Cutting Edge: Impaired Toll-Like Receptor Expression and Function in Aging

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Toll-like receptors (TLR) are pattern recognition receptors that recognize conserved molecular patterns on microbes and link innate and adaptive immune systems. We investigated whether the enhanced susceptibility to bacterial, yeast, and viral infections and poor adaptive immune responses in aging are a result of diminished expression and function of TLRs. We examined the expression and function of all murine TLRs on macrophages from young and aged mice. Both splenic and activated peritoneal macrophages from aged mice expressed significantly lower levels of all TLRs. Furthermore, macrophages from aged mice secreted significantly lower levels of IL-6 and TNF-α when stimulated with known ligands for TLR1 and 2, and 2 and 6, TLR3, TLR4, TLR5, and TLR9 when compared with those from young mice. These results support the concept that increased susceptibility to infections and poor adaptive immune responses in aging may be due to the decline in TLR expression and function. The Journal of Immunology, 2002, 169: 4697–4701.

Decline in immune function is a hallmark of aging, leading to increased susceptibility of elderly individuals to bacterial infections of lungs, urinary tract, skin and soft tissues and reactivation of inactive tuberculosis and herpes zoster (reviewed in Refs. 1 and 2). There is an increased severity of pneumococcal, influenza, and respiratory syncytial viral infections in the elderly population (3–6). For example, an estimated 90% of the 20,000 deaths that are attributed to influenza annually in the U.S. occur in persons aged ≥65 years (7). Age-related changes in the adaptive immune system are well-documented and include diminished and/or altered cytokine patterns, reduction in clonal expansion and function of Ag-specific T and B cells and a decline in Ag-presenting cell function (1, 2, 8, 9). The decline in adaptive immune function leads to decreased efficacy of preventive vaccination in the elderly. In the case of influenza, although the vaccine is ∼70–90% effective in preventing illness in healthy younger adults, it is only 30–40% effective in preventing influenza-like illness in frail elderly individuals (4). The reduced efficacy of influenza vaccines in the elderly is likely, at least in part, due to reduced immune responses to vaccination.

As the thymus atrophies with age, there are fewer naïve T cells available to respond to new pathogens and neoantigens. This shift from naïve to memory cells also causes a shift in the cytokine environment. The expression of cell adhesion molecules on APCs and T cells from the aged declines, possibly contributing to immune dysfunction. Humoral immunity also exhibits changes with age, but to a lesser extent, particularly the diminished ability to generate high-affinity protective Abs against infectious agents. It has been suggested that this may be due to inefficient somatic hypermutation in the V gene segments of the Abs, inefficient help by aged Th cells, and the altered cytokine environment.

Similar to the decline in adaptive immune function, the functions of NK cells, macrophages, and neutrophils, crucial cellular components of innate immunity, are decreased with aging (10–13). In the aged mouse, alveolar macrophages are decreased in number and are not efficient at presenting Ags to T cells, and more macrophages are needed to effectively activate a T cell (5). Neutrophils have impaired chemotaxis, degranulation, and phagocytosis. Because macrophages, NK cells, and neutrophils provide the first line of defense against bacterial and viral infections, the decline in function could possibly explain the increased incidence of bacterial and viral pneumonias and gastrointestinal and skin infections in the aged as well as diminished protective immune responses to pneumococcal and influenza vaccines.

This first line of defense is accomplished through evolutionarily conserved sets of molecules, namely Toll-like receptors (TLR) that recognize conserved molecular patterns associated with pathogens. Microbes, microbial products, and pharmaceuticals that are ligands for TLR2, 3, 4, 5, 6, 7 and 9 have been identified (14–17). Ligands for TLR1 and 2, and 2 and 6, are Gram-positive bacteria and yeast cell wall components, while the predominant Gram-negative bacterial product, LPS, is a ligand for TLR4. Recent reports have also documented that dsRNA (poly I:C), bacterial flagellin, imiquimod, and CpG oligodeoxynucleotides (ODN) are ligands for TLR 3, 5, 7, and 9, respectively. The interaction between a TLR and its ligand results in the secretion of anti-bacterial peptides, defensins, and proinflammatory cytokines such as TNF-α and IL-6, which initiate an inflammatory response to clear the invading organism. Furthermore, the inflammatory response results in the recruitment of cells of adaptive immunity to initiate clearance of the pathogens by generating a specific immune response. Hence, defects at the level of expression and function of TLRs with aging could contribute to poor recruitment of APCs, and T
and B cells at the site of inflammation, resulting in suboptimal adaptive immune responses leading to increased incidence of illness and complications from infection. To address this, we examined the expression of all known murine TLRs (TLR 1–9) on splenic macrophages and thioglycollate-elicited peritoneal macrophages from young and aged mice by real-time RT-PCR. These macrophages were cultured with microbial products that interact with TLR2, TLR3, TLR4, TLR5, and TLR9 to investigate changes in function with age. We show that macrophage TLR expression and function decline with aging, which may impact both the quality and the magnitude of both innate and adaptive immunity.

