Dysregulation of TLR3 Impairs the Innate Immune Response to West Nile Virus in the Elderly

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West Nile virus (WNV), a mosquito-borne flavivirus, has recently emerged in North America, and the elderly are particularly susceptible to severe neurological disease and death from infection with this virus. We have investigated the innate immune response of primary human macrophages to WNV in vitro and have found significant differences between the responsiveness of macrophages derived from younger donors and that from older donors. Binding of the glycosylated WNV envelope protein to the C-type lectin dendritic cell-specific intercellular adhesion molecule 3 (ICAM3) grabbing nonintegrin (DC-SIGN) leads to a reduction in the expression of Toll-like receptor 3 (TLR3) in macrophages from young donors via the signal transducer and activator of transcription 1 (STAT1)-mediated pathway. This signaling is impaired in the elderly, and the elevated levels of TLR3 result in an elevation of cytokine levels. This alteration of the innate immune response with aging may contribute to the permeability of the blood-brain barrier and suggests a possible mechanism for the increased severity of WNV infection in older individuals.

Here, we examine the effect of age on the innate immune response to WNV infection. Using human primary macrophages derived from healthy young subjects and older individuals, we show altered innate immune responses in macrophages from aged hosts. This altered response may contribute to increased susceptibility to WNV infection.

MATERIALS AND METHODS

Recombinant WNV-E protein, antibodies, and reagents. The first 406 amino acids of the envelope (E) protein of WNV (NCBI accession no. AF206518) were cloned into the pMTB/His vector (Invitrogen, CA) and expressed in the Drosophila melanogaster S2 expression system (Invitrogen, CA). The recombinant WNV-E (rWNV-E) protein was purified via its carboxyl His tag, using a nickel column (Qiagen, CA). The His-tagged rWNV-E protein was eluted with 10 ml of elution buffer containing 50 mM NaH2PO4, 300 mM NaCl, and 250 mM imidazole (pH 8) and dialyzed against 30 times its volume with sterile phosphate-buffered saline (PBS). The purity of the WNV-E protein was assessed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the concentration was estimated by the Bradford assay (2a). The endotoxin-free recombinant protein was used at the concentration of 30 ng per 10⁶ cells, unless indicated otherwise. Antibodies used in this study included anti-dendritic cell-specific intercellular adhesion molecule 3 (ICAM3) grabbing nonintegrin (DC-SIGN)/CD209 (clone 120507; R&D Systems), anti-macrophage mannose receptor (MMR)/CD206 (BD Pharmingen, CA), anti-phospho-abl (Tyr578), and -actin (Sigma-Aldrich, MO). Rabbit polyclonal anti-WNV-E was generated as described previously (16). Control mouse serum immunoglobulin G (IgG), EGTA, and Saccharomyces cerevisiae mannans were purchased from Sigma. F(ab)² fragments of anti-DC-SIGN, -L-SIGN, -MMR, and mouse serum IgG were prepared with an Immunopure Fab preparation kit according to the manufacturer’s instructions (Pierce, IL).

Viral isolates and cell lines. West Nile virus strain CT-2741 was provided by John Anderson, Connecticut Agricultural Experiment Station, New Haven, CT. This WNV strain is identical at the protein level to the NY99 strain (1). The glycosylation-deficient mutant strain NY99-E154 was a generous gift from Alan Barrett and David Beasley, University of Texas Medical Branch, Galveston, TX. All virus isolates were passaged once in Vero cells (African green monkey kidney...
cell line, ATCC CCL-81), as described previously (36). Viral PFU were quantified from culture supernatants by plaque assays with Vero cells to evaluate the production of infectious virus according to our routine methods. No significant differences were detected in the attachment of these strains to macrophages, as determined by densitometric scanning of Western blots of lysates of cells infected with equal numbers of PFU (the WNV-Eaucin ratio for CT-2741 was 0.43 ± 0.07; the ratio for NY99-E154 was 0.63 ± 0.11; n = 4 to 6; differences were not significant [NS]). Where indicated, viral stocks were obtained from infection of HeLa cell lines, using the same procedure. Viral stocks were 4.9 × 10^7 PFU/ml and 1.8 × 10^7/ml for Vero-grown (WNVVero) and HeLa-grown (WNVHeLa), CT-2741 cultures, respectively. Viral stocks were 6.3 × 10^9/ml for the NY99-E154 culture.

To obtain a multiplicity of infection (MOI) of 1, 1 × 10^6 viral particles were used to infect the same number of macrophages in a 35-mm well in a total volume of 2 ml of culture medium. All studies with WNV were conducted in a biosafety level 3 facility, licensed according to the regulations of the state of Connecticut and the Office of Environmental Health and Safety of Yale University.

