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Age-Associated Defect in Human TLR-1/2 Function[1]

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The effects of aging on human TLR function remain incompletely understood. We assessed TLR function and expression in peripheral blood monocytes from 159 subjects in 2 age categories, 21–30 and >65 years of age, using a multivariable mixed effect model. Using flow cytometry to assess TLR-induced cytokine production, we observed a substantial, highly significant defect in TLR1/2-induced TNF-α ($p = 0.0003$) and IL-6 ($p < 0.0001$) production, in older adults compared with young controls. In contrast to findings in aged mice, other TLR (including TLR2/6)-induced cytokine production appeared largely intact. These differences were highly significant even after correcting for covariates including gender, race, medications, and comorbidities. This defect in TLR1/2 signaling may result from alterations in baseline TLR1 surface expression, which was decreased by 36% in older adults ($p < 0.0001$), whereas TLR2 surface expression was unaffected by aging. Production of IL-6 ($p < 0.0001$) and TNF-α ($p = 0.003$) after stimulation by *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*R*,*S*)-propyl]-Cys-[*S*]-Ser1-[*S*]-Lys(4) trihydrochloride was strongly associated with TLR1 surface expression. Diminished TLR1/2 signaling may contribute to the increased infection-related morbidity and mortality and the impaired vaccine responses observed in aging humans. *The Journal of Immunology,* **2007, 178: 970–975.**

Human aging is associated with increased morbidity and mortality from infectious diseases and impaired responses to immunization, resulting in part from immunosenescence (1–3). Well-described alterations in adaptive immunity associated with aging include a shift from naive to memory phenotype T cells, as well as decreased CD28 expression and oligoclonality in the T cell repertoire (4). However, the influence of aging on components of the innate immune system, such as the family of TLRs, remains incompletely understood. TLRs are pattern recognition receptors that recognize structural components shared by many bacteria, viruses, and fungi (5). TLR activation results in a multitude of pro- and anti-inflammatory events, including cytokine production and costimulatory molecule up-regulation. These events are mediated in part by the translocation of NF-κB and signal transduction via MAPKs (6). By thus activating APCs, TLRs provide a crucial link between innate and adaptive immunity (7). Changes in human TLR signaling have been implicated not only in altered susceptibility to infections but also in other conditions such as atherosclerosis and autoimmune diseases (8).

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Much of the evidence on the influence of aging on TLR function is derived from murine systems. TLR function and expression were reported to be generally impaired in macrophages from aged mice (9). Other murine studies did not report a change in murine TLR2 or TLR4 surface expression but noted a decrease in proinflammatory cytokine production after LPS and zymosan stimulation that correlated with decreased levels of p38 and JNK MAPK (10, 11). In humans, several reports have examined LPS-induced cytokine production in older adults, mostly using ELISA-based assays following stimulation of bulk PBMCs or whole blood and yielding conflicting results (12–16). We therefore evaluated the function and expression of a broad range of TLRs recognizing components of Gram-positive and Gram-negative bacteria, mycobacteria, fungi, and viruses in a cohort of 161 younger (21–30 years) and older (>65 years) adults. In these studies, we used flow cytometry, which in contrast to ELISA-based approaches allowed us to evaluate TLR expression and cytokine expression at the single-cell level in specific cell populations such as monocytes. In analyzing these results, we used a repeated measures mixed effects model that allowed the evaluation of the effect of age while accounting for heterogeneous baseline characteristics of our older and younger participants, a challenge for studies in human immunology (17). Our findings represent the first broad analysis of the influence of human aging on TLR function.

Materials and Methods

Human volunteers

Older (>65 years) and young (21–30 years) adults were enrolled from Yale Health Services. Immunocompromised subjects were excluded, including individuals with HIV infection, those with diabetes mellitus requiring medication, and those taking immunomodulating medications. Subjects with self-reported symptoms of recent infection (within 2 wk before enrollment) were excluded. This study was approved by the Human Investigations Committee at the Yale University School of Medicine. Informed consent was obtained from all volunteers.

