure 4. Differences of TLR1, 2, 4 and 6 expression on PBMCs between healthy volunteers and oral candidiasis patients. The expression levels were compared in

various cell populations (gate 1-7), and are shown as relative MFIs. Thick black line shows average level in each group.

Figure 5. Amounts of TNF-α in culture supernatants of PBMCs stimulated with FSL-1 or LPS. Cell suspension of PBMCs (1×10^6) from healthy volunteers and oral candidiasis patients were stimulated with FSL-1 (10 ng/ml, 100 ng/ml) or LPS (100 ng/ml, 1000 ng/ml) for 16 h at 37 °C. PBS was used as a control. Thick black line shows average amount in each group. *$p<0.05$.

Figure 6. NK activity of PBMCs from healthy volunteers and oral candidiasis patients. PBMCs (2×10^4 / 100 μl) were incubated for 4 h with K562 cells at E/T cell ratios of 5:1, 10:1, and 20:1, and then LDH released in the supernatants was measured. The activity was evaluated by calculating slope of the lines on the basis of data obtained from three different E/T ratio. Thick black line shows average amount in each group.

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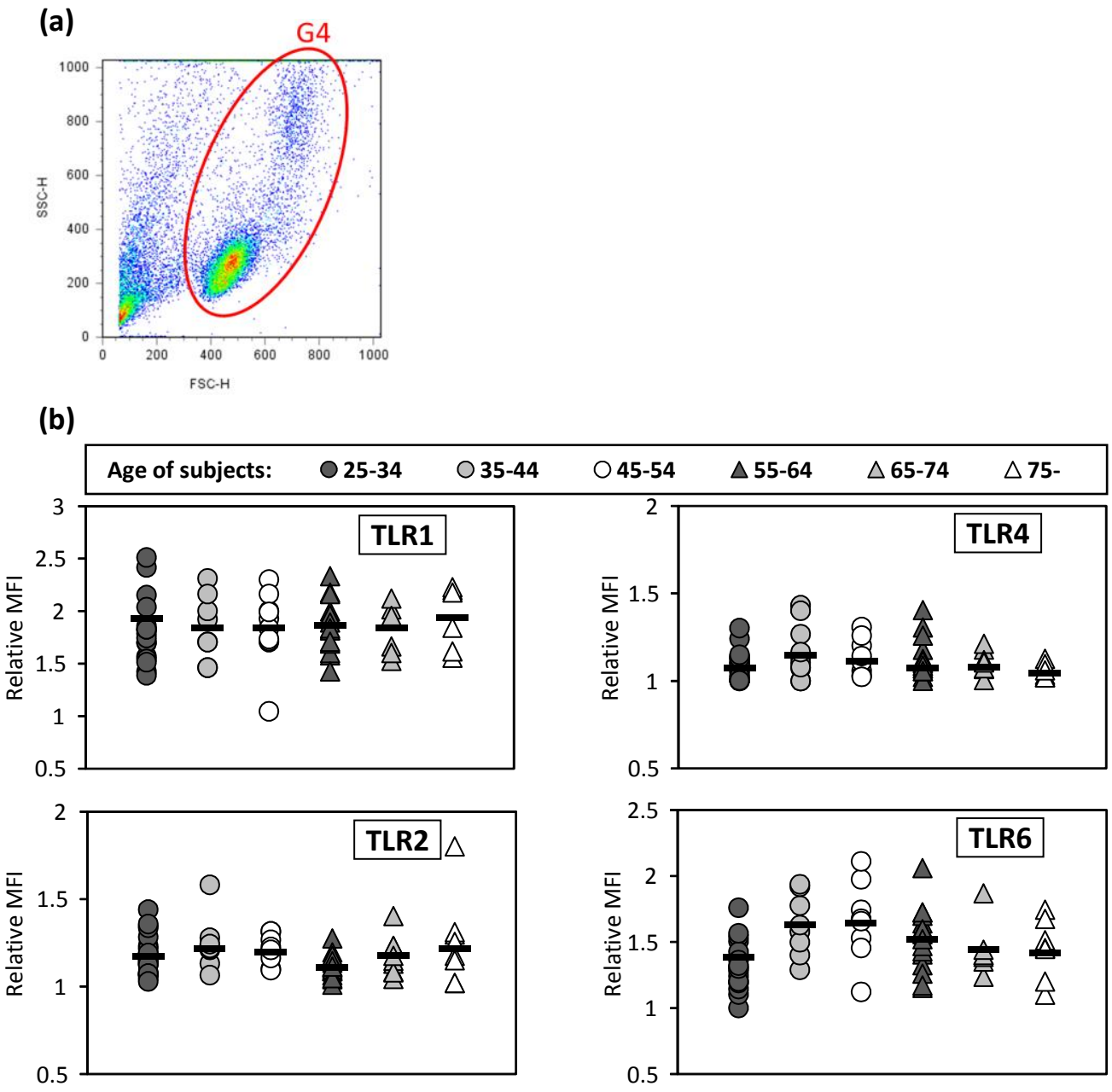
(a)
G4
SSC-H
FSC-H
(b)
Age of subjects:
25-34
35-44
45-54
55-64
65-74
75-
TLR1
TLR4
TLR2
TLR6
Relative MFI

