

Spleen Tyrosine Kinase (Syk) Mediates IL-1 β Induction by Primary Human Monocytes during Antibody-enhanced Dengue Virus Infection*

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Background: Insights into the mechanisms of elevated cytokine production during severe dengue disease are needed.

Results: Dengue virus immune complexes induce inflammatory cytokine expression by activating spleen tyrosine kinase (Syk) during antibody-dependent enhancement of infection.

Conclusion: Syk-mediated activation of ERK1/2 elevates cytokine production during antibody-enhanced dengue infection.

Significance: Targeting Syk and ERK1/2 has therapeutic potential during antibody-enhanced dengue infection.

Approximately 500,000 people are hospitalized with severe dengue illness annually. Antibody-dependent enhancement (ADE) of dengue virus (DENV) infection is believed to contribute to the pathogenic cytokine storm described in severe dengue patients, but the precise signaling pathways contributing to elevated cytokine production are not elucidated. IL-1 β is a potent inflammatory cytokine that is frequently elevated during severe dengue, and the unique dual regulation of IL-1 β provides an informative model to study ADE-induced cytokines. This work utilizes patient-derived anti-DENV mAbs and primary human monocytes to study ADE-induced IL-1 β and other cytokines. ADE of DENV serotype 2 (DENV-2) elevates mature IL-1 β secretion by monocytes independent of DENV replication by 4 h postinoculation (hpi). Prior to this, DENV immune complexes activate spleen tyrosine kinase (Syk) within 1 hpi. Syk induces elevated *IL1B*, *TNF*, and *IL6* mRNA by 2 hpi. Syk mediates elevated IL-1 β secretion by activating ERK1/2, and both Syk and ERK1/2 inhibitors ablated ADE-induced IL-1 β secretion. Maturation of pro-IL-1 β during ADE requires caspase-1 and NLRP3, but caspase-1 is suboptimally increased by ADE and can be significantly enhanced by a typical inflammasome agonist, ATP. Importantly, this inflammatory Syk-ERK signaling axis requires DENV immune complexes, because DENV-2 in the

presence of serotype-matched anti-DENV-2 mAb, but not anti-DENV-1 mAb, activates Syk, ERK, and IL-1 β secretion. This study provides evidence that DENV-2 immune complexes activate Syk to mediate elevated expression of inflammatory cytokines. Syk and ERK may serve as new therapeutic targets for interfering with ADE-induced cytokine expression during severe dengue.

Dengue virus (DENV)² is transmitted by the bite of infected *Aedes* spp. mosquitoes and is the most burdensome arthropod-borne virus in the world. The World Health Organization estimates that the four distinct serotypes of DENV (DENV-1 to DENV-4) combine to cause 50–100 million infections per year worldwide (1). However, a recent cartography-based analysis of DENV prevalence estimates that the true number of global infections is ~390 million per year (2). Approximately 96 million of these cases present with disease symptoms annually. Most symptomatic cases present with mild to severe flu-like symptoms, but a small percentage of patients progress to life-threatening severe dengue (1).

Severe dengue almost always occurs in conjunction with a secondary infection with a heterologous serotype, leading to the hospitalization of ~500,000 people yearly (1, 3). The association of severe disease with a secondary infection is due to cross-reactive immunity from the original infection. Initially cross-protective against other serotypes in the months following a primary infection, the cross-reactive DENV immune response wanes in strength over time (4). Eventually, this cross-

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² The abbreviations used are: DENV, dengue virus; ADE, antibody-dependent enhancement; Fc γ Rs, Fc γ receptors; NLR, nucleotide-binding domain, leucine-rich repeat-containing protein; hpi, hours postinoculation; Syk, spleen tyrosine kinase; PBMC, peripheral blood mononuclear cell; FBS-HI, 90% heat-inactivated FBS; MOI, multiplicity of infection; ffu, focus-forming units; Z, benzyloxycarbonyl; FMK, fluoromethyl ketone; ANOVA, analysis of variance; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; FSC, forward scatter; SSC, side scatter.

reactive response becomes too weak to neutralize heterologous serotypes and paradoxically can enhance both viral replication and the production of dangerous inflammatory mediators (3, 5).

It is believed that cross-reactive antibodies contribute to severe dengue during infection with a heterologous DENV serotype by a process termed antibody-dependent enhancement (ADE) of infection (4, 6). The molecular mechanisms underlying ADE are incompletely understood. Cross-reactive antibodies may bind heterologous DENV serotypes, forming immune complexes when a DENV virion is bound by multiple antibodies (4). IgG immune complexes trigger endocytosis by cross-linking at least two activating Fc γ receptors (Fc γ R) on Fc γ R-bearing cells (7). Antibody-mediated neutralization of flaviviruses has strict antibody occupancy thresholds that must be reached to prevent endocytosed DENV from fusing with the endosome (8). When immune complexes are formed that do not meet the neutralization threshold, endocytosis of the complexes can promote increased cellular infection with DENV (9–11). Circulating blood monocytes are thought to be the primary target of ADE *in vivo* and are largely resistant to DENV infection in the absence of enhancing antibodies (12–15).

Immune dysfunction during severe dengue can progress to life-threatening hypovolemic shock due to hemorrhage and leakage of vascular fluid (16). It is believed that a “cytokine storm,” a massive and aberrant production of cytokines, contributes to this deadly pathology (17). Although much progress has been made in recent years elucidating the mechanisms of cytokine production during severe dengue, our understanding is incomplete, with many studies providing conflicting results (18).

IL-1 β is an extremely potent cytokine that is tightly regulated and can be induced by DENV in macrophages and monocytes (19–21). Induction of *IL1B* mRNA expression leads to the translation of inactive pro-IL-1 β , which must undergo post-translational cleavage to mature IL-1 β by activated caspase-1 (22, 23). Caspase-1 activation is regulated by a second, independent stimulus, a multiprotein complex termed the inflammasome (24, 25). Several nucleotide-binding domain, leucine-rich repeat-containing proteins (NLRs), and other innate immune receptors, such as AIM2, can serve as basic building blocks of the inflammasome, and each shows differing specificity to pathogen- and danger-associated molecular patterns (26–28). With both transcriptional and post-translational regulatory mechanisms, IL-1 β thus serves as an interesting cytokine to study differential cytokine modulation between unenhanced DENV infection and ADE.

Evidence continues to mount that IL-1 β may contribute to severe dengue pathology. Serum cytokine or gene expression profiles of many severe dengue patients exhibit elevated IL-1 β (29–31). IL-1 β increases vascular permeability, especially in conjunction with TNF α and IFN γ , two other cytokines elevated in many severe dengue profiles (29, 32–34). Exaggerated vascular permeability, caused by biologic mediators and not structural damage, is a key feature of severe dengue (16). A recent study described a mouse model of severe dengue disease, and antagonism of IL-1 β and caspase-1 or genetic knockout of caspase-1 and NLRP3 provided protection against disease (35).

Further, IL-1 β production by DENV-infected platelets was recently shown to correlate with vascular permeability in patients and promote permeability in endothelial cell layers *in vitro* (36). Thus, production of IL-1 β by DENV-infected monocytes could be expected to enhance vascular leakage as well.

Because monocytes circulate in the blood, they represent a first line of defense within the battleground of severe dengue. Human monocytes have long been known to secrete IL-1 β in response to DENV inoculation, with elevated IL-1 β secretion induced by ADE (21). However, no study has compared how ADE modulates the transcriptional *versus* post-translational regulation of IL-1 β in primary monocytes. DENV inoculation in the absence of ADE has been studied and is found to activate caspase-1 in primary human monocytes 4 days after infection (37). However, monocytes secrete IL-1 β within 4 h of inoculation with DENV (21), suggesting that DENV may induce IL-1 β secretion before it causes this delayed caspase-1 activation. Consistent with this, monocytes are known to partially process synthesized pro-IL-1 β in the absence of additional caspase-1 agonists (38–42), which may account for rapid IL-1 β secretion after DENV inoculation. A study of the pathways activated by DENV immune complexes in human monocytes is needed to elucidate the regulation of IL-1 β secretion during ADE.