PBMC isolation and stimulation

PBMC were isolated using Histopaque (Sigma-Aldrich) and were adjusted to 2×10^6 cells/ml. For TLR surface expression, the cells were stained, fixed,

and frozen until analysis. The TLR ligands *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*R,S*)-propyl]-Cys-[*S*]-Serl-[*S*]-Lys(4) trihydrochloride (Pam_3CSK_4)[3] (10 μg/ml), lipoteichoic acid (LTA; 2 μg/ml), LPS (1 μg/ml), flagellin (5 μg/ml), and polyuridylate (poly(U); 0.5 μg/ml) were used. RPMI 1640 alone served as a control. All ligands were obtained from InvivoGen. Cells were incubated for 18 h, with Golgi-stop (BD Pharmingen), containing brefeldin A, present for the last 12 h for intracellular cytokine staining assays.

Flow cytometry and Abs

Cell suspensions were stained with Abs for surface staining (CD11c (PE-Cy5, clone B-ly6), TLR1 (PE, clone GD2.F4), TLR2 (FITC, clone TL2.1), TLR4 (PE, clone HTA 125)), washed in 1% FBS, resuspended in 4% paraformaldehyde, washed again in 1% FBS, resuspended in 10% DMSO, 90% FBS, and stored at −80°C. On the day of analysis, cells were thawed, washed in 1% FBS, resuspended in BD Perm/Wash (BD Pharmingen), spun, and resuspended in BD Perm/Wash containing Abs for intracellular staining (IL-6 (FITC, clone MQ2-13A5), TNF-α (PE, clone Mab11)). Data were acquired on an LSR II instrument (BD Biosciences) and analyzed using FlowJo software (Tree Star); we acquired 30,000 events per sample. All Abs were from eBioscience except anti-CD11c (BD Pharmingen).

IL-8 ELISA

ELISA for IL-8 production was performed on supernatants of PBMC cultures after 18 h of stimulation. Supernatants were stored at −80°C until use. An OptEIA ELISA kit was used according to the manufacturer's instructions (BD Biosciences). Briefly, ELISA plates were coated overnight with anti-IL-8 capture mAb. After a washing and blocking, samples, controls and standards were incubated for 1 h. After incubation with Vectastain Elite ABC reagent (Vector Laboratories), ODs were read at 405 nm.

p38 MAPK phosphorylation

PBMC isolated as above were subjected to negative bead isolation to isolate monocytes as per the manufacturer's instructions (Dynabeads, Invitrogen). Monocytes were then resuspended in RPMI 1640; media, and stimulated with Pam_3CSK_4 (10 μg/ml). At the indicated time points, cells were placed on ice, washed, and further treated as described in *Western blot* below.

Western blot

Monocytes were lysed in 50 mM Tris (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM PMSF, 1 mM sodium orthovanadate, 10 mM NaF, and Complete Mini Protease Inhibitors (Roche) on ice for 20 min with intermittent vortexing. Lysates were then spun at 10,000 rpm for 15 min, analyzed via SDS-PAGE, and transferred to Immobilon-P membranes (Millipore). Membranes were blocked with 5% TBST containing 5% nonfat milk powder for 30 min at 20°C and then incubated overnight with rabbit anti-human TLR1 (Imgenex), monoclonal anti-human CD14 (Abcam), and anti-actin (Sigma-Aldrich) Abs or with rabbit anti-phospho-p38 MAPK (Thr^{180}/Tyr^{182}) Ab (Cell Signaling Technology). After washing, probed blots were developed using ECL Western Blotting Detection Reagents (Amersham Biosciences).

Statistics

We assessed whether the IL-6 and TNF-α responses were approximately normally distributed by estimating the first four moments and through examination of normal probability plots. The normality assumption was satisfied; consequently, we calculated descriptive statistics for participants at baseline and compared young and old participants using *t* tests for normally distributed covariates and χ^2 tests for categoric covariates. We calculated descriptive statistics for participants at baseline and compared young and old participants using *t* tests for normally distributed covariates and χ^2 tests for categoric covariates. The data were analyzed with a mixed effects model that used each ligand as a repeated measure (17, 18). The mixed model accounts for the correlation between ligand-specific TLR stimulation and cytokine responses in the same participant (19). Specifically, we used an unstructured covariance structure that allowed each participant to have their own specific correlation structure for each TLR, thus accounting for the inherent variation of each participant. The full models test for the fixed effect of age group (old vs young) and ligand, the interaction between age group and ligand, and the covariates gender, race, medications or vaccination (aspirin, nonsteroidal anti-inflammatory drugs (NSAID), statin use in the past 2 wk, influenza vaccination in previous year), and comorbid conditions (heart disease, stroke, peripheral vascular disease) using restricted maximum likelihood. Next, the interaction term was tested by re-estimating the model using the maximum likelihood method. In all models, the interaction term was significant and retrained. Least squared means were estimated for the fixed effects of age group and age group by ligand interaction, and the differences were tested. Subset analyses (Fig. 2) were tested by bivariate analysis only (Student's *t* test). To determine temporal variance of TLR1 surface expression, variance components estimated for date, assessor, and subject. Furthermore, a mixed model testing whether date were associated with TLR1 surface expression found no significant difference between dates. All statistical tests were two-tailed, and $p < 0.05$ was considered to indicate statistical significance. All analyses used SAS version 9.1 (SAS Institute).