### Materials and Methods

#### Mice

Young (2–3 mo old) and aged (18–24 mo old) female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and the National Institute of Aging (Bethesda, MD), respectively, and were maintained in an environmentally controlled facility.

#### Isolation of splenic and thioglycollate-elicited peritoneal macrophages

Splenic macrophages were isolated by incubating spleen cells at 3 × 10^6 cells/ml in a final volume of 15 ml in tissue culture-treated Petri plates for 90 min at 37°C with 5% CO₂. Nonadherent cells were removed and the plates were washed twice with complete medium (DMEM (Life Technologies, Grand Island, NY) containing 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM glutamine, and 5% FBS (HyClone Laboratories, Logan, UT)). To induce activated peritoneal macrophages, 1 ml of 3% thioglycollate (Difco, Irvine, CA) was injected i.p. into young or aged mice. Five days later, peritoneal exudate cells, consisting mostly of macrophages, were collected in 10 ml of PBS. Macrophages were washed with complete medium. Nonadherent cells were removed by incubating the cell suspensions for 90 min at 37°C with 5% CO₂. The purity of macrophage preparations was assessed by flow cytometry using allophycocyanin-conjugated anti-mouse CD11b (Mac-1) mAb (BD Biosciences, San Diego, CA). Cell surface expression of TLR4 was assessed with PE-conjugated mAb against murine TLR4 (e-Bioscience, San Diego, CA).

#### TLR expression by real-time RT-PCR

RNA was isolated with Tri-Reagent (Sigma-Aldrich, St. Louis, MO) from splenic macrophages and thioglycollate-elicited peritoneal macrophages. RNA samples were quantified by spectrophotometric analysis and were treated with DNase before cDNA synthesis with amplification grade deoxyribonuclease I (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. cDNA synthesis was performed with 1 μg of total RNA using 1.6 μg oligo(dT)15 primer, 20 nM of each dNTP, and 1× reaction buffer (all from Roche, Indianapolis, IN) in a final volume of 20 μl. cDNA reactions were incubated at 70°C for 10 min to denature the RNA templates and were quenched-cooled for 5 min. AMV reverse transcriptase (40 U) was added and reactions were incubated at 42°C for 60 min. cDNA reactions were diluted 1/10 and 2 μl of the diluted cDNA reaction was added to a 18-μl Light Cycler PCR containing 0.5 μM of each primer (Table I), 1× Light Cycler Fast Start DNA Master SYBR-Green mix containing Fast Start Taq polymerase and appropriate MgCl₂ (Table I). Reactions were conducted in glass capillaries (Roche) in the Light-Cycler instrument (Roche), subjected to a 10-min initial hot-start activation of the Taq polymerase at 95°C, followed by 40 cycles of amplification (95°C for 10 s, 56°C for 5 s, and 72°C for 10 s). The correct size of the amplified PCR products (Table I) was confirmed by gel electrophoresis. Amplification of accurate targets was confirmed by sequence analysis. For real-time analysis, samples were quantified by a standard curve generated by amplifying three serial dilutions in duplicate of each cDNA template with GAPDH primers as listed in Table I and the same reaction conditions as described above. Relative units were determined by generating a GAPDH curve, amplifying three serial 10-fold dilutions from 2 μl of the diluted cDNA and allowing the software to accurately determine the relative units of expression for each of the TLRs amplified as compared with the GAPDH curve. Removal of contaminating DNA was verified by adding 2 μl of diluted RNA before cDNA synthesis to an 18-μl Light Cycler PCR containing GAPDH primers.