This report studies IL-1 β synthesis and caspase-1 activation during ADE by using clinically and physiologically relevant human anti-DENV mAbs isolated from previously infected patients. This is particularly informative because DENV does not cause relevant disease in immunocompetent mice unless extremely high inoculums are used (43–51). Using these valuable antibodies in ADE studies with primary human monocytes allows identification of important signaling pathways that represent potential therapeutic targets for the treatment of severe dengue disease. Here, we find that ADE rapidly enhances expression of *IL1B* and pro-IL-1 β , leading to significant IL-1 β secretion by 4 h postinoculation (hpi). Enhanced IL-1 β secretion is independent of viral replication but instead requires activation of spleen tyrosine kinase (Syk) by DENV immune complexes. Pharmacologic inhibition of caspase-1 and NLRP3 and genetic inhibition of NLRP3 significantly reduced DENV-induced IL-1 β secretion. Importantly, ADE also induced Syk-dependent elevation of *TNF* and *IL6* expression, suggesting that activation of Syk by DENV immune complexes may have broad implications for the cytokine storm. Finally, activation of ERK1/2 by Syk is required for an IL-1 β response to DENV immune complexes, thus identifying two potential points of intervention for interfering with ADE-induced cytokine production.

Experimental Procedures

Ethics Statement—Mobilized peripheral blood mononuclear cells (PBMCs) were isolated from the blood of leukapheresed patients enrolled in a study approved by the University of North Carolina Institutional Review Board (Study 05-2860) after providing written informed consent. Samples were anonymized and provided as de-identified samples prior to use in the described studies. The University of North Carolina Office of Human Research Ethics determined that the use of the de-identified samples does not constitute human subjects research as

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defined under federal regulations (45 CFR 46.102 (d or f) and 21 CFR 56.102(c)(e)(l)) and does not require further Institutional Review Board approval.

Cell Isolation and Cell Culture—Mobilized PBMCs were isolated from leukapheresed blood using a 1.073 g/ml Ficoll-Hypaque Premium gradient (GE Healthcare) according to the manufacturer's instructions. After collection, mixed PBMCs were cryopreserved in 90% heat-inactivated FBS (FBS-HI) with 10% DMSO, or CD14⁺ monocytes were purified prior to cryopreservation via negative selection (to prevent preactivation by signaling through CD14) using the Dynabeads[®] Untouched[™] Human Monocytes kit (Life Technologies, Inc.). Cells were thawed, washed twice, and placed at 37 °C for 1–2 h in RPMI with 10% FBS-HI, 1% L-glutamine, 1% nonessential amino acids, 1% penicillin/streptomycin, 20 mM HEPES buffer, and 30 units/ml DNase. For thawed mixed PBMCs, CD14⁺ monocytes were immediately isolated before a rest period. Cells were then counted and resuspended as needed in the same RPMI mixture (without DNase) for experiments. In selected experiments, cells were resuspended in DNase-free culture medium 3 h after purification and allowed to rest until 24 h postpurification at 37 °C in vented polyethylene conical tubes to prevent adherence-induced cellular changes.

Virus Stock Growth—DENV-2 strain 16681 was kindly provided by Dr. Robert Tesh of the University of Texas Medical Branch-Galveston. To generate large stocks for experiments, nearly confluent T150 flasks of mosquito cell monolayers were inoculated with DENV supernatant at a multiplicity of infection (MOI) of 0.5 focus-forming units (ffu) per cell diluted into 2 total ml of minimum essential medium with 1% FBS-HI, 1% nonessential amino acids, 1% penicillin/streptomycin, and 20 mM HEPES buffer. Flasks were placed in a 30 °C incubator with 5% CO₂ and were rocked every 15 min. At 2 hpi, 15 ml of medium was added (minimum essential medium + 10% FBS-HI, 1% penicillin/streptomycin, and 20 mM HEPES buffer). At various days, typically days 3, 7, and 10, medium was collected from the flasks and spun down at 4,000 rpm for 10 min to clear the supernatant of cells, and the debris-cleared, infectious supernatant was aliquoted into tubes for storage. Fresh medium replaced the collected infectious supernatant. Except for in Fig. 1, all virus was derived from C6/36 *Aedes albopictus* mosquito cells (ATCC). Experiments in Fig. 1 used virus derived from C7/10 cells, a line related to the C6/36 cell line. C7/10 cells were provided by Dr. Frank Scholle (North Carolina State University).

To enumerate the concentration of virus, we modified a protocol described previously (52). Briefly, Vero cells were inoculated with 50 μ l of sequential 10-fold dilutions of infectious supernatants. 2 hpi, 150 μ l of a 1.6% carboxymethylcellulose overlay (diluted 1:1 in Gibco 2 \times minimum essential medium and supplemented with 1% FBS-HI, 10 mM HEPES, and 1 \times antibiotics) was added to each well to limit the spread of virus. 72 hpi, supernatants were aspirated, and cells were fixed with 200 μ l of a 1:1 mixture of acetone and methanol. Viral foci positive for DENV E protein were visualized with the VIP peroxidase substrate kit (Vector Laboratories) and expressed as ffu/ml.

DENV-specific Antibodies—Crude mAb 4G2 supernatant for use in IHC viral titrations was provided by Dr. Frank Scholle. The generation and purification of human mAbs 5G22 (α -DENV-1-DENV-4 prM used for enhancement) and 1F4 (α -DENV-1 virion for isotype in DENV-2 experiments or enhancement of DENV-1) were described in detail previously (53–55). Purified mouse mAb 4G2 for flow cytometry was produced by the Antibody Core Facility at the University of North Carolina (Chapel Hill, NC).

Reagents—All IL-1 β ELISAs were measured using the BD OptEIA[™] human IL-1 β ELISA set II (BD Biosciences catalog no. 557953). LPS (tlrl-eblps), ATP, glybenclamide, and the ERK1/2 inhibitor PD98059 were all purchased from Invivogen. The Caspase-Glo 1 assay was kindly provided by Dr. Martha O'Brien of Promega to assist with product development. Z-YVAD-FMK and Z-WEHD-FMK were purchased from R&D Systems. Syk inhibitors BAY 61-3606 and R406, p38 inhibitor SB 203580, and JNK inhibitor SP600125 were all purchased from Santa Cruz Biotechnology.

Transfection of siRNAs—The FlexiTube GeneSolution GS114548 for NLRP3 (Qiagen) was used to interfere with *NLRP3* expression. Each of the four included siRNAs were diluted in combination in 0.5 ml of serum-free RPMI. AllStars Negative Control siRNA (Qiagen) was used as the negative control. HiPerFect transfection reagent (Qiagen) was added to siRNAs, and tubes were vortexed and allowed to incubate at room temperature for 10 min for complex formation. Complexes were then mixed into cell suspensions, and cells were allowed to incubate with the siRNA complexes for 40 h prior to washing and inoculation with DENV-2.

For transfection, cells were resuspended to a final concentration (after siRNA complex addition) of 1×10^6 cells/ml in vented polyethylene conical tubes. Each of the four NLRP3-specific siRNAs was used at 25 nM final concentration (after addition to cells). The AllStars negative control siRNA was used at 100 nM to maintain equal siRNA concentration. 10 μ l of HiPerFect transfection reagent was used per 1 ml of final volume (after addition to cells).