**Blood donors and isolation of monocyte-derived macrophages.** Blood was collected in accordance with the regulations of the Human Investigation Committee of Yale University. In this study, young individuals were defined as people 20 to 36 years of age (average, 27.2 ± 4.6 years; n = 45; 71% female; 82% white), and elderly subjects were defined as individuals 56 years of age and older (average, 72.3 ± 8.8 years; n = 42; 67% female; 93% white). Donors were healthy and had no acute illness, took no antibiotics or non-steroidal antiinflammatory drugs, and were screened for exposure to WNV infection. Peripheral blood mononuclear cells (PBMC) isolated from these donors were randomly chosen for different experiments over the period of 1 year. No experiments were done using one donor repeatedly. PBMC were isolated by Ficoll-Hypaque density gradient centrifugation of blood from blood donors with antibodies, as previously described (23). PBMC were suspended in endotoxin-free RPMI 1640 medium containing 20% human sera (Cambrex, MD), 1,000 U/ml penicillin, and 1,000 µg/ml streptomycin (Invitrogen, CA). Cells were plated at 5 × 10^5 cells/35-mm well or 1 × 10^6 cells/60-mm plate. After 2 h, nonadherent cells were removed by washing, and cells were incubated for 6 to 8 days to obtain primary monocyte-derived macrophages.

**Pericellular flow cytometry of human primary macrophages.** Mature primary human monocyte-derived macrophages were detached from the culture dishes by using 100 µM of EDTA for 30 min at 37°C. Detached cells were washed once in PBS and labeled for 1 h at 4°C in PBS containing 2% fetal bovine serum with surface-staining monoclonal antibodies, as follows: DC-SIGN–fluorescein isothiocyanate (FITC), L-SIGN–FITC, CD1a–phycoerythrin (PE), CD3–allophycocyanin (APC)-Cy7, CD19–APC-Cy7 (BD Biosciences, CA), and CD14–PE-Cy7 (eBioscience, CA). Cells were fixed in 1% paraformaldehyde for fluorescence-activated cell sorter (FACS) analysis. Data were acquired on an LSRII system unit (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR). Macrophage cultures from both age groups contained fewer than 1% of cells positive for CD1a, CD3, and CD19, showing that there were few or no contaminating dendritic cells or B or T lymphocytes.

**Immunoprecipitation and Western blot analysis.** Mature primary human monocyte-derived macrophages were incubated with antibodies or inhibitors for 30 min at 37°C before they were infected with WNV at an MOI of 1 or treated with WNV-E protein at a concentration of 30 ng/10^6 cells for 1 h. F(ab)_2 fragments of anti-DC-SIGN, L-SIGN, -MMR, mouse serum IgG, and EGTA and mannan were each used at a concentration of 5 µg/ml. The concentration of agents was maintained throughout the incubation with WNV or WNV-E.

**RNA interference.** Adherent monocyte-derived macrophages were dislodged using 100 µM EDTA at 37°C for 30 min and washed twice with Ca^2+–Mg^{2+}-free PBS at room temperature. Macrophages (5 × 10^6) were transfected with 6 µg of pooled small interfering RNAs (siRNAs) targeting DC-SIGN or L-SIGN (Ambion Inc, CA) (Table 1), using the nucleofection technology (Amaxa Inc., MD). Electroporated cells without siRNA were used as a mock control. Cells transfected with L-SIGN siRNA were used as nontargeting controls because human macrophages express little L-SIGN (≤10%; 21). Cells were washed after 6 to 8 h, and treatment was carried out for 24 h. Total proteins from two replicates were harvested and pooled for immunoblotting, using anti-DC-SIGN monoclonal antibody to assay the efficiency of RNA interference.

**Infection with WNV.** Infection with WNV followed a time course described previously, with some modifications (24). Mature primary human monocyte-derived macrophages were chilled on ice for 10 min before warm medium containing WNV-E protein (30 ng/ml) or recombinant WNV-E protein (30 ng/ml) was added, and cells were incubated at 37°C for 0, 5, 10, 20, and 30 min. At each harvest time point, cells were placed on ice, medium was removed, and cells were washed twice with cold PBS containing a kinase inhibitor cocktail (1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride) and a protease inhibitor cocktail (Roche Diagnostics, NJ). Total proteins were harvested using a modified radioimmunoprecipitation assay (RIPA) buffer containing the same kinase and protease inhibitor cocktails.

**Chromatin immunoprecipitation.** Chromatin immunoprecipitation from 10^7 mature macrophages treated with WNV-E protein for 1 h was performed using the method described by Thomas et al. (33), with some modifications. Cells were fixed with paraformaldehyde at a final concentration of 1% for 10 min at room temperature before cross-linking was quenched with glycine (150 mM) for 5 min. Cells were washed twice with ice-cold PBS containing a protease inhibitor cocktail, harvested with modified RIPA lysis buffer, and sonicated to shear the chromatin. Following centrifugation of the cells at 12,000 × g for 5 min at 4°C, protein concentrations were determined by measuring absorbance at 280 nm. Equal protein concentrations of the supernatants were incubated overnight with 5 µg of anti-STAT1 antibody. A fraction of the supernatant was immunoblotted to ensure that equal amounts of STAT1 protein were used in the immunoprecipitation. Aliquots of equal amounts of supernatants were subjected to DNA purification and used as “input.” The antibody-protein/DNA complexes were incubated with protein A/G Sepharose agarose beads (Santa Cruz Biotechnol, CA) for an additional 3 h at 4°C and pelleted at 12,000 × g for 5 min at 4°C. The bound antibody-protein/DNA complexes were washed six times with ice-cold PBS containing 0.05% Tween-20 (PBST) and twice with 0.1% PBST and heated overnight at 65°C. Supernatants were collected and treated with proteinase K for 2 h at 37°C. DNA was purified as “output,” using a Qiagen PCR purification kit (Qiagen, CA). Input and output DNA was used in PCR amplifications with primers specific to promoters of TLR3, interferon regulatory factor 1 (IRF1), IFI16, and β-actin (Table 1).