#### TLR function

One million cells, from the same populations used for the RNA extraction as described above, were stimulated in a 24-well plate in a final volume of 2 ml with 200 ng of LPS (Sigma-Aldrich), 200 μg of poly(I:C) (Sigma-Aldrich), 3 × 10⁵ BioParticles of zymosan A (Saccharomyces cerevisiae), 3 × 10⁶ BioParticles of Staphylococcus aureus (both from Molecular Probes, Eugene, OR), 20 μg of ODN control (TCCATGAGCTTCTGGAT CCT), ODN containing CPG motifs (TCCATGACGTCTGGCTTT) obtained from GENSET (La Jolla, CA), or 1 μg of flagellin from Salmonella typhimurium (18). The optimum dose for each ligand indicated above was determined in a dose-response study to ensure that it is the TLR expression that is limiting but not the ligand. Cultures were incubated at 37°C with 5% CO₂ for 48 h. Supernatants were collected and IL-6 and TNF-α levels were determined by ELISA using OptiEIA kits (BD PharMingen, San Diego, CA).

### Results and Discussion

Deterioration of immune function and the increased incidence and lethality of infectious diseases in the elderly is well-documented (1–3). Macrophages are important cellular constituents of innate immunity that influence the priming environment in addition to

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Table I. Primer sequences of TLRs and GAPDH for real-time RT-PCR using the Light Cycler

<table>
<thead>
<tr>
<th>TLR</th>
<th>Primer Sequence (5'–3’)</th>
<th>MgCl₂ (mM)</th>
<th>Fragment Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Forward, CAATGGGAAACACGTTGGA</td>
<td>3</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>Reverse, TGTAACTTTGGGGGGGAGCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Forward, AAGAGGAAAGCCCAAGAAAGC</td>
<td>3</td>
<td>199</td>
</tr>
<tr>
<td></td>
<td>Reverse, CGATGGAATCTCGATGTGTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Forward, CACAGGCCGTGAGCAGTTGGA</td>
<td>3</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td>Reverse, TTTGGCCTTCTTTGATGCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Forward, ACCTGCGCTGTTTACAAGTC</td>
<td>3</td>
<td>201</td>
</tr>
<tr>
<td></td>
<td>Reverse, CTGCCAGAGACATTGCAGAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Forward, AAGTTCGGGGGAATCTGTTT</td>
<td>3</td>
<td>201</td>
</tr>
<tr>
<td></td>
<td>Reverse, GCATAGGCTGAGCTGTTTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Forward, TTCCCAATACCGCTGTTTTC</td>
<td>5</td>
<td>201</td>
</tr>
<tr>
<td></td>
<td>Reverse, CTATGCGCTTGGAGGGTGCAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Forward, AATCCACAGGCTTCCACATA</td>
<td>3</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>Reverse, CAGGTAACCAAGGGATGCTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Forward, GACATGGCCCTTTAATTTCTC</td>
<td>3</td>
<td>195</td>
</tr>
<tr>
<td></td>
<td>Reverse, GACCAGAATCTCCTGATGGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Forward, ACTGACACCCCGTGTTTCTA</td>
<td>3</td>
<td>198</td>
</tr>
<tr>
<td></td>
<td>Reverse, AGATTAGTACCGCCAGGAA</td>
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</tr>
<tr>
<td>GAPDH</td>
<td>Forward, CTCATGACCAAGCTTCCATGC</td>
<td>3</td>
<td>201</td>
</tr>
<tr>
<td></td>
<td>Reverse, CATATTGGGGAAGGACAC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
their phagocytic and tumoricidal functions. In this report, we examined the effect of age and the expression and function of various TLRs on splenic and thioglycollate-elicited macrophages.

Splenic macrophages and thioglycollate-elicited macrophages from young and aged C57BL/6 mice, which were over 90% pure based on FACS analysis, were used to assess the expression of TLRs 1–9 by RT-PCR as described in Materials and Methods. TLR and GAPDH PCR products were amplified and confirmed by gel electrophoresis (Fig. 1) and sequence analysis. Fig. 2 shows the relative units of expression of splenic and thioglycollate-elicited macrophages from young and aged mice relative to GAPDH determined by the Light Cycler.