Inoculation of Monocytes—1 h preinoculation, dilutions of enhancing antibodies, isotype-matched control antibodies, or control medium were plated into 96-well, round-bottom, non-tissue culture-treated plates. Crude supernatant from mosquito cells infected with DENV-2 strain 16681 or DENV-1 strain West Pac 74 was diluted to the appropriate MOI and mixed with antibodies or control medium. Plates were incubated for 1–1.5 h at 37 °C with 5% CO₂ to allow immune complex formation. For mock infection, spent culture medium from uninfected mosquito cell cultures was used instead of infectious supernatant. After immune complex formation, purified primary monocytes were added and mixed by pipetting. For Fig. 1, at 1 hpi, cells were washed two times with PBS and resuspended in fresh culture medium until 24 hpi. Once IL-1 β secretion was confirmed to be an early event, samples were collected at the earlier indicated time points without prior removal of inoculum and washing of cells unless otherwise noted. Supernatants were assayed immediately by ELISA, stored at 4 °C short term, or stored at –20 °C until ELISAs could be run. For flow cytometry, cells were washed once with PBS and fixed with Cytotfix/Cy-

topermTM fixation/permeabilization solution (BD Biosciences) for 20 min at 4 °C. For immunoblots, cells were washed once with PBS and lysed with radioimmune precipitation assay buffer (Boston BioProducts) with 1 \times cComplete protease inhibitor mixture (Roche Applied Science) and 1 \times PhosSTOP (Roche Applied Science) for at least 20 min at 4 °C with rotation. For RNA isolation, unwashed cell samples were mixed at a ratio of 1 volume of culture supernatant to 5 volumes of RNeasy Protect cell reagent (Qiagen) to immediately protect and stabilize mRNA. All inoculations were done with an MOI of 50 Vero cell ffu of DENV/monocyte, unless otherwise noted. Vero cell cultures are highly susceptible to DENV infection, whereas primary monocytes are not. Thus, this measurement is used to keep doses consistent between virus stocks and does not translate exactly to 50 times the inoculum required to infect one monocyte.

Virus Inactivation—Crude infectious supernatant was inactivated using shortwave UV exposure. 100 μ l/well of virus stock was added to each well of a 24-well plate, placed on ice, and exposed to shortwave UVB (254 nm) irradiation for 2 min at a distance of \sim 5 cm.

Flow-cytometric Analysis—After fixation, cells were washed twice with Cytotfix/Cytoperm Perm/Wash buffer. Nonspecific binding was blocked by incubating cells for 15 min with human Fc receptor binding inhibitor (eBioscience) diluted 1:20 in 1 \times Perm/Wash buffer. DENV E-protein was then stained with the pan-flavivirus E-protein mAb 4G2 conjugated to Alexa Fluor[®] 647 diluted into Perm/Wash buffer for 30 min at 4 °C. Cells were analyzed on a Cyan ADP flow cytometer. For experiments utilizing inhibitors, cells were stained with LIVE/DEAD[®] fixable violet dead cell stain (Life Technologies) per the manufacturer's instructions prior to fixation. Cells were initially gated on FSC-area versus SSC-area, with single cells positively selected by gating cells on FSC-lin versus pulse width followed by selecting cells on the diagonal of FSC-lin versus FSC-area. When used, cells were then gated for low fluorescence of LIVE/DEAD dye on the Pacific Blue channel. Cells positive for DENV antigen were detected on the allophycocyanin channel for intracellular E-protein expression, with positive gates set based on mock-infected cells.

Immunoblot Analysis—Lysates (or supernatants to be assayed by immunoblot) were recollected into fresh tubes, and appropriate volumes of 4 \times NuPAGE[®] LDS Sample Buffer (Life Technologies) and DTT reducing reagent were added to yield final concentrations of 1 \times sample buffer and 50 mM DTT. Samples were vortexed and heated at 97 °C for 5 min. Protein samples were loaded into Novex NuPAGE[®] SDS-PAGE 4–12% Bis-Tris precast gels (Life Technologies) using 1 \times NuPAGE[®] MES SDS running buffer (Life Technologies) and separated via SDS-PAGE. Gels were transferred onto Bio-Rad 0.2- μ m nitrocellulose membranes under wet transfer conditions for 45–60 min at 100 V using 1 \times transfer buffer (Boston BioProducts) containing 20% methanol. Blots were blocked for at least 1 h at room temperature with 5% nonfat dry milk dissolved in TBS-T or SuperBlock T20 blocking buffer (Thermo Scientific). The following primary antibodies were used for 2 h at room temperature or overnight at 4 °C: 1:2,000 dilution α -IL-1 β (Santa Cruz Biotechnology, sc-7884), 1:1,000 α -caspase-1 (Santa Cruz Bio-

technology, sc-56036) for pro-caspase-1, 1:500 α -phospho-Syk (Tyr-323) (Santa Cruz Biotechnology, sc-293118), 1:1,000 α -Syk (Cell Signaling Technology, catalog no. 13198), 1:1,000 α -p-ERK1/2 (Cell Signaling Technology, catalog no. 4370), 1:2,000 α -ERK1 (Santa Cruz Biotechnology, sc-93), 1:2,000 α -ERK2 (Santa Cruz Biotechnology, sc-154), 1:2,000 α -phospho-NF- κ B p65 (Ser-536) (Cell Signaling Technology, catalog no. 3033), 1:2,000 α -NF- κ B p65 (Cell Signaling Technology, catalog no. 8242), 1:1,000 α -phospho-JNK (Santa Cruz Biotechnology, sc-12882), or 1:1,000 α -JNK (Cell Signaling Technology, catalog no. 9252). Membranes were incubated for 1–2 h at room temperature with HRP-conjugated goat α -mouse (Jackson ImmunoResearch, 115-035-062), goat- α -rabbit (Jackson ImmunoResearch, 111-035-144), or donkey- α -goat (Santa Cruz Biotechnology, sc-2056) secondary antibodies. Concentrations varied from 1:10,000 to 1:50,000, depending on primary antibody. Actin was measured with 1:15,000 α -actin conjugated to HRP (Santa Cruz Biotechnology, sc-1615 HRP). Caspase-1 p10 was measured using SuperSignal Western blot Enhancer (Thermo Scientific, catalog no. 46640) according to the manufacturer's instructions, with α -caspase-1 (Santa Cruz Biotechnology, sc-522) diluted 1:250 in the primary antibody diluent contained in the kit. Membranes were developed after 5-min incubations with SuperSignal West Pico (Thermo Scientific, catalog no. 34080) or SuperSignal West Femto (Thermo Scientific, catalog no. 34096) chemiluminescent substrates.

Real-time PCR—RNA was purified using the RNeasy Plus minikit per the manufacturer's instructions (Qiagen, catalog no. 74134). Cell samples preserved in RNeasy Protect were stored at 4 °C until processing. RNA was eluted in a volume of 30 μ l of RNase/DNase-free water. To generate cDNA, 16 μ l of RNA eluate was mixed with 4 μ l of 5 \times iScriptTM reverse transcription supermix (Bio-Rad, 170-8841) and incubated in a thermal cycler as detailed in the manufacturer's protocol.

For real-time PCR analysis, 4.5 μ l of cDNA was added to 0.5 μ l of 20 \times ABI TaqMan[®] gene expression assay mix (Life Technologies, catalog no. 4331182) and 5 μ l of ABI 2 \times TaqMan Universal PCR Master Mix (Life Technologies, catalog no. 4304437) in a 384-well plate. Plates were run on the ABI ViiATM 7 real-time PCR machine using the following parameters: 50 °C for 2 min, 95 °C for 10 min, and 40 repeats of 95 °C for 15 s followed by 60 °C for 1 min. -Fold change values were calculated using the $\Delta\Delta C_t$ method, normalized to a control value set at 1. For all samples, 18S rRNA was used as the housekeeping gene control.

The following ABI TaqMan[®] Gene Expression Assays were used for real-time PCR analysis of gene expression: assay Hs01555410_m1 for *IL1B*, Hs00918082_m1 for *NLRP3*, Hs01113624_g1 for *TNF*, Hs00985639_m1 for *IL6*, and Hs03928985_g1 for *RN18S1*.

Caspase-Glo 1 Assay—The assay reagent was mixed per the manufacturer's instructions. At the indicated timepoints, unprocessed cell samples were mixed at a 1:1 ratio of culture supernatant to assay reagent in 96-well opaque plates. Each sample had a matching well in which 1 μ M Ac-YVAD-CHO was added to the assay reagent, blocking caspase-1-mediated cleavage of the substrate. Cell-free controls were also run to control for background signal. After 1 h of incubation at room tempera-

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ture, luminescence was read on a standard plate reader. The appropriate cell-free background luminescence value was first subtracted from each sample. Then YVAD control values (measuring caspase-1-independent peptide cleavage) were subtracted from the full assay values. The control-adjusted values represent relative caspase-1 activity.