**Immunoprecipitation and Western blot analysis.** Total proteins were harvested and treated with proteinase K, then immunoprecipitated with monoclonal antibodies, using modified RIPA lysis buffer. Equal amounts of protein lysates were incubated with 5 µg of anti-DC-SIGN antibody for 3 h and then precipitated with protein A/G Sepharose agarose beads at 4°C overnight. The antibody-protein bead complexes were washed six times with PBST and twice with 0.1% PBST, and then proteins were denatured and eluted with Laemmli buffer. Immunoprecipitates or whole-cell lysates were electrophoresed on a 4 to 12% polyacrylamide gel (Invitrogen, CA) and blotted. Immunoblots were developed using a Western Lighting chemiluminescence kit (Pierce, IL).

**Confocal imaging.** PBMC (2 × 10^5) were plated on 12-mm round glass coverslips, washed after 2 h, and incubated for 6 to 8 days to obtain macrophages, as described previously (23). Primary macrophages were incubated in the absence and presence of WNV-E (30 ng/ml) cells for 3 h at 37°C. Samples were fixed and permeabilized in 4% paraformaldehyde containing 0.2% Triton-X at 37°C for 30 min and blocked in PBS-10% fetal bovine serum for 1 h. Cells were labeled with rabbit anti-STAT1 primary antibody (1:100) overnight, staining was detected by tetramethyl rhodamine isothiocyanate-conjugated anti-rabbit secondary antibody (1:500; Molecular Probes, CA) for 1 h, and nuclei were counterstained with TO-PRO3 (1:1,000; Molecular Probes, CA) for 20 min. A minimum of 100 cells was counted from each sample, and the percentage of STAT1 staining was determined by measuring the area of staining in the nucleus/(total number of cells) × 100. Images were collected with an LSM 510 laser scanning confocal microscope (Carl Zeiss MicroImaging Inc., NY), as previously described (23).

**qPCR analysis.** Total RNA was harvested from macrophages, using the RNeasy mini-kit according to the manufacturer’s instructions (Qiagen, CA), and cDNA was synthesized according to standard protocols. Primers and probes for quantitative PCR (qPCR) assays were either synthesized according to customized sequences or obtained from Applied Biosystems, CA (Table 1). Amplification was performed in an iCycler (Bio-Rad, CA) for 60 cycles with an annealing temperature at 60°C. All qPCR assays were done with one RNA isolation and two duplicate qPCR runs. Cells were processed over the period of more than 1 year. RNA samples from young and old donors in one experiment were isolated simultaneously, and the qPCR was assayed together under the same conditions. To maximize the consistency of qPCR measurements over the time period of our studies, the standard curves for all genes were standardized, and the same lots of primers and probes were used for consistent assessment. The qPCR results were considered valid when the efficiency of the standard curve was between 90% and 110% and the R^2 value was ≥0.9. Values for each gene were calculated from the accompanying standard curve in each qPCR plate. Each duplicate measurement was divided by the corresponding measurement for actin and then averaged. Values from each age group are presented as averages ± the standard errors of the means.

**Cytokine-specific ELISA.** Culture supernatants from macrophages were harvested, and production of the cytokine interleukin-6 (IL-6), IL-8, and TNF-α was quantified with enzyme-linked immunosorbent assays (ELISA) using cytokine-specific ELISA.
specific capture human cytokine standards, and recombinant human cytokine standards, according to the manufacturer's instructions (BD Pharmingen, CA). The cytokine level in each sample was determined using one-way analysis of variance (ANOVA) with Bonferroni correction. The statistical significance was determined using Analyse-it software (Analyse-it Ltd., United Kingdom). The statistical significance was determined using one-way analysis of variance (ANOVA) with Bonferroni correction. Values of \( P < 0.05 \) were considered statistically significant.

RESULTS

WNV downregulates the expression of TLR3 in macrophages from young but not elderly donors. Older individuals are more susceptible to WNV encephalitis (10), and mice lacking TLR3 are more resistant to the development of WNV encephalitis (36). Therefore, we examined the responsiveness of TLR3 to WNV infection in primary macrophages derived from young and older individuals, using three MOI levels: 0, 0.1, 1, and 10, for 1 h. The baseline level of TLR3 was higher in macrophages from young donors. Infection with WNV led to a dramatic decrease of the mRNA level of TLR3 in macrophages of young adults in a dose-dependent manner (Fig. 1A). However, the expression of TLR3 in macrophages of the elderly remained unchanged in response to WNV infection. This change appears to be specific to TLR3 because there were no changes in mRNA levels of CD14 and CD36, receptors for lipopolysaccharide and oxidized low-density lipoproteins, respectively (data not shown). The decrease in the mRNA level in young adults was followed by a corresponding reduction of the TLR3 protein level after 3 h of infection with WNV, whereas the TLR3 protein level in older individuals increased during that time (Fig. 1B). The rapid downregulation of TLR3 levels suggests that suppression is likely to be due to the initial recognition or binding of WNV, rather than to viral replication, which does not occur until ~12 h postinfection (30).