The pattern of expression of TLRs in splenic macrophages differs from that of thioglycollate-elicited macrophages indicating that TLR expression varies with the state of activation and anatomical location as well. The most predominant TLR expressed by splenic macrophages from young mice is 2, followed by 9, 7, 6, 5, 8, 1, 3, and 4 (Fig. 2A). Unlike the splenic macrophages, thioglycollate-elicited peritoneal macrophages expressed TLR7 followed by 9, 2, 8, 5, 6, 3, 1, and 4 (Fig. 2B). These patterns have been confirmed by three independent experiments consisting of 5–10 mice per experiment. The pattern of expression of TLRs 1–9 on the splenic macrophages from the aged mice from the highest to the lowest was 2, 7, 9, 6, 1, 4, 5, and 8 and the levels were reduced when compared with those from young mice. Expression of TLR3 was barely detectable in the aged splenic macrophages. The maximum decline on splenic macrophages from aged mice was in TLR9 expression followed by TLR 5, 3, 2, 6, 8, 7, 1, and 4 when compared with those from young mice. The pattern of expression of all TLRs on thioglycollate-elicited peritoneal macrophages from aged mice from the highest to the lowest was 7, 2, 1, 3, 8, 5, 4, 9, and 6 as shown in Fig. 2B. There was a maximum decline in TLR9 expression followed by TLR5, 3, 2, 6, 8, 7, 1, and 4 when compared with splenic macrophages from young mice. The pattern of expression of all TLRs on thioglycollate-elicited peritoneal macrophages from aged mice from the highest to the lowest was 7, 2, 1, 3, 8, 5, 4, 9, and 6 as shown in Fig. 2B. There was a maximum decline in TLR9 expression followed by TLR5, 3, 2, 6, 8, 7, 1, and 4 when compared with those from young mice. The surface expression of TLR4 on the splenic and thioglycollate-elicited macrophages from the aged mice also declined when compared with those from young mice (Fig. 3). These data indicate that TLRs are differentially expressed on splenic and thioglycollate-elicited macrophages and the expression declines with aging. In addition, translation efficiency differences, mRNA stability, signal transduction defects, and other factors may also contribute to differences in surface expression and also function.

Ligation of TLRs with their ligands leads to NF-kB activation and proinflammatory cytokine secretion by MyD88-dependent and -independent pathways. To correlate the expression of TLRs with function, macrophages from aged and young mice were activated with ligands for TLR1 and 2, TLR2 and 6, TLR3, TLR4, TLR5,
These components could contribute to poor in
formation of CD14, LPS binding protein, and LPS and defects in any of
phages express very low levels of TLR5 and/or the expression of
flammatory cytokine secretion suggesting that splenic mac-
fold). However, TLR5 ligation with
flammatory cytokines, IL-6 and TNF-
and secreted signi
sentially higher amounts of proin
S. aureus
zymosan A (2.7-fold), flagellin (6.2-fold), Cpg ODN
(3.1-fold), and S. aureus (1.4-fold) stimulation. These data indicate
that proinflammatory cytokine responses decline with aging when
TLR2, 3, 4, 5, and 9 on splenic or thioglycollate-elicited macro-
phages are stimulated with their ligands. TNF-α enhances class I
and class II MHC expression and decreased levels in aging could
affect Ag processing and presentation, thus affecting T cell re-
sponses (22). Reduced TNF-α levels in aging may also contribute
to reduced phagocytic activity, reduced NO, reduced tumor cell
killing, and delayed tissue repair process.

Decreased expression and function of various TLRs may pre-
dispose the elderly to various bacterial and yeast infections. Due to
the decline in proinflammatory cytokines, the cardinal signs of
inflammation such as fever are absent in the elderly patients. Hence,
the increased mortality rates are attributed to lack of pre-
sentation of clinical signs at the onset of infection due to poor
inflammatory response. S. aureus is the fourth most common
pathogen and one of the top 10 causes of death in persons aged
>65 years. In addition, the elderly are also highly susceptible to
pneumonia and soft tissue infections caused by S. aureus (23). The
results of this study indicate that decreased expression and func-
tion of TLRs 1, 2, and 6, when ligated with S. aureus contribute to
enhanced susceptibility of the elderly to S. aureus infections. Fur-
thermore, decreased function of TLR 2 when ligated with zymosan
A (yeast) may help to explain the enhanced susceptibility to in-
fec tion with C. albicans, Cryptococcus neoformans, Coccidioides
immitis, and Aspergillus fumigatus in the elderly individuals (24,
25). The reduced function of macrophages in response to LPS from
Escherichia coli used in the current study and LPS from other
Gram-negative bacteria which trigger TLR4, may also explain why
the elderly are highly susceptible to urinary tract infections caused
by E. coli, and Proteus, Klebsiella, and Enterobacter species. The
poor inflammatory response induced by the macrophages from the
aged mice when stimulated TLR5 through the ligation by flagellin
from S. typhimurium coupled with poor responses with LPS may
explain why the aged mice and elderly humans are highly suscep-
tible to enteric infections caused by E. coli, and Salmonella, Shi-
gella, and Enterobacter species (26, 27). dsRNA (poly(I:C)) has
been shown to be a ligand for TLR3 and poly(I:C) has been used
to stimulate NK cells. The decline in TLR3 may be contributing to
decreased NK cell function in aging and predisposes the elderly to
viral, and intracellular, bacterial infections. An altered cytokine
pattern with a bias toward Th2 is a hallmark of aging. Bacterial
DNA containing Cpg sequences has been shown to interact with
TLR9 and induces a Th1 response (Refs. 1, 2, and 14 and unpub-
lished results). Hence, reduced expression and function of TLR9
on aged macrophages could be responsible for the altered priming/