Table I. *Baseline characteristics*

	Young ($n = 78$)	Older ($n = 81$)	p^a
Age, mean year (range)	25.7 (22–30)	74.6 (65–49)	<0.0001
Gender, female	55 (71%)	39 (48%)	0.0059
Race			
% Caucasian	50 (64%)	76 (94%)	<0.0001
% African American	5 (6%)	3 (4%)	0.0498
% Asian	16 (21%)	2 (2%)	0.0003
% Hispanic	7 (9%)	0 (0%)	0.0059
Comorbidities			
None	77 (99%)	66 (80%)	0.0003
Stroke (%)	0 (0%)	4 (5%)	0.1205
Heart disease (%)	0 (0%)	12 (15%)	<0.0001
PVD[b] (%)	0 (0%)	3 (4%)	0.2455
COPD (%)	0 (0%)	3 (4%)	0.2455
PUD (%)	1 (1%)	4 (5%)	0.1205
Medications			
Mean no. (range)	1.6 (0–8)	4.1 (0–11)	<0.0001
Aspirin	0 (0%)	45 (56%)	<0.0001
NSAID	25 (32%)	7 (9%)	<0.0001
Statin	0 (0%)	27 (33%)	<0.0001

[a] *p* values are based on *t* tests for normally distributed characteristics and χ^2 tests for categoric characteristics.

[b] PVD, Peripheral vascular disease; COPD, chronic obstructive pulmonary disease; PUD, peptic ulcer disease.

Results

TLR1/2-induced cytokine responses are reduced in older adults

To determine the influence of aging on human TLR function, we enrolled 159 community-dwelling adults in two groups: 78 between 21 and 30 years; and 81 ≥65 years of age (Table I). Our cohort represents relatively healthy older adults, with 83% listing no major comorbid conditions. We used a multivariate mixed modeling approach to account for characteristics of participants potentially associated with TLR responses. All demographic and clinical characteristics that were significantly different between the two groups (including race and gender) are corrected for in the reported analyses. After isolation of PBMCs, we assessed TLR surface expression in $CD11c^+$ monocytes and in parallel tested in vitro responses to ligands for TLRs expressed on monocytes, including those for the TLR1/2 heterodimer (Pam_3CSK_4), the TLR2/6 heterodimer (LTA), TLR4 (LPS), TLR5 (flagellin), and TLR7 and/or TLR8 (poly(U)). We then measured cytokine responses via intracellular staining and flow cytometry.

We found that TLR1/2-mediated IL-6 and TNF-α responses induced by Pam_3CSK_4 were diminished in $CD11c^+$ monocytes of older adults when compared with young individuals (Fig. 1). These least squared mean differences were highly significant (IL-6 $p < 0.0001$; TNF-α $p = 0.0003$), even after adjusting for gender, race,

[3] Abbreviations used in this paper: Pam_3CSK_4, *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*R,S*)-propyl]-Cys-[*S*]-Ser1-[*S*]-Lys(4) trihydrochloride; LTA, lipoteichoic acid; NSAID, nonsteroidal anti-inflammatory drug; poly(U), polyuridylate; OspA, outer surface lipoprotein A.