Fig. 1

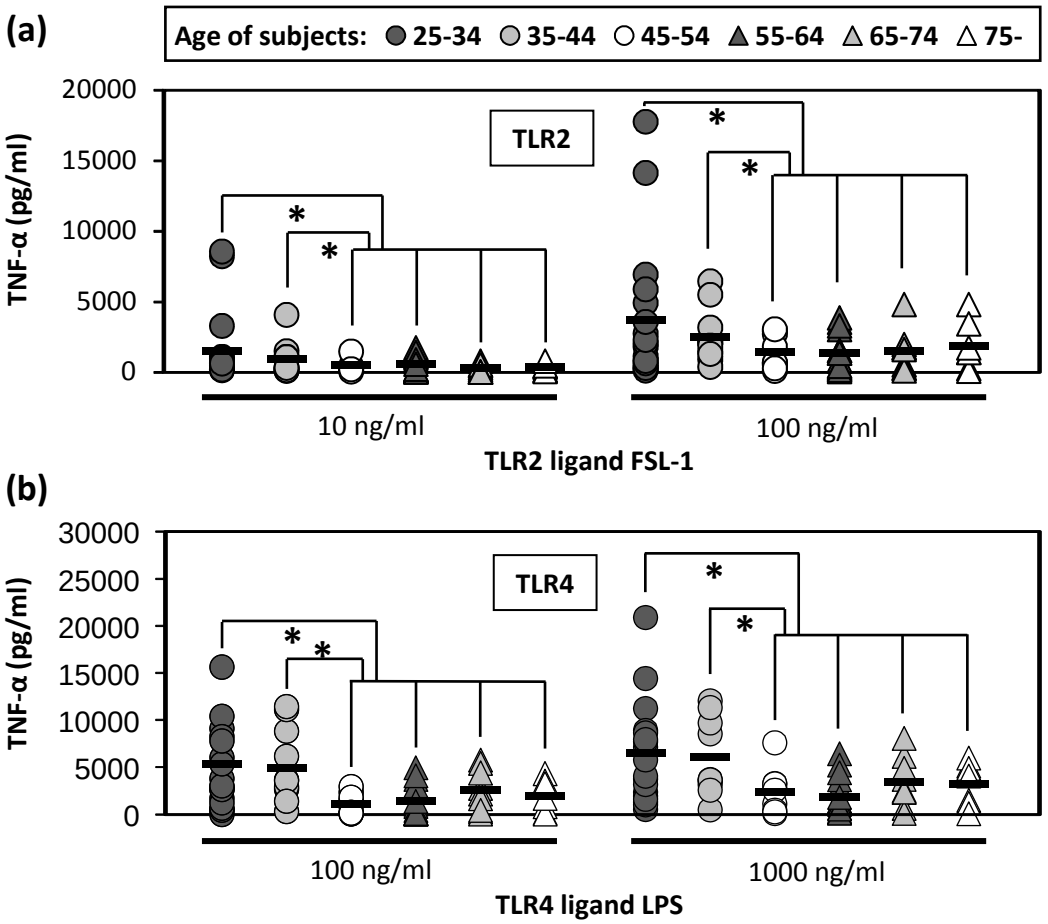
(a)
Age of subjects: 25-34 35-44 45-54 55-64 65-74 75-
TLR2
TNF-α (pg/ml)
20000
15000
10000
5000
0
*
*
*
*
10 ng/ml
100 ng/ml
TLR2 ligand FSL-1
(b)
TLR4
TNF-α (pg/ml)
30000
25000
20000
15000
10000
5000
0
*
*
*
*
100 ng/ml
1000 ng/ml
TLR4 ligand LPS