Data Presentation and Statistical Analyses—Unless otherwise noted, all graphs shown represent the mean \pm S.E. of three or more values. For all assays except real-time PCR, each biological replicate was pipetted only once. For real-time PCR, each biological replicate was pipetted with 2–3 technical replicates. Statistical analyses were performed only when three or more biological replicates were present using Prism version 6.0 (GraphPad). Statistical significance was defined as $p < 0.05$. When unpaired, two-tailed t tests were used; *asterisks* represent actual p values. Ordinary one-way ANOVAs were used for comparisons within one independent variable. Ordinary two-way ANOVAs were used for comparisons containing two independent variables (e.g. presence or absence of virus and presence or absence of mAb 5G22). For figure panels using ANOVA comparisons, *asterisks* represent the multiplicity-adjusted p values within the multiple comparisons. Dunnett's test was used to compare all values with a single control value. Tukey's test was used when all means were compared, and Bonferroni's test was used when only means within one independent variable were compared (two-way ANOVAs only). For all cases, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Results

Antibody-dependent Enhancement of Dengue Virus Infection Increases Viral Replication in Primary Human Monocytes—Purified CD14⁺ monocytes were inoculated with mosquito cell-derived supernatant containing an MOI of 50 DENV-2 16681 Vero cell ffu/monocyte. 1 h prior to inoculation, DENV-2 was incubated with 2 μ g/ml anti-DENV prM human mAb 5G22 to form DENV-2 immune complexes or with control medium for non-ADE conditions. Mock-infected cells were inoculated with spent culture supernatant from uninfected mosquito cell cultures. At 1 hpi, cells were washed and resuspended in fresh medium until 24 hpi. Cells were stained with the anti-E-protein (pan-flavivirus) mouse mAb 4G2 conjugated to AlexaFluor-647 to measure intracellular expression of DENV E protein by flow cytometry. Detecting DENV antigen intracellularly by flow cytometry is a measure of viral replication (13). Inoculation of monocytes with DENV-2 alone caused little shift in DENV E-protein expression compared with mock conditions (Fig. 1, A and B). However, inoculation with DENV-2 that had been complexed with mAb 5G22 significantly increased the percentage of monocytes expressing DENV E protein. This agrees with previous literature that monocytes are largely resistant to DENV infection in the absence of anti-DENV-enhancing antibodies (14, 15, 56).

ADE Increases Secretion of IL-1 β by Primary Human Monocytes Independent of Viral Replication—Culture supernatants collected at 24 hpi from the above experiment were then assessed for the presence of human IL-1 β by ELISA. Consistent with literature reports (21), inoculation of monocytes with DENV-2 alone significantly enhanced secretion of IL-1 β , and

forming DENV-2 immune complexes with mAb 5G22 further increased IL-1 β secretion (Fig. 1C). This confirms that DENV alone induces IL-1 β secretion by primary human monocytes and that ADE further elevates IL-1 β secretion. Interestingly, the degree of IL-1 β enhancement by mAb 5G22 is not proportional to the enhancement of cells expressing DENV E protein. This suggests that DENV-2 alone induces IL-1 β production in primary monocytes by signaling through a pathway that does not promote increased cellular infection and DENV replication.

To determine the requirement for viral replication, we subjected DENV-2 to shortwave UV exposure for 2 min, rendering the virus replication incompetent. UV inactivation decreased the infectious titer of DENV-2, as measured by immunoassay on Vero cells, by 10⁵-fold (Fig. 1D). Consequently, UV inactivation prevented the expression of DENV E protein in primary monocytes at 24 hpi (Fig. 1E). However, UV inactivation only modestly reduced ADE-induced IL-1 β secretion at 24 hpi (Fig. 1F). These data indicate that ADE-induced IL-1 β secretion by primary monocytes can occur independently of viral replication.

ADE of DENV-2 Induces Early Elevated IL1B and Pro-IL-1 β Expression—Detection of elevated IL-1 β in culture supernatants could be due to elevated synthesis of pro-IL-1 β , elevated caspase-1 activation, or both. To determine which steps were modulated by ADE, inoculated and control monocytes were assessed for *IL1B* gene expression differences at 2 hpi by real-time PCR. Inoculation of monocytes with DENV-2 alone induced significant elevation of *IL1B* expression, whereas DENV-2 complexed with mAb 5G22 further increased *IL1B* expression (Fig. 2A). These results were measured against the *IL1B* expression induced by a known agonist LPS. The *IL1B* expression induced by ADE at 2 hpi was even greater than the levels induced by stimulation with 100 ng/ml LPS for 2.5 h.

Because elevated *IL1B* expression should increase pro-IL-1 β synthesis, we assessed pro-IL-1 β expression by monocytes after DENV-2 inoculation. Monocytes were lysed at 4 hpi and subjected to gel electrophoresis and immunoblot. As with *IL1B* expression, inoculation with DENV-2 alone induced elevated pro-IL-1 β expression, whereas DENV-2 complexed with mAb 5G22 further increased this expression (Fig. 2B). Supernatants collected at 4 hpi for the corresponding experiment were assessed for IL-1 β by ELISA (Fig. 2C). DENV-2 complexed with mAb 5G22 induced significant elevation of IL-1 β secretion by this early time point, whereas that induced by DENV-2 alone fell just short of significance. Because monocytes can secrete both pro-IL-1 β and mature IL-1 β (57), assaying supernatants by immunoblot is a way of specifically assessing the 17-kDa mature IL-1 β protein. Thus, we again inoculated monocytes and collected supernatants at 4 hpi but assayed specifically for the presence of mature IL-1 β by immunoblot (Fig. 2D). There was faint detection of mature IL-1 β in the supernatant of cells inoculated with DENV-2 alone. However, DENV-2 complexed with mAb 5G22 induced robust mature IL-1 β secretion. These data confirm that ADE-induced expression of *IL1B* and pro-IL-1 β lead to elevated mature IL-1 β secretion by 4 hpi.

As Fig. 2C shows, even mock-inoculated cells secrete a small amount of IL-1 β . Thus, we wanted to assess whether allowing