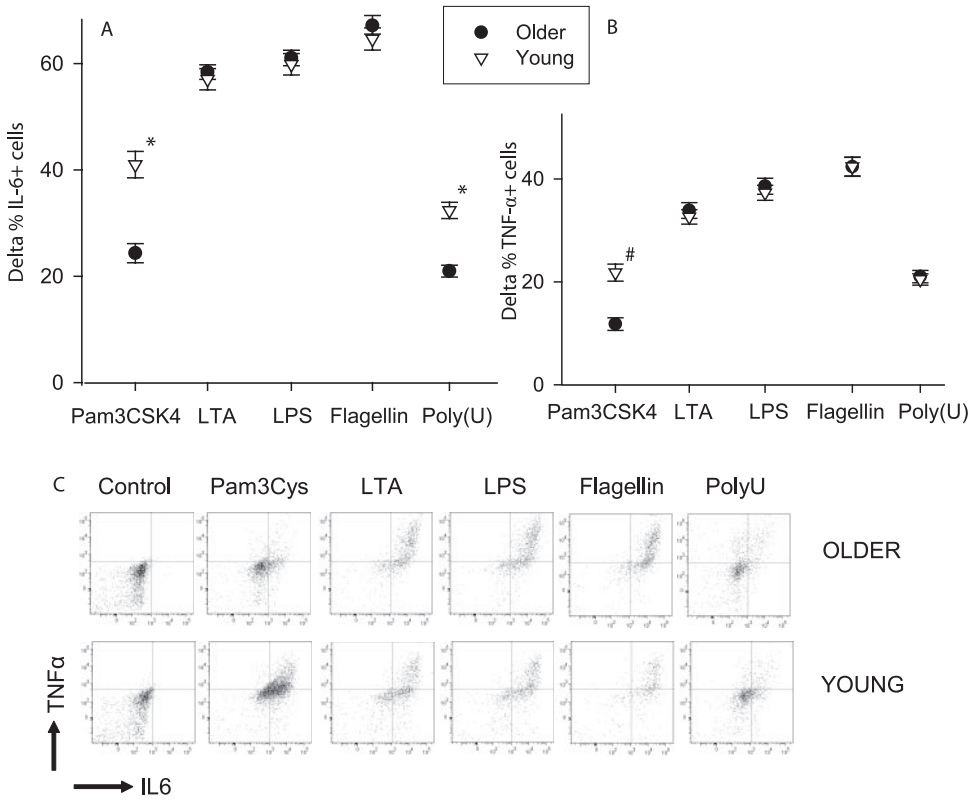

FIGURE 1. TLR1/2-induced TNF-α and IL-6 production is decreased in older vs younger adults. The difference in the percentage of cells with detectable intracellular IL-6 (*A*) and TNF-α (*B*) in unstimulated vs TLR ligand-stimulated CD11c$^+$ monocytes (relative to isotype control) is depicted. Values indicate the mean $\pm$ SEM of the young ($n = 78$; $\triangledown$) and older adults ($n = 81$; ●). $*$, $p < 0.0001$; #, $p = 0.0003$. p values are adjusted for covariates in the multivariable mixed model. *C*, Representative flow cytometry plots of intracellular TNF-α and IL-6 in monocytes from a young and older participant.

medications, or vaccination history (i.e., aspirin, NSAID, statin use in the past 2 wk, influenza vaccination in previous year) and co-morbid conditions (e.g., heart disease, stroke, peripheral vascular disease) using the multivariable mixed effect regression model. In some participants in the older age group, Pam_3CSK_4-induced IL-6 and TNF-α responses were virtually absent (Fig. 1).

No significant differences between older and young adults were observed for IL-6 and TNF-α responses after stimulation with