Fig. 2

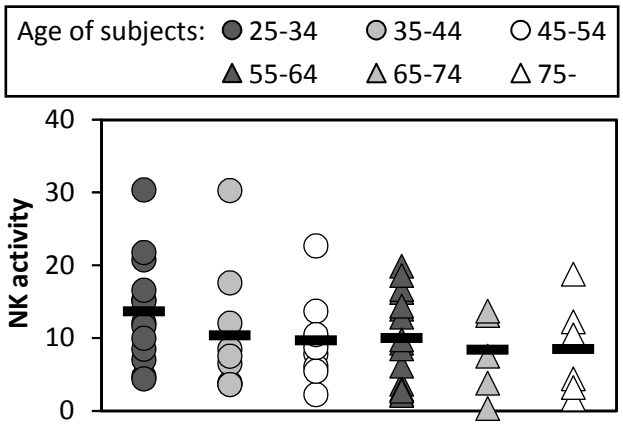
Age of subjects:
25-34
35-44
45-54
55-64
65-74
75-
NK activity
40
30
20
10
0

Fig. 3

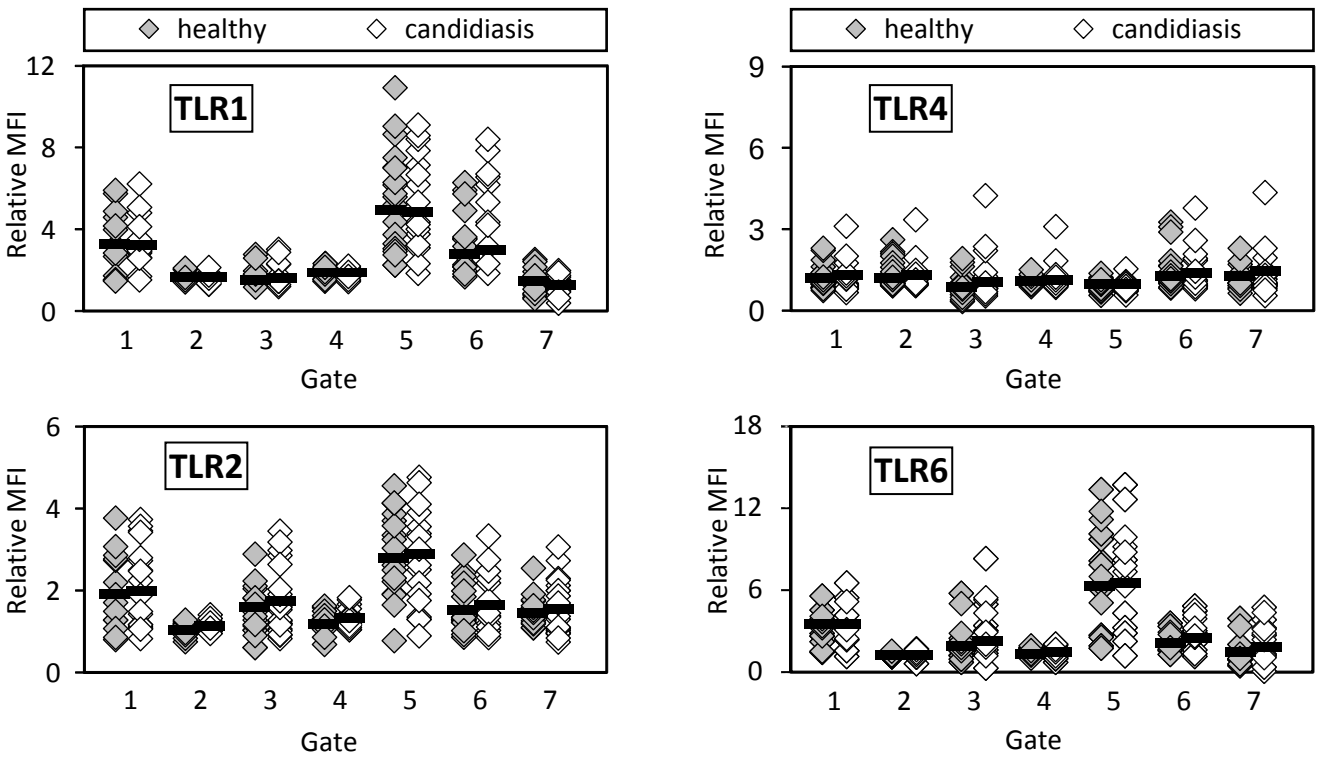
healthy
candidiasis
TLR1
Relative MFI
12
8
4
0
1
2
3
4
5
6
7
Gate
healthy
candidiasis
TLR4
Relative MFI
9
6
3
0
1
2