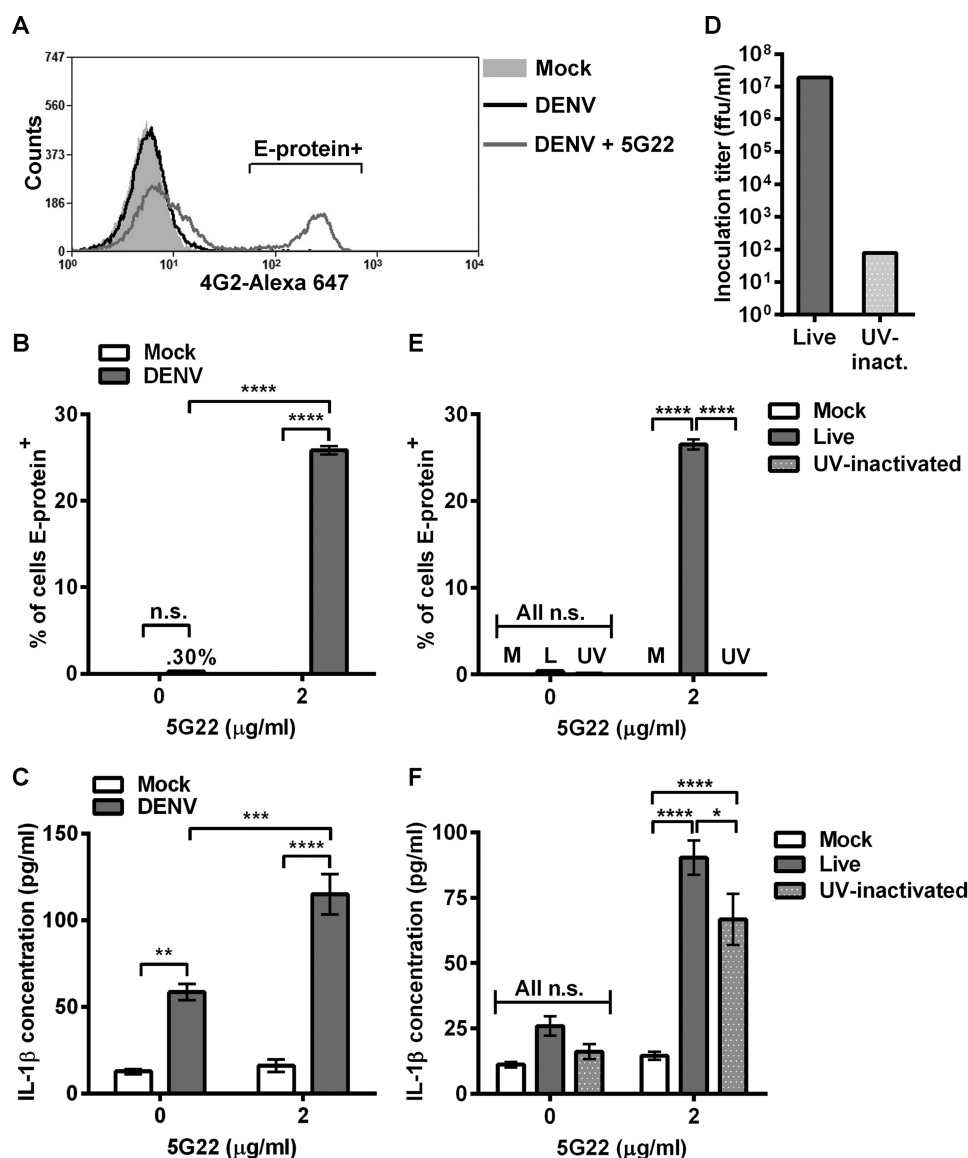


FIGURE 1. ADE of DENV-2 infection induces elevated IL-1 β secretion independent of viral replication. *A*, representative flow-cytometric histogram overlay showing DENV E-protein expression in primary monocytes. Cells were inoculated with mock supernatant (light gray region), DENV-2 alone (black line), or DENV-2 complexed with mAb 5G22 (dark gray line) for 1 h at 37 °C before being washed, resuspended in fresh medium, and incubated until 24 hpi, at which point cells were prepared for flow cytometry as described under "Experimental Procedures." *B*, cumulative percentages of all samples from the experiment described in *A*. *C*, IL-1 β ELISA of 24 hpi supernatants from experiment described in *A* and *B*. *D*, measurement of infectious titer of DENV-2 stock before and after exposure to shortwave UV irradiation for 2 min. Titer was determined based on immunoassay in Vero cells as described under "Experimental Procedures." *E*, flow-cytometric detection of DENV E-protein expression within monocytes 24 hpi after inoculation with live and UV-inactivated DENV-2. Cells again were exposed to inoculum for 1 h prior to washing and resuspension in fresh medium. *M*, mock-treated; *L*, live virus; *UV*, UV-inactivated virus. *F*, IL-1 β ELISA of 24 hpi supernatants from the experiment described in *D* and *E*. All *n.s.*, all comparisons were not statistically significant. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; *n.s.*, not significant. Ordinary two-way ANOVA (Tukey's) was used for all comparisons. See "Experimental Procedures" for detailed information on statistical analyses. *A–C* are representative of more than three independent experiments. *D–F* are representative of two independent experiments. Error bars, S.E.

the primary monocytes to rest overnight would reduce baseline IL-1 β secretion. Unexpectedly, DENV-induced pro-IL-1 β expression in the absence or presence of mAb 5G22 was dramatically reduced at 4 hpi in cells rested for 24 h postpurification compared with cells rested for 2 h postpurification (Fig. 2*E*). This was reflected by dramatically reduced IL-1 β secretion by monocytes rested for 24 h (Fig. 2*F*). Interestingly, although monocytes rested for 24 h still secrete significantly more IL-1 β when inoculated with DENV-2 compared with mock inoculation, the enhancement by ADE is lost. These data suggest that primary human monocytes aged in culture have a reduced abil-

ity to produce IL-1 β and an impaired response to immune complexes.

ADE of DENV-2 Does Not Cause Detectable Elevated Caspase-1 Activation in Primary Human Monocytes—We next determined whether ADE could enhance intracellular processing of pro-IL-1 β to mature IL-1 β by activating the inflammasome. The caspase-1 p10 subunit is an indicator of inflammasome activation and can be detected by immunoblot. However, we could not detect caspase-1 p10 by immunoblot under any mock or viral condition in 2 hpi lysates (Fig. 3*A*), despite the use of different sources of antibodies and protocols.

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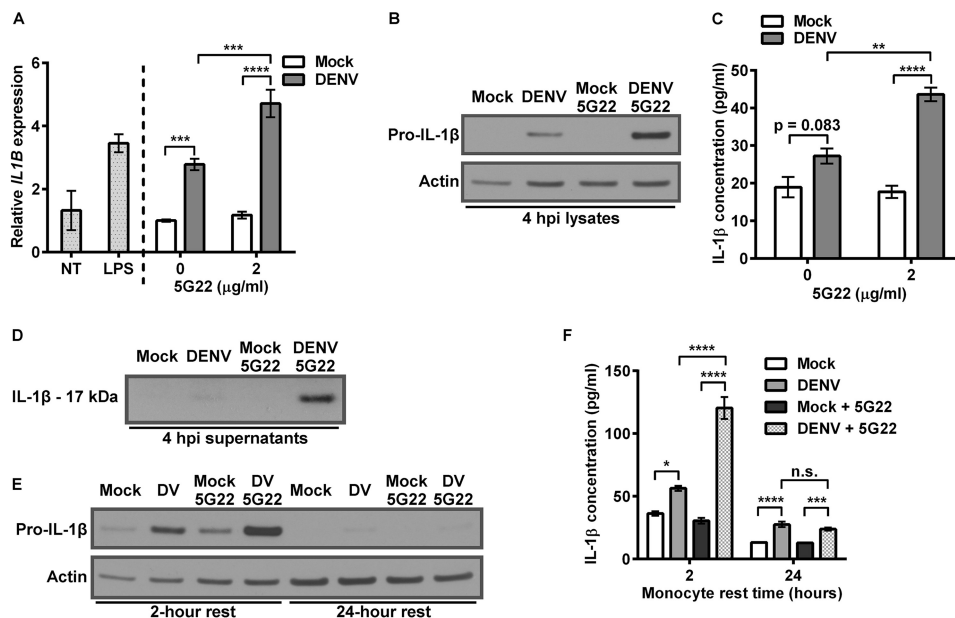


FIGURE 2. ADE rapidly induces *IL1B* and pro-IL-1 β expression to elevate mature IL-1 β secretion by 4 hpi. *A*, monocytes were lysed at 2 hpi after inoculation with DENV-2, and RNA was purified. No treatment (NT) and LPS (100 ng/ml) samples were collected at 2.5 h poststimulation. Samples were prepared as described under "Experimental Procedures." *IL1B* expression was measured using semiquantitative real-time PCR. All data are normalized to mock inoculation with no mAb 5G22. NT and LPS conditions contain two values each and represent mean \pm S.D. (error bars). *B*, at 4 hpi, DENV-2-inoculated monocytes were pelleted, washed, and lysed. Pro-IL-1 β protein levels were then measured via immunoblot. *C*, 4 hpi supernatants corresponding to *B* were assessed by ELISA for IL-1 β . *D*, in a separate experiment, supernatants from monocytes inoculated with DENV-2 were collected at 4 hpi and assessed for mature IL-1 β by immunoblot. *E*, monocytes were allowed to rest at 37 $^{\circ}$ C with 5% CO₂ for either 2 or 24 h following thawing and purification. After resting, monocytes were inoculated with DENV-2, collected at 4 hpi, and lysed for protein analysis. Intracellular pro-IL-1 β expression was assessed by immunoblot. *F*, supernatants collected at 4 hpi for the experiment described in *E* were assessed by ELISA for IL-1 β . Statistical tests were as follows. Ordinary two-way ANOVA (Tukey's) was used in *A*, *C*, and *E*. For *E*, separate two-way ANOVA tests were run within each rest time. *A* contains pooled values from two independent experiments (mock and DENV conditions). *B*–*D* are representative of three or more independent experiments. *E* and *F* are representative of two independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; n.s., not significant. Error bars, S.E., except as noted for NT and LPS conditions in panel *A*.