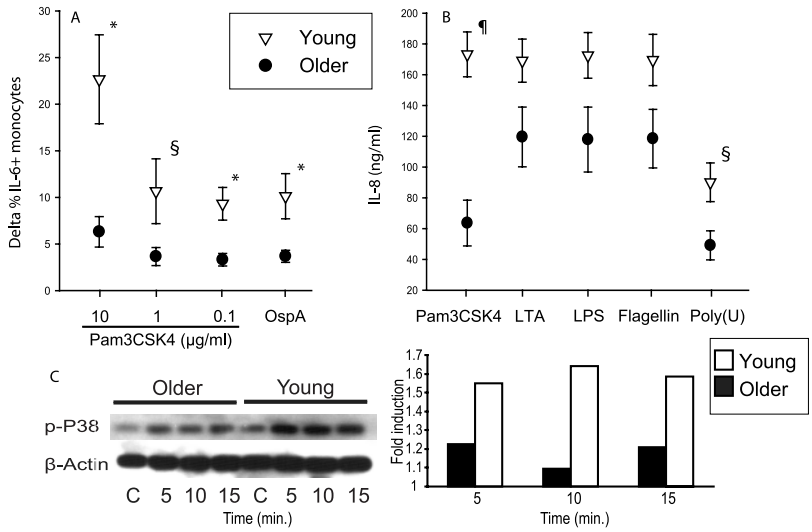

FIGURE 2. Age-associated decrease in TLR1/2 function. *A*, IL-6 production as measured by intracellular staining of a subset of older ($n = 9$) and young ($n = 5$) adults in response to OspA and various concentrations of Pam_3CSK_4 are shown. *B*, Results of IL-8 ELISA performed on supernatants from young ($n = 10$) and older ($n = 10$) individuals. Values indicate the mean $\pm$ SEM; p values of these subsets were calculated in bivariate analyses by t test. ¶, $p < 0.0001$; $*$, $p < 0.01$; §, $p < 0.05$. *C. Left*, Western blot of p38 phosphorylation in a young and an old subject after stimulation with Pam_3CSK_4 in bead-isolated monocytes (representative result of four experiments on samples from two pairs of young and older individuals). Phosphorylation levels at 5, 10, and 15 min are compared with unstimulated control (*C*) samples. Actin is shown as a control. *Right*, Results of densitometry of the Western blot at left, shown as fold induction of phospho-p38 MAPK relative to control, with each value normalized for actin content.