Binding of WNV envelope protein to DC-SIGN attenuates the production of TLR3. Macrophages express pathogen recognition receptors, including TLRs and C-type lectin receptors (CLRs) (12). Glycosylated WNV-E is recognized by the CLR DC-SIGN (8), which recognizes a wide range of glycosylated proteins found on viruses, bacteria, and fungi (35). In humans, DC-SIGN is expressed in vivo on CD11b+ cells and in vitro on monocyte-derived dendritic cells and macrophages (21, 22, 32). To determine whether CLRs are involved in the suppression of TLR3 upon WNV infection, macrophages derived from young individuals were incubated with EGTA and S. cerevisiae mannan, agents that are known to block the Ca\(^{2+}\)-dependent carbohydrate-binding capacity of CLRs (37). Blocking CLRs also blocked the suppression of TLR3 triggered by WNV (data not shown), suggesting that a CLR may be involved in the attenuation of TLR3.

As an initial identification of the specific CLR, macrophages

<table>
<thead>
<tr>
<th>Assay</th>
<th>Gene</th>
<th>Primers and probe or gene identity</th>
</tr>
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<tr>
<td>Quantitative PCR</td>
<td>β-Actin</td>
<td>Forward: 5'-ATCCGTCGCTGCCTGTCGAC-3' ( \star ) ( \star ) Revers: 5'-GGCCGCAGCTGCTAC-3' ( \star ) ( \star ) Probe: 5'-6FAM TCCACGAGATGGTAGCAAGCA TAMRA-3'</td>
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<tr>
<td>DC-SIGN</td>
<td>Forward: 5'-CAGGAGCGCGTCGCTGACGAC-3' ( \star ) ( \star ) Revers: 5'-AGGAGCAGGACGCGAAGTAACGA TAMRA-3'</td>
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<tr>
<td>IFN-β1</td>
<td>Forward: 5'-CAGCAGTTTGTAAAGTGGPACTGCTA-3' ( \star ) ( \star ) Revers: 5'-TCATCAGTCCTGAGGCCGACTG-3'</td>
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<tr>
<td>IL-8</td>
<td>Forward: 5'-GCCAACAGAAAATATGTTAAGCTT-3' ( \star ) ( \star ) Revers: 5'-AAATTCTCAGGCTCCTTCAAAACT-3'</td>
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<tr>
<td>TLR3</td>
<td>Forward: 5'-CCTGAGTGTGTTAAAGATGGAACGA-3' ( \star ) ( \star ) Revers: 5'-TGAGGTGAGTGGTGGAGCAAGG-3'</td>
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<tr>
<td>WNV-E</td>
<td>Forward: 5'-TCTTCAAGAGCGGACAGCTG-3' ( \star ) ( \star ) Revers: 5'-CCGCCTCCATATTCATCACTC-3'</td>
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<tr>
<td>CITA</td>
<td>Hs0172094_m1</td>
<td>Forward: 5'-CGGTGCGTCTCGGAAATAGAAAAA-3' ( \star ) ( \star ) Revers: 5'-ATGAGCTGAGCTGAGCTGAGC-3'</td>
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<tr>
<td>IL-6</td>
<td>Hs0174131_m1</td>
<td>Forward: 5'-CCTGCGGTGAGGTGTAGGCAGG-3' ( \star ) ( \star ) Revers: 5'-GCTCCGGGTGGCCTCGGTTCG-3'</td>
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<td>OAS1</td>
<td>Hs0242943_m1</td>
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<td>RnasL</td>
<td>Hs0221692_m1</td>
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<tr>
<td>TNF-α</td>
<td>Hs0174128_m1</td>
<td>Forward: 5'-CAGCAATTTTCAGTGTCAGAAGCT-3' ( \star ) ( \star ) Revers: 5'-CCGCCTCCATATTCATCACTC-3'</td>
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<td>Chromatin immunoprecipitation</td>
<td>ICAM1</td>
<td>Forward: 5'-CCTGCTGAGTCCCGAATAGA-3' ( \star ) ( \star ) Revers: 5'-ATGAGCTGAGCTGAGCTGAGC-3'</td>
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<td>IRF1</td>
<td>Forward: 5'-AGTACGACGAGAGCTGACGA-3' ( \star ) ( \star ) Revers: 5'-GCTCCGGGTGGCCTCGGTCG-3'</td>
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<td>TLR3</td>
<td>Forward: 5'-TGAAACGCTTCTTGAGTT-3' ( \star ) ( \star ) Revers: 5'-GCGAACATAGCGGACTC-3'</td>
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<td>β-Actin</td>
<td>Forward: 5'-CAGAACTTTCCGGAACG-3' ( \star ) ( \star ) Revers: 5'-AAATTCTCAGCCCTCTTCAAAAACT-3'</td>
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<td>RNA interference</td>
<td>DC-SIGN</td>
<td>5'-CGACGAGGAAAUGUAACUUU-3' ( \star ) ( \star ) GGGCUUUGCUUCGGAGUCAUA-3'</td>
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<td>L-SIGN</td>
<td>5'-GGCUCGCAUGACCAAUCUUC-3' ( \star ) ( \star ) GGAACACUCCAGCAAGAC-3'</td>
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TABLE 1. Primers and probes used in this study