3
4
5
6
7
Gate
TLR2
Relative MFI
6
4
2
0
1
2
3
4
5
6
7
Gate
TLR6
Relative MFI
18
12
6
0
1
2
3
4
5
6
7
Gate

Fig. 4

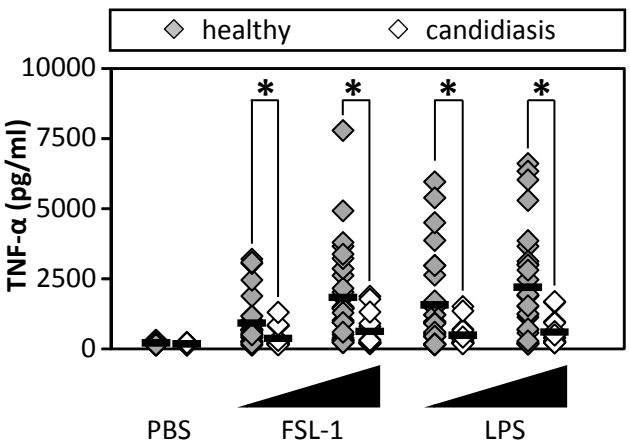
healthy
candidiasis
10000
7500
5000
2500
0
TNF-α (pg/ml)
*
*
*
*
PBS
FSL-1
LPS

Fig. 5

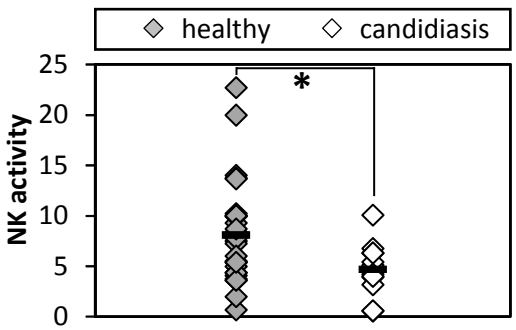

Fig. 6