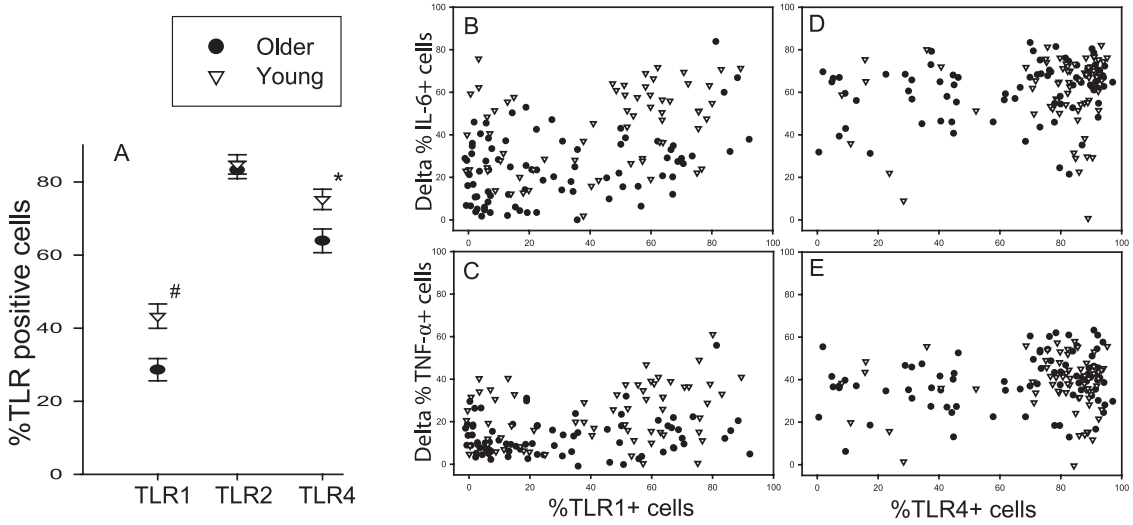

FIGURE 3. Age-associated decrease in TLR1 surface expression correlates with decreased cytokine production. *A*, Baseline percentage of $CD11c^+$ monocytes (compared with isotype control) with detectable surface TLR1, TLR2, or TLR4. Values indicate the mean ± SEM of the young adults (n = 66; ▽) and older adults (n = 80; ●). #, p = 0.0006; ∗, p = 0.0026. *B* and *C*, Individual outcomes for percentage of $CD11c^+$ monocytes positive for cell surface TLR1 were correlated with the change in percentage of positive cells for IL-6 (*B*), and TNF-α (*C*) after Pam_3CSK_4 stimulation. A statistically significant association was observed for both outcomes in young and older adults for IL-6 production and TLR1 surface expression (overall: Pearson correlation coefficient, 0.425, $p < 0.0001$; young: Pearson correlation coefficient, 0.355, p = 0.0035; older: Pearson correlation coefficient, 0.371, p = 0.0007), whereas for TNF-α and TLR1 surface expression a significant correlation was found in the young group, and a trend in older adults (overall: Pearson correlation coefficient, 0.324, p = 0.0003; young: Pearson correlation coefficient, 0.279, p = 0.0235; older: Pearson correlation coefficient, 0.210, p = 0.059). *D* and *E*, For TLR4 surface expression in $CD11c^+$ monocytes and LPS-induced IL-6 and TNF-α (*D* and *E*, respectively), a weak association was observed that was nonsignificant in the young cohort (IL-6 overall: Pearson correlation coefficient, 0.218, p = 0.048; young: Pearson correlation coefficient, 0.159, p = 0.2; older: Pearson correlation coefficient, 0.236, p = 0.03; TNF-α overall Pearson correlation coefficient, 0.277, p = 0.04; young: Pearson correlation coefficient, 0.087, p = 0.48; older: Pearson correlation coefficient, 0.226, p = 0.04).

LTA, LPS, or flagellin, recognized by TLR2/6, TLR4, and TLR5, respectively. Stimulation of TLR7/8 by poly(U) resulted in a significantly decreased IL-6 response in the older group, although no significant difference in poly(U)-induced TNF-α production was observed (Fig. 1).

We also evaluated responses in a subset of our older and young cohort to outer surface lipoprotein (OspA; n = 14), a lipopeptide derived from *Borrelia burgdorferi* which also specifically engages TLR1/2 (20). As with Pam_3CSK_4, we found a statistically significant decrease in IL-6 production in older, compared with young, adults after OspA stimulation (p = 0.0064; Fig. 2*A*). We performed a Pam_3CSK_4 dose-response analysis for IL-6 production in a subset of older and young individuals and observed both an expected dose-response relationship as well as a statistically significant, age-associated decrease in IL-6 production at all doses of Pam_3CSK_4 tested (Fig. 2*A*). For these analyses, we focused on IL-6 and TNF-α production, cytokines classically associated with TLR engagement. We extended these findings and measured IL-8 production, also a result of TLR engagement in monocytes by ELISA in a subset of individuals (Fig. 2*B*; n = 20). Using this approach, we again observed a decrease in IL-8 production in older adults after stimulation with Pam_3CSK_4 that was highly significant despite the small sample size (173 ± 15 ng/ml vs 63 ± 15 ng/ml in young and older adults, respectively, $p < 0.0001$ by two-sided t test). Poly(U) stimulation also resulted in significantly less IL-8 in older adults, although the difference was smaller (91 ± 13 vs 50 ± 9 in young and older adults, respectively, p = 0.02). Marginal differences at the limits of statistical significance were seen for other ligands (p = 0.05 for LTA and LPS; p = 0.06 for flagellin). Consistent with these findings, monocytes purified from young and older individuals with detectable and low levels of surface TLR1 respectively revealed decreased levels of p38 phosphorylation after Pam_3CSK_4 treatment of samples from older adults (Fig. 2*C*). Taken together, these findings indicate that human TLR1/2 function is substantially diminished in the context of aging.

TLR1 surface expression is decreased in older adults

We assessed baseline TLR1, TLR2, and TLR4 surface expression on monocytes to determine whether a concomitant age-associated

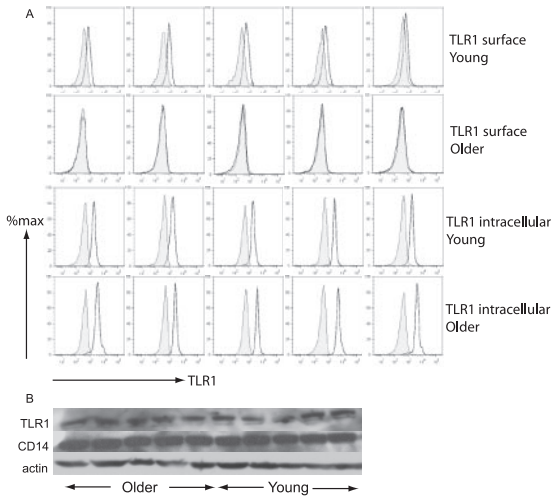

FIGURE 4. Total cell TLR1 protein levels are unaffected by aging. *A*, Flow cytometry histograms from five young and five older adults (isotype, gray tinted; TLR1, black line). *Top two rows*, TLR1 surface staining in young (top) vs old (*second row*). *Bottom two rows*, TLR1 staining in permeabilized cells (young, *third row*; old, *fourth row*). *B*, Western blot for TLR1 protein in whole cell lysates of PBMC of the same individuals, with CD14 and actin shown as controls.