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were incubated with F(ab)₂ fragments of specific antibodies blocking DC-SIGN, L-SIGN/CD299, and MMR/CD206, three well-characterized CLRs that are known to serve as attachment factors for viruses (22). The mouse serum F(ab)₂ fragment was used as a negative control. After cells had been infected for 1 h, WNV infection reduced the level of TLR3 expression when cells were treated with mouse serum F(ab)₂ fragment and F(ab)₂ fragment derived from L-SIGN- and MMR-specific antibodies. However, blocking DC-SIGN reduced the inhibition of TLR3 in the presence of WNV (data not shown), suggesting a role for DC-SIGN in WNV-induced reduction of TLR3 levels.

DC-SIGN has been reported to recognize the glycosylation of WNV envelope protein (8). We infected macrophages from young individuals with the glycosylation-proficient WNV strain CT-2741 and glycosylation-deficient WNV strain NY99-E154. Only infection with the glycosylation-proficient WNV strain CT-2741 led to a decrease in the expression of TLR3 in macrophages; infection with the glycosylation-deficient WNV strain NY99-E154 failed to downregulate the expression of TLR3 in macrophages derived from young individuals (Fig. 2A). Glycosylation of WNV E protein (30 ng/10⁶ cells) for 1 h and assessed by qPCR (young, n = 6; elderly, n = 8, data are means ± standard errors of the means; **, ANOVA, P < 0.05 for both untreated versus E154). (B) Primary macrophages from young and older adults were treated with WNV-E (30 to 120 ng/10⁶ cells) for 1 h and assessed by qPCR (young, n = 6; elderly, n = 8; data are means ± standard errors of the means; **, ANOVA, P < 0.05 for macrophages of the young vs. the elderly. (C) Primary macrophages from young and older adults were treated with WNV-E protein (30 ng/10⁶ cells) for 0, 1, and 3 h. Protein data are representative of three subjects in each cohort.

The downregulation of TLR3 by WNV was also analyzed with human primary macrophages lacking DC-SIGN. Using RNA interference technology, we determined that the expression of DC-SIGN on macrophages (TLR3/actin levels for untreated, 822 ± 17.4 ng/ng; for WNV₉Vent, 252 ± 28.8 ng/ng; for WNV₉HeLa, 854 ± 39.9 ng/ng; P < 0.01 for both untreated versus WNV₉Vent cells and for WNV₉Vent versus WNV₉HeLa cells; differences, NS for untreated versus WNV₉HeLa cells). This difference is most likely due to altered glycosylation patterns of WNV proteins cultivated in human cells, which lead to reduced interactions with DC-SIGN (9). In addition, downregulation of TLR3 was not observed for murine macrophages infected with WNV (data not shown), as could be expected from the species-specific regulatory mechanisms for TLR3 expression and signaling (13).

The downregulation of TLR3 by WNV was also analyzed with human primary macrophages lacking DC-SIGN. Using RNA interference technology, we determined that the expression of DC-SIGN was efficiently knocked down in human primary macrophages, with reductions of 86% and 75% in mRNA and protein levels, respectively, as determined by qPCR and densitometric quantitation of the immunoblot (Fig. 3A and B). The DC-SIGN level was not affected by electroporation in mock-transfected cells or nontargeting siRNA (using se-
Ligation of DC-SIGN by WNV-E attenuates STAT1 activity through Lyn in a Jak-independent pathway. Macrophages express a moderate level of DC-SIGNs, which was not significantly different between the age cohorts. As assessed by FACS analysis of primary human monocyte-derived macrophages labeled with specific monoclonal antibodies, age had no effect on the expression of DC-SIGN (37.55% ± 5.24% and 41.94% ± 7.00% in macrophages from young and elderly subjects, respectively; n = 6/age group; differences, NS) or CD14 (88.59% ± 3.32% and 83.14% ± 3.53% in macrophages from young and elderly subjects, respectively; n = 6/age group; differences, NS). This finding suggests that the dysregulation of TLR3 may be due to a difference in the signaling downstream of DC-SIGN. The cytoplasmic domain of DC-SIGN contains the YXXL motif, a consensus sequence for the phosphorylation by Src family kinases (32). Thus, we first determined whether an Src kinase coprecipitates with DC-SIGN. Human primary macrophages derived from young and old donors were treated with the WNV-E protein for 0, 5, 10, 20, and 30 min and immunoprecipitated with anti-DC-SIGN-specific antibody. Proteins immunoprecipitated with DC-SIGN were immunoblotted to identify interacting molecules. As shown in Fig. 4A, DC-SIGN coprecipitated with Lyn. WNV-E protein was detected in macrophages from both age groups, although binding appeared to be more efficient with proteins from young donors (data not shown). The interaction between DC-SIGN and Lyn appeared to be specific because there was no detectable Syk or Lck that coprecipitated with DC-SIGN in primary macrophages (data not shown). In macrophages from young donors, ligation of the WNV-E protein to DC-SIGN led to an increase in the phosphorylation of the carboxyl-terminal Tyr507 that inactivates Lyn. In contrast, in macrophages from elderly donors, the phosphorylation of the carboxyl-terminal Tyr507 was reduced at 30 min (Fig. 4A). The phosphorylation of Tyr416 in the activation loop of Lyn was not affected in either age group (Fig. 4A). These data suggest that the engagement of DC-SIGN by WNV-E protein downregulates the kinase activity of Lyn in macrophages from young donors but not in those from elderly donors.