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Splenic Peritoneal</th>
<th>Splenic Peritoneal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>Aged</td>
<td>Young</td>
</tr>
<tr>
<td>LPS</td>
<td>2.860 ± 0.28</td>
<td>536 ± 34</td>
</tr>
<tr>
<td>Poly(I:C)</td>
<td>1.600 ± 0.09</td>
<td>208 ± 0.3</td>
</tr>
<tr>
<td>Zymosan A</td>
<td>920 ± 57</td>
<td>204 ± 6</td>
</tr>
<tr>
<td>S. aureus</td>
<td>570 ± 42</td>
<td>240 ± 34</td>
</tr>
<tr>
<td>Flagellin</td>
<td>≥ 20</td>
<td>≥ 20</td>
</tr>
<tr>
<td>ODN CpG</td>
<td>280 ± 0</td>
<td>≥ 20</td>
</tr>
<tr>
<td>ODN control</td>
<td>44 ± 3</td>
<td>≥ 23</td>
</tr>
<tr>
<td>LPS</td>
<td>730 ± 14</td>
<td>514 ± 20</td>
</tr>
<tr>
<td>Poly(I:C)</td>
<td>540 ± 28</td>
<td>220 ± 28</td>
</tr>
<tr>
<td>Zymosan A</td>
<td>1,568 ± 45</td>
<td>480 ± 28</td>
</tr>
<tr>
<td>S. aureus</td>
<td>16,320 ± 1,358</td>
<td>7,776 ± 1,222</td>
</tr>
<tr>
<td>Flagellin</td>
<td>≥ 20</td>
<td>≥ 20</td>
</tr>
<tr>
<td>ODN CpG</td>
<td>525 ± 92</td>
<td>83 ± 18</td>
</tr>
<tr>
<td>ODN control</td>
<td>53 ± 18</td>
<td>47 ± 2</td>
</tr>
</tbody>
</table>

* Proinflammatory cytokine (IL-6 and TNF-α) secretion by splenic and thioglycollate-elicited peritoneal macrophages from young and aged mice when cultured with ligands for TLR1 and 2, 3, 4, 5, and 9 as determined by ELISA.

One million splenic and thioglycollate-induced peritoneal macrophages were cultured in a 2-ml final volume with 200 ng of LPS, 200 ng of poly(I:C), 3 × 10⁶ BioParticles of S. aureus or 20 μg of ODNs, or 1 μg of flagellin from S. typhimurium. Cultures were incubated at 37°C with 5% CO₂ for 48 h and supernatants were collected to measure IL-6 and TNF-α levels. The values represent mean ± SD and the statistical significance (p > 0.05) was tested by the Wilcoxon rank sum test. Each group consisted of cells from 5–10 mice.
recall microenvironment resulting in a Th2 bias in aging. Reduced expression and function of TLRs with aging thus impacts both the quality and magnitude of host innate and adaptive immune responses to bacterial and fungal infections by the altered inflammatory and priming environment. Hence, modulation of innate immunity through the up-regulation of TLR expression and function may be important for successful therapeutic and preventive immune intervention strategies for the elderly.

Acknowledgments
We thank Drs. Chin-Yih Ou and Stephen Lindstrom for their assistance with the quantification of real-time RT-PCR and sequence analysis, respectively, and Thomas Rowe and Nancy Cox for their critical reading of the manuscript.

References