decrease in TLR1 surface expression might be observed in the context of diminished cytokine responses to TLR1/2 engagement, (Fig. 3*A*). TLR1 surface expression was 36% lower on monocytes of older adults than on those of young adults ($p = 0.0006$), but TLR2 surface expression was not significantly altered between the age groups. In contrast, although a small (18%) but statistically significant decrease in expression was observed for TLR4 in the older group (Fig. 3*A*), no accompanying alteration in LPS-mediated cytokine production was observed (Fig. 1).

To evaluate the stability over time of TLR1 surface expression on human monocytes, we measured TLR1 surface expression by flow cytometry three times each from five additional individuals over a 3-wk period. All measurements were analyzed by two independent assessors. Intersubject variation accounted for 91.6% of observed variation, whereas date and assessor accounted for only 8 and 0.4%, respectively (data not shown). Therefore, temporal variation in TLR1 surface expression levels is unlikely to account for our findings.

Furthermore, a strong, statistically significant association was observed between TLR1 surface expression and the production of both IL-6 ($p < 0.0001$) and TNF-α ($p = 0.003$) following Pam_3CSK_4 stimulation (Fig. 3, *B* and *C*). This supports a link between our observations of decreased TLR1/2 function and TLR1 surface expression; by contrast, a much weaker association was observed between TLR4 surface expression and cytokine responses (IL-6 $p = 0.048$; TNF-α $p = 0.039$) after LPS stimulation (Fig. 3, *D* and *E*). Thus, the decreased TLR1 surface expression that we have observed is strongly associated with the functional outcome of decreased cytokine production upon TLR1/2 engagement in older adults.

We then measured intracytoplasmic TLR1 by flow cytometry in monocytes from an additional five older adults and five young adults to evaluate whether total cell TLR1 protein is also decreased in the setting of human aging. These older adults were selected on the basis of low TLR1 surface expression by flow cytometry. We found levels of intracellular TLR1 staining in these older adults comparable with those in young adults. Additionally, Western blot analysis on whole cell lysates from PBMCs derived from these same subjects revealed comparable total cell TLR1 protein levels (Fig. 4). These results suggest that the observed defect in TLR1 surface expression results from posttranslational mechanisms mediating membrane expression of TLR1.

Discussion

Our findings represent the most comprehensive determination to date of TLR function in the setting of human aging. We found that older adults have impaired responses to TLR1/2-specific stimulation, with decreased TLR1/2-induced TNF-α and IL-6 production in older adults when compared with younger participants; these differences remained highly significant after correcting for covariates, including gender, race, medication use, and comorbidities. Moreover, we extended these findings in a subset of our cohort to demonstrate significant decreases in IL-6 production by a distinct TLR1/2 agonist, OspA; in addition, we observed decreased Pam_3CSK_4-induced IL-8 production in older individuals and a dose-dependent relationship between Pam_3CSK_4 and IL-6 production. These results strongly suggest that TLR1/2 function is diminished in monocytes from older individuals.

We found that monocytes of older adults express decreased levels of TLR1 on monocytes, but unchanged levels of TLR2, compared with younger individuals. This baseline TLR1 surface expression was a strong predictor of subsequent TLR1/2-induced cytokine production, suggesting that decreased TLR1 expression on the surface of aged human monocytes may contribute to the observed defects in TLR1/2 stimulation. We found that total cell TLR1 protein, as determined by flow cytometry and Western blot analyses, was unaffected in older adults, indicating that posttranslational events mediating the transport of TLR1 to the cell surface may be responsible for the age-associated defect in TLR1 surface expression.