Lyn kinase has been shown to participate in the activation of STAT in the engagement of the B-cell antigen receptor (25, 26). We determined whether the reduced phosphorylation of Lyn after the ligation of DC-SIGN by WNV-E reduces STAT1 activity. Macrophages were examined for the phosphorylation of the carboxyl-terminal Tyr507 that inactivates Lyn. In contrast, in macrophages from elderly donors, the phosphorylation of STAT1 at positions Tyr701 and Ser727 was diminished at 20 min after treatment with the WNV-E protein (Fig. 4B). In contrast, in macrophages from elderly donors, the phosphorylation of STAT1 at positions Tyr701 and Ser727 was diminished at 20 min after treatment with the WNV-E protein (Fig. 4B). In contrast, in macrophages from elderly donors, the phosphorylation of STAT1 at positions Tyr701 and Ser727 was diminished at 20 min after treatment with the WNV-E protein (Fig. 4B).
utilizes DC-SIGN to facilitate viral entry, reduced the phosphorylation of STAT1 via an increase in the phosphorylation at the carboxyl terminus of Lyn in macrophages from the young donors; the glycosylation-deficient WNV strain NY99-E154 did not modify the phosphorylation profiles of Lyn, STAT1, Jak1, or Tyk2 in macrophages derived from the young or old individuals (Fig. 4C).

Ligation of DC-SIGN by WNV-E reduces induction of antiviral response in an age-dependent manner. We next determined whether the downregulation of TLR3 induced by WNV-E protein at a concentration of 30 ng/10^6 cells. Total proteins were harvested at 0, 5, 10, 20, and 30 min. Cell lysates were immunoprecipitated with anti-DC-SIGN monoclonal antibody, and the products were immunoblotted with antibodies against phospho-Lyn (Tyr507), phospho-Src (Tyr416), and total Lyn. DC-SIGN was used as a control to ensure equal loading. Data are representative of three subjects from each cohort. (B) Lysates of infected macrophages were immunoblotted with monoclonal antibodies against phospho-STAT1 (Tyr701), phospho-STAT1 (Ser727), total STAT1, phosphor-Jak1 (Tyr1022/1023), total Jak1, phospho-Tyk2 (Tyr1054/1055) and Tyk2. Data are representative of three subjects from each cohort. (C) Primary macrophages derived from young and older individuals were treated with the WNV strain NY99-E154 at an MOI of 1. Total proteins were harvested at 0, 5, 10, 20, and 30 min. Cell lysates were immunoblotted with monoclonal antibodies as for panel B. Data are representative of three subjects in each cohort.

TLR3 expression is mediated through STAT1. Promoters of IRF1 and ICAM1, two STAT1-regulated gene products, were also reduced after treatment with WNV-E protein from young but not from elderly donors (Fig. 5A), suggesting that the WNV-E structural protein reduces the induction of antiviral genes through the SFK-STAT pathway in the young but that this signaling pathway is impaired in macrophages from elderly donors.

STAT1 is a pivotal transcriptional regulator for the induction of antiviral responses (17), including regulation of RNase L (RNaseL), 2′,5′-oligoadenylate synthase 1 (OAS1), and major histocompatibility class II transactivator (CITA). Although young donor samples expressed a higher level of STAT1-regulated genes, we found that infection with WNV led to a downregulation of STAT1-regulated genes in a dose-dependent manner in macrophages from young donors (Fig. 5B). However, the expression level of STAT1-regulated genes remained unchanged or elevated upon infection with WNV in macrophages from the elderly (Fig. 5B). In contrast, macrophages from both age groups showed an upregulation of 16- to 33-fold in the expression of RNaseL, OAS1, and CITA when stimulated with IFN-γ, which utilizes the Jak/STAT1 pathway (25).
Impaired signaling between DC-SIGN and STAT1 leads to an early and sustained elevation of cytokines in the elderly. We expect that the differences between the signaling in macrophages from young individuals and that from elderly individuals, as shown upon the binding of WNV to macrophages, would lead to different outcomes at a later phase of infection. When proteins were examined later, after 3 h of infection with WNV, there was a decrease in the phosphorylation of STAT1 at positions Tyr701 and Ser727 in macrophages from young adults, whereas the phosphorylation of STAT1 at positions Tyr701 and Ser727 was markedly elevated in macrophages from elderly donors (Fig. 6A). (Densitometric quantitation for Tyr701/STAT1 from young donors were as follows: at 0 h, 1.0; 1 h, 0.96 ± 0.004; 3 h, 0.85 ± 0.05. Those for elderly donors were as follows: at 0 h, 1.0; 1 h, 1.05 ± 0.035; 3 h, 1.24 ± 0.07. Densitometric quantitation for Ser727/STAT1 from young donors were as follows: at 0 h, 1.0; 1 h, 0.93 ± 0.006; 3 h, 0.88 ± 0.01. Those for elderly donors were as follows: at 0 h, 1.0; 1 h, 0.98 ± 0.012; 3 h, 1.17 ± 0.046). In addition, phosphorylation of pTyk2 and pJak1, which were unchanged for both the young and the elderly donors at 30 min of infection, was increased at 3 h in macrophages from elderly but not young donors. This suggests a role for the Jak/STAT pathway in the viral response. This effect on STAT1 phosphorylation was also observed for the treatment with WNV-E protein alone (data not shown).