Caspase-1 p10 could only be detected in the positive control, consisting of lysates of cells that had been primed with 200 ng/ml LPS for 4 h and then pulsed with a 5 mM concentration of the NLRP3 agonist ATP for 45 min. Importantly, we could not detect caspase-1 p10 in response to viral inoculation even at very long exposures and in multiple experiments.

To measure caspase-1 activity in a different manner, we employed a caspase-1 activity assay in development by Promega (Fig. 3*B*). The assay uses a lytic reagent that contains a proteasome inhibitor and the caspase-1 substrate Z-WEHD conjugated to aminoluciferin. A luminescent signal develops when caspase-1 cleaves the substrate. At 2 and 3 hpi, caspase-1 activity in mock- and ADE-inoculated cells was on par with that in cells treated with LPS only. In contrast, LPS and ATP induced a large increase in caspase-1 activity. These data confirm that ADE-induced caspase-1 activity in primary human monocytes was below the level of detection by the assays used here during the early hours following inoculation. However, this does not exclude the involvement of caspase-1.

ADE-induced IL-1 β Secretion Requires Caspase-1 and NLRP3—Our results to this point are consistent with reports on IL-1 β processing in human monocytes after induction of pro-IL-1 β synthesis (38–42). This endogenous processing is inefficient, with less than 30% of synthesized pro-IL-1 β cleaved to mature IL-1 β (40), and processing is greatly enhanced by inflammasome agonists (40, 42). More recent studies support these findings, indicating that primary monocytes and mono-

cyte-like THP-1 cells have low levels of caspase-1 activity in the absence of inflammasome agonists (58, 59).

This suggests the possibility that low levels of caspase-1 activity may mediate the processing of IL-1 β in the current system. To test for this, we employed two irreversible caspase-1 inhibitors, Z-YVAD-FMK and Z-WEHD-FMK at low concentrations of 1 μ M each. Both caspase-1 inhibitors ablated ADE-induced elevation of mature IL-1 β secretion, as measured by immunoblot (Fig. 3*C*).

Because NLRP3 is known to respond to a wide array of stimuli, we tested whether pharmacologic inhibition of NLRP3 with the potassium efflux inhibitor glybenclamide would affect DENV-induced IL-1 β secretion. Glybenclamide ablated IL-1 β secretion induced by all conditions (Fig. 3*D*). To confirm the dependence on the inflammasome, we interfered with *NLRP3* expression by siRNA transfection. Real-time PCR confirmed that siRNA transfection targeting *NLRP3* reduced *NLRP3* expression 43.5% compared with transfection with a negative control siRNA (Fig. 3*E*). As expected, knockdown of *NLRP3* expression led to a significant reduction in IL-1 β secretion induced by the positive control consisting of LPS followed by stimulation with ATP (Fig. 3*F*). Additionally, the partial knockdown of *NLRP3* expression proportionately and significantly reduced the secretion of IL-1 β induced by DENV-2 alone and DENV-2 complexed with mAb 5G22 (Fig. 3*G*). Notably, ADE-induced elevation of IL-1 β secretion was lost in the knockdown experiments. However, as shown in Fig. 2, this is to be expected

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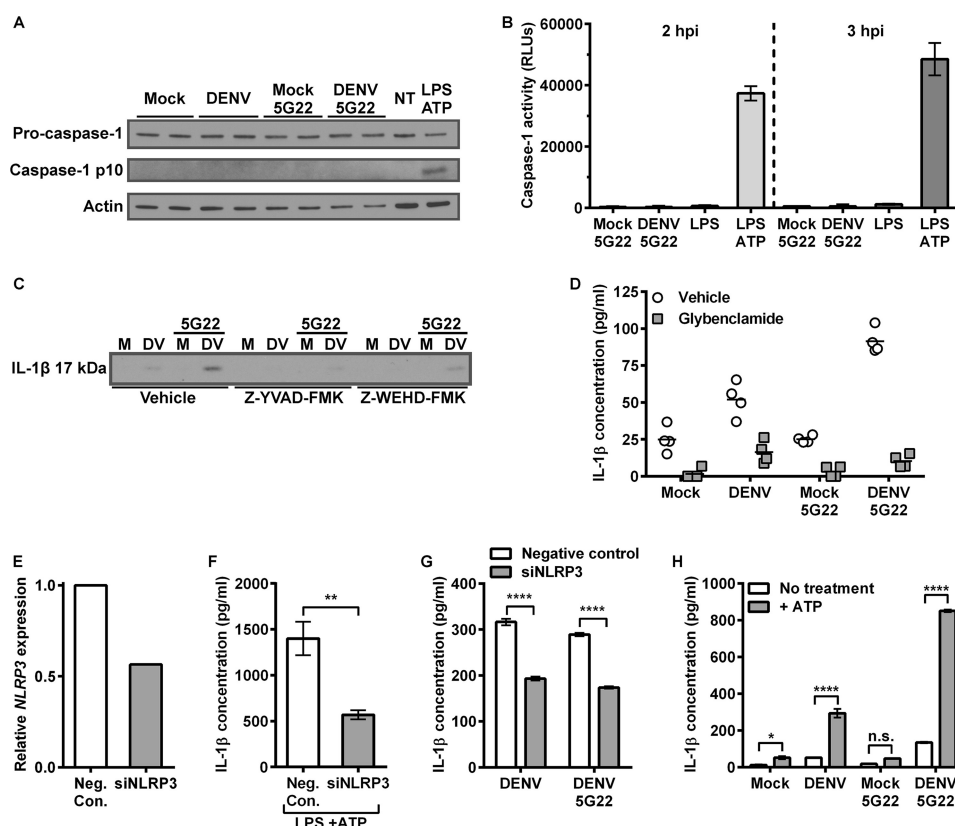


FIGURE 3. Suboptimal NLRP3 and caspase-1 activity mediates DENV-induced IL-1 β processing. *A*, monocytes were inoculated with the indicated mock or DENV-2 conditions, collected at 2 hpi, and lysed for protein analysis. No treatment (NT) and LPS (200 ng/ml priming for 4.25 h) plus ATP (5 mM pulse for final 0.75 h) control samples were collected and lysed at 5 h poststimulation in a separate experiment. Intracellular pro-caspase-1 and caspase-1 p10 were measured via immunoblot. *B*, monocytes were inoculated with DENV-2 and mixed at a 1:1 ratio with Caspase-Glo 1 reagent or Caspase-Glo 1 reagent with YVAD (to block cleavage of substrate by caspase-1) at the indicated time points. Graph represents relative caspase-1 activity after subtraction of cell-free background and YVAD controls. 100 ng/ml LPS was given to indicated cells. Cells receiving ATP received a 3 mM pulse 45 min prior to the addition of assay reagent. *C*, monocytes were treated with DMSO vehicle or a 1 μ M concentration of the caspase-1 inhibitor Z-YVAD-FMK or Z-WEHD-FMK at the time of inoculation with DENV-2. Supernatants were collected at 4 hpi and assessed for mature IL-1 β secretion by immunoblot. *D*, monocytes were pretreated with DMSO vehicle or 25 μ g/ml glybenclamide for 30 min prior to inoculation with DENV-2. Supernatants were collected at 4 hpi and assessed for IL-1 β by ELISA. All conditions had four biological replicates. Values placed at 0 fell below the limit of detection. *E–G*, monocytes were transfected with a negative control siRNA or siNLRP3 and allowed to incubate for 40 h. Knockdown of *NLRP3* expression at the time of inoculation with DENV-2 was assessed by real-time PCR (*E*). Control and knockdown cells were primed with 100 ng/ml Ultrapure LPS for 5 h followed by the addition of 2.5 mM ATP for the final hour prior to collection at 6 h. Supernatants were collected and assessed for IL-1 β by ELISA (*F*). Control and knockdown cells were inoculated with DENV-2, and supernatants were collected at 6 hpi and assessed for IL-1 β by ELISA (*G*). *H*, supernatants from monocytes inoculated with DENV-2 were collected at 4 hpi after adding 2 mM ATP (final concentration) to the indicated samples 45 min before collection. Supernatants were then assayed for IL-1 β by ELISA. Statistical tests used were as follows: unpaired, two-tailed *t* test (*F*) and ordinary two-way ANOVA (Bonferroni's) (*G* and *H*). *A*, *B*, and *D–H* are representative of three or more independent experiments, and *C* is representative of two independent experiments. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$; n.s., not significant. Error bars, S.E.

for monocytes that have aged, because monocytes were transfected with siRNA complexes for 40 h prior to inoculation with DENV-2. In conclusion, these data confirm that IL-1 β secretion induced by DENV-2 requires NLRP3-mediated activation of caspase-1, supporting a role of the inflammasome in this process.