We did not observe a generalized defect in TLR function and expression in this group of older individuals; in particular, we did not observe an alteration in the expression or function of TLR2, with which TLR1 associates. Given the role of TLR2 in forming heterodimers with both TLR1 and TLR6, it is attractive to speculate that defective TLR2 function would have severe consequences for innate immune signaling such that substantial decrement in expression or function would be rare in humans. As an alternative explanation for the observed isolated TLR1 defect, it is conceivable that changes in TLR1 surface expression have more profound effects on signaling as a consequence of low baseline surface expression of TLR1 and in view of observations indicating that the threshold for TLR1/2 engagement of intracellular signaling pathways is increased compared with other TLRs (21).

We observed a difference between young and older individuals in TLR4 surface expression; however, the magnitude of this difference was substantially smaller than that noted for TLR1, and TLR4 surface expression was a poor predictor of LPS-induced cytokine production, in contrast to the robust association observed for Pam_3CSK_4 engagement of TLR1/2, (Figs. 1 and 3). In this regard, it is conceivable that the amount of TLR4 on the cell surface in older individuals, notwithstanding the marginal decrease in surface expression, meets the threshold for normal cytokine production. Although age-associated changes in TLR4 function have been previously reported in studies using ELISA-based assays (13–16), we chose a flow cytometry-based approach over ELISA-based assays to detect cytokine production specifically in monocytes. Because this method allowed us to assess cytokine production in specific cell populations and account for relative differences in numbers of monocytes, we believe that this approach offers more specific information compared with analysis via ELISA of supernatants from mixed populations of PBMCs. Although it remains possible that alterations in TLR4 function in monocytes might be observed at other concentrations of LPS, at the very least our results indicate that a dose-response effect as we have observed for Pam_3CSK_4-mediated TLR1/2 stimulation is unlikely for human TLR4.

Whether the function of any other TLRs is altered in the context of human aging remains to be determined; our older cohort of participants was remarkably free of comorbid conditions, and it is possible that age-associated TLR functional defects are observed in populations with increased levels of disability and/or disease. TLR7/8 function may also be impaired as a function of aging, given the observed decreased poly(U)-induced IL-6 and IL-8 production in older adults. Additional studies in large human cohorts are needed to address these issues. However, the data in the present study incorporate a variety of experimental approaches that all substantiate the conclusion that TLR1/2 function and TLR1 surface expression are defective in human aging.

In this context, our finding of an age-associated TLR1/2 defect is reminiscent of the characterization of TLR1 defects in seven human non-responders to the OspA vaccine against Lyme disease; while in mice both TLR1 and TLR2 were required for cytokine responses to OspA, all seven human nonresponders had a defect in TLR1 function but preserved TLR2 function (22), analogous to the findings we now report in a large cohort of older and younger adults. Furthermore, our analyses of a cohort of older individuals with a low prevalence of comorbid medical conditions indicate

that decreased TLR1/2 function in monocytes is associated with TLR1 surface expression. Although the Lyme vaccine study did not address the effects of aging on TLR function, all seven nonresponders in this study were older than 65 years of age (V. Thomas and E. Fikrig, unpublished observation).

We believe that our use of multivariable mixed effect modeling to account for heterogeneity in aged human populations is critical for determining the significance of immunological studies in highly diverse human cohorts. Consistent with this approach, we did not choose to apply restrictive enrollment criteria, such as the SENIEUR protocol, which is based on clinical data and laboratory measurements that typically result in the exclusion of >70% of subjects older than 65 years of age (23, 24). Hence, our participants are more representative of the community-dwelling human population, and our results therefore potentially more readily generalizable.

TLR1/2 ligands are present in a wide range of pathogens, including bacteria, spirochetes, and mycobacteria. Examples of these include *Neisseria meningitidis*, *B. burgdorferi*, *Mycobacterium tuberculosis*, and *Mycobacterium leprae* (22, 25–31). The age-associated TLR1/2 defect that we observed may therefore have substantial consequences in the ability of older individuals to mount effective innate and adaptive immune responses to these organisms. It is likely that engagement of multiple TLRs by an organism occurs in the context of infection; for example, *M. tuberculosis* is known to signal through TLR4 and TLR9 in addition to TLR1/2 (32–34). Nonetheless, in the setting of an aged immune system, the defect in TLR1/2 signaling may contribute to the impairment in immune responses to both infectious diseases and vaccination in the elderly.

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Disclosures

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