Once STAT1 is phosphorylated in the cytoplasm, it translocates to the nucleus and acts as a transcription factor via binding to target genes (31). Using confocal microscopy, we found that STAT1 remained cytosolic in macrophages from young donors (4% ± 2% translocated), whereas STAT1 translocated to the nucleus in macrophages derived from older donors (77% ± 4% translocated) (Fig. 6B), suggesting that STAT1 is activated in macrophages from older individuals upon infection with WNV.

We determined the expression levels of cytokines at 3 h after infection with WNV in human macrophages derived from young and old individuals. Even with comparable infection rates, macrophages derived from older donors expressed significantly higher levels of IFN-β and IL-6, both of which are regulated by STAT1, at the mRNA and protein levels (Fig. 6C). However, there were no significant differences between the IL-8 and TNF-α levels, regulated by NF-κB, of the young and older individuals (Fig. 6C). The differences between cytokine production levels in macrophages derived from younger and older individuals were also observed when cells were treated with WNV-E protein. Higher levels of phosphorylated to as output, and samples collected prior to immunoprecipitation are noted as input. Differential binding in the absence and presence of WNV-E was determined by PCR using primers specific to the promoters of TLR3, IRF1, and ICAM1 (Table 1). Data are representative of three subjects from each cohort. (B) The expression of the STAT1-regulated RNaseL, CIITA, and OAS1 genes was measured by qPCR using RNA obtained from primary macrophages of healthy young and older individuals infected with WNV (MOI, 0, 0.1, 1, and 10) for 1 h. Stimulation in the presence of 1 ng/ml of IFN-γ was used as a positive control (young, n = 7; elderly, n = 7; data are means ± standard errors of the means; **, ANOVA, P of <0.05 for the young vs. the elderly at the MOI indicated).
STAT1 in older individuals resulted in an early increase of the production of STAT1-regulated IFN-β and IL-6. The released cytokines in turn bind to their cognate receptors and phosphorylate Jak1 and Tyk2 (Fig. 6A), resulting in an activation loop of STAT and further expression of cytokines in the elderly (31).

To assess the possible contribution of the different signaling pathways between younger and older individuals in response to WNV infection, macrophages derived from each age group were infected for 3 days and assayed for the production of cytokines. There was a significant increase in the production of IL-6 and TNF-α at both the mRNA and protein levels in macrophages from older individuals from day 1 to day 3 of infection (Fig. 7A and B), and this was accompanied by a higher level of WNV in those macrophages (Fig. 7C). We have shown previously that infection with WNV does not inhibit the production of IL-8 (14), and thus, it is not surprising that there were no apparent differences in IL-8 production between younger and older individuals over the 3-day course of infection with WNV (Fig. 7A and B). The expression levels of IFN-β and TLR3 were also elevated significantly in the elderly compared to those in the young (Fig. 7A and 7C). These long-term effects suggest that macrophages from older individuals may be either more permissive to WNV replication or hyperresponsive to infection with WNV.

DISCUSSION

Sensing molecules such as TLRs are a critical component of the innate immune system’s surveillance for invading pathogens. The endosomal TLR3 plays an important role in the pathogenesis of WNV infection (36). Since the development of severe WNV encephalitis is increased in people who are >55 years old, we were interested in determining whether TLR3 plays a role in the pathogenesis of WNV in the aging population. In resting macrophages, the expression of TLR3 in macrophages of the older cohort was reduced in comparison to that of the younger counterparts. The decrease in expression of TLR3 is in agreement with our recent findings of decreased levels and functional defects of TLR1 in humans in the aging
The reduced expression of TLR3 in resting macrophages from the more-susceptible aged cohort did not initially correlate with the mouse model, where animals lacking TLR3 are more resistant to the development of WNV (36). However, during WNV infection, we have shown that the expression of TLR3 in the young cohort was reduced, whereas the expression of TLR3 was increased in macrophages from the older counterpart. This reduction of the mRNA level of TLR3 in macrophages from young donors could be detected as early as 1 h postinfection with WNV and may reflect pivotal signaling pathways mediated by WNV during viral entry into phagocytes. One of the characterized surface molecules that has been demonstrated to mediate WNV entry to phagocytes is DC-SIGN (8). Using RNA interference and a glycosylation-deficient mutant WNV, we have shown here that the interaction between DC-SIGN and the glycosylated WNV-E protein leads to the downregulation of TLR3. Downregulation of TLR3 was not noted in murine macrophages incubated with WNV, as expected, due to species-specific regulation of TLR3 (13).