Processing of DENV-induced Pro-IL-1 β Can Be Enhanced with ATP—To assess whether processing of DENV-induced pro-IL-1 β can be enhanced, we pulsed monocytes with 2 mM ATP to activate the NLRP3 inflammasome after DENV-2 inoculation with and without mAb 5G22 (Fig. 3H). Exogenous ATP addition significantly increased the secretion of IL-1 β induced by DENV-2 and DENV-2 immune complexes but had less impact on IL-1 β secretion induced by mock conditions. These data confirm that caspase-1 activation is suboptimal in monocytes inoculated with DENV-2 alone or DENV-2 immune complexes.

ADE-induced Syk Activation Is Dispensable for DENV Replication—To this point, our data indicate that ADE enhances transcription of *IL1B* mRNA and the synthesis of pro-IL-1 β protein but does not significantly enhance caspase-1 activation. To determine the pathway utilized by ADE to elevate *IL1B* expression, we considered early signaling pathways likely to be activated by DENV-2 immune complexes. Syk is a critical protein for initiating signaling cascades downstream of many classical immunoreceptors, including Fc receptors (60, 61). Cross-linking of both Fc γ RI and Fc γ RIIa strongly activates Syk, which induces expression of numerous cytokines in monocytes (62, 63). Thus, we first assessed whether ADE of DENV-2 infection induces elevated Syk activation in primary monocytes. At 4 hpi, inoculation with DENV-2 complexed with mAb 5G22 induced strong Syk phosphorylation at tyrosine 323 (Fig. 4A). Tyrosine 323 of Syk serves as a docking site for Cbl (64), and Cbl docking leads to ubiquitination and down-regulation of Syk activity in

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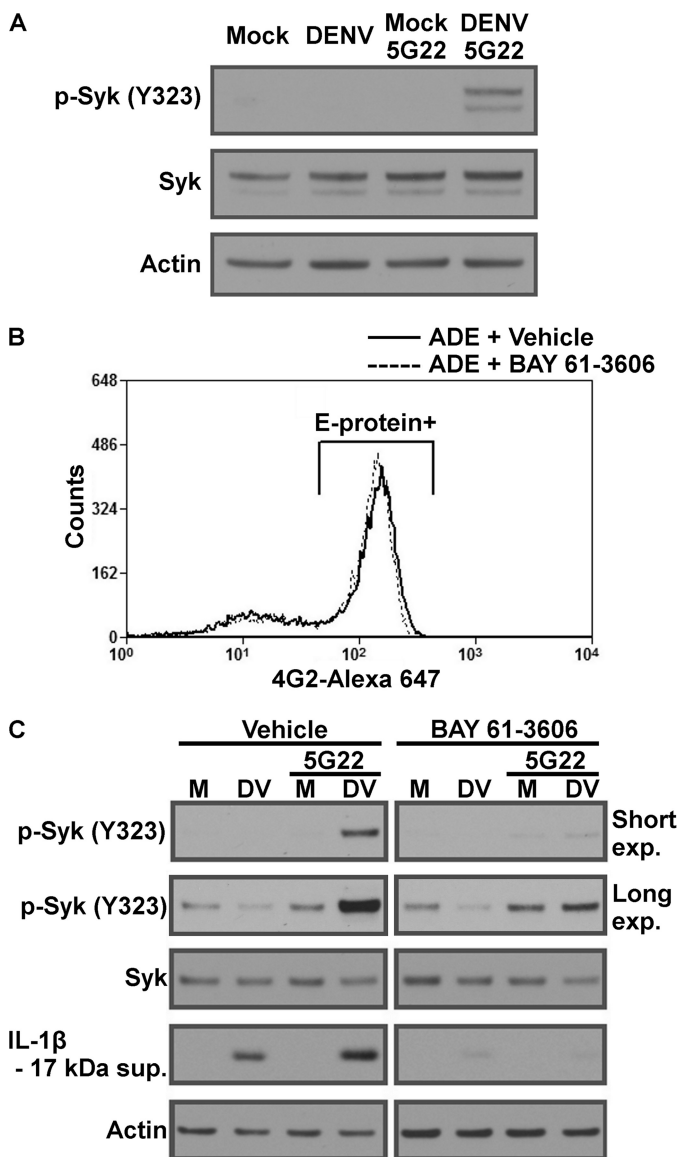


FIGURE 4. Syk mediates DENV-induced IL-1 β secretion. *A*, at 4 hpi, monocytes inoculated with DENV-2 were collected and lysed, with subsequent measurement of phospho-Syk and Syk via immunoblot. *B*, monocytes were inoculated with DENV-2 complexed with mAb 5G22 after pretreatment for 30 min with DMSO vehicle (solid line) or 0.5 μ M BAY 61-3606 (dashed line). Cells were allowed to incubate without removal of inoculum until 24 hpi, at which point cells were prepared for DENV E-protein measurement by flow cytometry. *C*, monocytes were pretreated with DMSO vehicle or 0.5 μ M BAY 61-3606 for 30 min before inoculation with DENV-2. Monocytes were allowed to incubate until 8 hpi without removal of inoculum. Supernatants were then collected, and cells were washed and lysed. Lysate phospho-Syk, lysate Syk, and supernatant mature IL-1 β levels were then measured via immunoblot. Boxes indicate that samples are non-adjacent lanes, but all samples are from the same blot and exposure. All panels are representative of three or more independent experiments.

response to strong Syk activity (64–67). Thus, Syk phosphorylation at tyrosine 323 serves as an indirect measure of Syk activation because it is used to down-regulate Syk activity after strong activation.

To assess the importance of Syk activation in ADE-induced infection and cytokine production, we interfered with Syk activation by employing the potent, cell-permeable Syk inhibitor BAY 61-3606. Importantly, BAY 61-3606 is very selective for Syk at the concentrations used (68). We first assessed the effects

of Syk inhibition on DENV-2 replication by measuring intracellular DENV E-protein expression in ADE-inoculated monocytes at 24 hpi (Fig. 4*B*). Syk inhibition with BAY 61-3606 did not reduce the detection of intracellular DENV E protein, indicating that Syk is dispensable for viral replication. This is in agreement with the report that Syk activation is not required for Fc receptor-mediated endocytosis (69).

Syk Mediates IL-1 β Induction by DENV Immune Complexes—We next sought to determine the impact of Syk inhibition on ADE-induced IL-1 β secretion. Treatment with BAY 61-3606 decreased ADE-induced Syk phosphorylation at tyrosine 323 (Fig. 4*C*). Additionally, overexposing the blot revealed that lower levels of Syk phosphorylation at tyrosine 323 were present in all other conditions. This suggests that Syk is modestly active under all conditions but is significantly elevated by DENV-2 immune complexes. This is not surprising due to the association of Syk with a multitude of signaling pathways (60).

We additionally assessed the impact of Syk inhibition on IL-1 β maturation by assessing the supernatants from the above experiment by immunoblot (Fig. 4*C*). Interestingly, Syk inhibition almost completely ablated mature IL-1 β secretion induced by both DENV-2 and DENV-2 complexed with mAb 5G22. The Syk pathway has been found to be important for DENV-induced inflammasome activation through C-type lectin receptors but has not been described for Fc γ Rs (20, 70–72). A recent study reported that Syk phosphorylates the inflammasome adapter ASC to promote caspase-1 activation (73). Thus, Syk activity may be critical for maintaining the suboptimal caspase-1 activation in the current study.