DC-SIGN is a CLR described in the binding and transmission of human immunodeficiency virus type 1 (5, 11) and mediates cell responses via downstream kinase signaling (4). Consistent with an earlier report (4), we found that Lyn, an Src family kinase, was recruited after DC-SIGN ligated with the glycosylated WNV-E protein and that ligation of DC-SIGN increased the phosphorylation of Lyn at the carboxyl terminus, while the phosphorylation at the activation loop of Lyn was unaffected. While the phosphorylation at the carboxyl terminus of Src kinases is mediated by the C-terminal Src kinase, and this change inactivates the activity of Src kinases (26), the details of how the carboxyl terminus of Lyn is phosphorylated and whether C-terminal kinase is involved in this signaling pathway are currently under investigation.

STAT1 has been implicated as a transcriptional modifier downstream of Src kinases after the engagement of B-cell antigen receptors in a Jak family kinase (JFK)-independent pathway (25, 26). Since the ligation of DC-SIGN with WNV-E leads to the inactivation of Lyn, we investigated whether this interaction also led to an inactivation of STAT1. Indeed, we found that the WNV-E protein led to a reduction in the phosphorylation of STAT1, consistent with the carboxyl-terminal phosphorylation of Lyn. This alteration is independent of Jak1 and Tyk2 at early time points, strongly suggesting that DC-SIGN utilizes this unconventional Src kinase-STAT pathway to exert its downstream effects.

One of the downstream effects of the Src kinase-STAT pathway is to regulate the expression of TLR3. Our finding that the level of STAT1-bound TLR3 promoter was reduced in mac-

FIG. 7. Infection with WNV produces significantly higher levels of cytokines from macrophages of the elderly than from those of the young. Primary macrophages from young and older individuals were infected with WNV (MOI, 1) over the course of 3 days, and cytokines were measured by qPCR using total RNA (A) and by ELISA using culture supernatants (B) (young, n = 7; elderly, n = 7; data are means ± standard errors of the means; **, ANOVA, P of <0.05 for macrophages of the young vs. the elderly for the time points indicated). (C) The infection rate of primary macrophages represented by the WNV-E gene level and TLR3 level were measured using qPCR. Poly(I:C) was used as a positive control (young, n = 7; elderly, n = 7; data are means ± standard errors of the means; **, ANOVA, P of <0.05 for macrophages of the young vs. the elderly at the time points indicated).
rophages from younger donors after DC-SIGN ligated with WNV-E protein indicates that TLR3 is regulated by STAT1 and that the STAT1-mediated transcription is reduced in the presence of WNV-E protein. In addition, we also found that the Src kinase-STAT pathway is able to modulate the expression of other STAT1-regulated genes, as the mRNA levels of the Src kinase-STAT pathway is able to modulate the expression of WNV nonstructural proteins reported previously (19). Our group has recently shown that WNV infection attenuates the activation of human macrophages (14) and that the WNV-E protein also inhibits murine cellular responses through interaction with RIP1 kinase (2).

We were interested in understanding how this pathway may contribute to an age-dependent susceptibility to WNV encephalitis. Interestingly, we found that the association between DC-SIGN and Lyn is impaired when macrophages derived from older individuals were infected with WNV or treated with WNV-E protein. Instead of inactivating Lyn as demonstrated in the young cohort, the ligation of DC-SIGN with glycosylated WNV-E protein led to a decrease of the phosphorylation of Lyn at the carboxyl terminus. The impaired signaling contributes to the unaltered expression of TLR3 and other STAT1-regulated genes within the first hour of infection. However, significant differences between the young and older cohorts were observed at 3 h after infection with WNV. While the inactivation of the Src kinase-STAT pathway downstream of DC-SIGN continues to inhibit the activation of STAT1-regulated genes and the release of cytokines in young individuals, macrophages derived from older individuals induce the release of STAT1-regulated cytokines, including IL-6 and IFN-β. The absence of this effect when macrophages are incubated with WNV cultivated in human cell lines suggests that only the presence of WNV-E protein. These data suggest that the structural E protein of WNV may regulate the antiviral response in addition to the entry of WNV to the brain (36). Impaired innate immune responses noted in aging, such as reduced basal levels of certain immune response genes (27), may provide an early window with which to establish infection. In addition, macrophages from older adults had a higher viral burden, possibly because they are more permissive to the replication of WNV. The mechanism of permissiveness of the WNV is currently unknown and could be due to the differences in the intracellular trafficking of the WNV-containing vacuoles in macrophages derived from young versus older donors or to other unidentified deficits of cells from elderly donors. Taken together, these results demonstrate one mechanism that may contribute to increased susceptibility in the elderly to infection with WNV or other infectious agents and which may provide clues for enhancing resistance in susceptible populations.

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