Antibody-enhanced IL-1 β Secretion Requires Isotype-specific Antibody—Because Syk is important for antibody-mediated signaling, we sought to verify that mAb 5G22 elevates DENV-induced IL-1 β due to immune complex formation with the virus. This is especially important because Fc γ RI can bind monomeric IgG that is not bound to antigen (74). Thus, we employed an isotype-matched control antibody for side-by-side comparison with mAb 5G22. Serotype-specific for DENV-1, mAb 1F4 does not bind DENV-2 (55). Thus, as expected, mAb 1F4 did not enhance DENV-2 infection in monocytes (Fig. 5*A*). When assessing IL-1 β secretion by ELISA, inoculating monocytes with DENV-2 in the presence of mAb 1F4 only modestly increased IL-1 β secretion over the mock condition, just as was seen earlier with DENV-2 alone (Fig. 5*B*).

In contrast, DENV-1 complexed with mAb 1F4 significantly enhanced IL-1 β secretion compared with inoculation with DENV-1 alone (Fig. 5*C*). Interestingly, enhancement of IL-1 β secretion was even stronger when DENV-1 was complexed with mAb 1F4 compared with mAb 5G22, which recognizes all four serotypes of DENV. In total, these data suggest that anti-DENV mAb enhances IL-1 β secretion when mAb and DENV form immune complexes.

Syk Mediates Elevated Inflammatory Cytokine Expression Induced by ADE—We next sought to determine whether Syk mediates the ADE-induced IL1B expression described earlier. Monocytes were inoculated with DENV-2 after pretreatment with DMSO vehicle or BAY 61-3606, and RNA was collected at 2 hpi for assessment by real-time PCR for IL1B expression (Fig. 6*A*). Syk inhibition prevented ADE-induced elevation of IL1B

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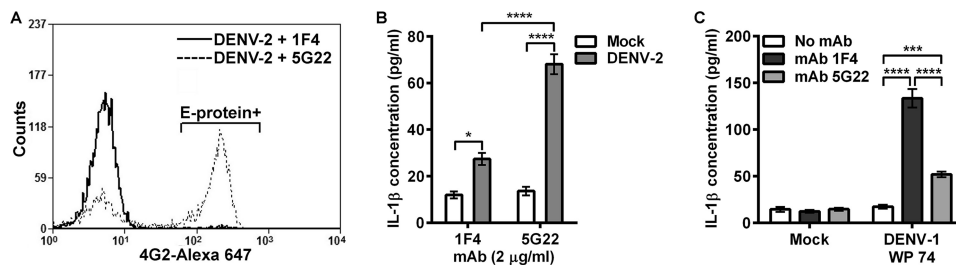


FIGURE 5. Serotype-specific anti-DENV antibodies enhance IL-1 β secretion. *A*, DENV-2 was complexed with mAb 5G22 (dashed line) or incubated with an equal concentration of DENV-1-specific mAb 1F4 (solid line) prior to inoculation onto monocytes. At 4 hpi, cells were washed twice and resuspended in fresh medium until collection at 24 hpi and staining for flow-cytometric detection of intracellular DENV E protein. *B*, cells were inoculated as indicated, and supernatants were collected at 4 hpi. IL-1 β was then assessed by ELISA. *C*, monocytes were inoculated with MOI 2.5 of DENV-1 that had been complexed with 1 μ g/ml mAb 1F4 or mAb 5G22. Supernatants were collected at 24 hpi and assessed for IL-1 β by ELISA. Statistical tests used were as follows: ordinary two-way ANOVA (Tukey's) (*B* and *C*). *A* and *B* are representative of more than three independent experiments. *C* is representative of two independent experiments. *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$. Error bars, S.E.

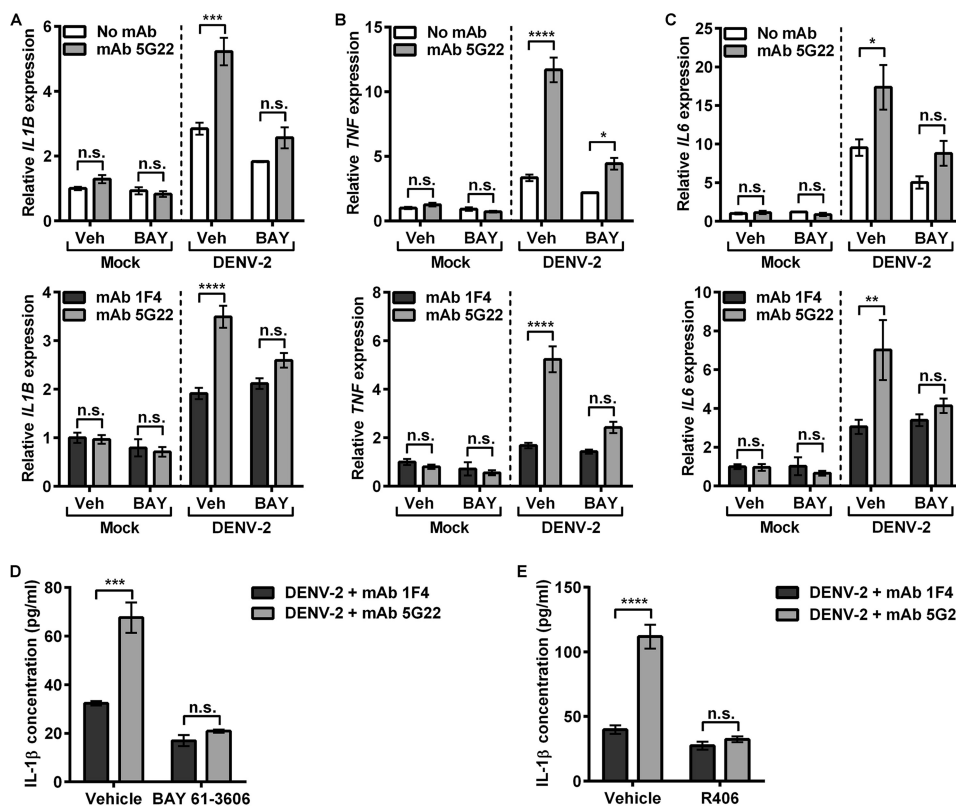


FIGURE 6. Syk mediates inflammatory cytokine expression induced by ADE. *A*, monocytes were pretreated with DMSO vehicle or 0.5 μ M BAY 61-3606 prior to inoculation with DENV-2. At 2 hpi, cells were lysed, and RNA was purified. IL1 β expression was assessed by semiquantitative real-time PCR, with normalization to mock and vehicle treatment. In the *top graph*, DENV-2 was incubated with medium as a non-ADE control, whereas in the *bottom graph*, DENV-2 was incubated with isotype mAb 1F4. Vehicle-treated values in the *top graph* are part of the pooled values in Fig. 2*A*. Expression of TNF (*B*) and IL6 (*C*) were similarly measured. *D* and *E*, monocytes were pretreated with DMSO vehicle, 1 μ M BAY 61-3606 (*D*), or 1 μ M R406 (*E*) prior to inoculation with DENV-2 in the presence of mAb 1F4 or complexed with mAb 5G22. Supernatants were collected at 5 hpi (*D*) or 4 hpi (*E*) and assessed for IL-1 β by ELISA. Statistical tests used were as follows. Two separate ordinary two-way ANOVAs were used for each graph for *A*–*C* with Bonferroni's post-test, one for mock inoculation and one for DENV inoculation, with antibody and inhibitor conditions set as independent variables. Ordinary two-way ANOVAs (Bonferroni's) were used for *D* and *E*. The *top graphs* in *A*–*C* are representative of two independent experiments, and the *bottom graphs* in *A*–*C* are pooled values from two independent experiments. *D* and *E* are representative of three or more independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; n.s., not significant. Error bars, S.E.

expression compared with inoculation with DENV-2 alone (Fig. 6*A*, *top*) and compared with inoculation with DENV-2 in the presence of isotype mAb 1F4 (Fig. 6*A*, *bottom*). Additionally, ADE-induced TNF (Fig. 6*B*) and IL6 (Fig. 6*C*) expression was also sensitive to Syk inhibition. These data suggest that Syk mediates ADE-induced inflammatory cytokine expression and that impairment of Syk activity reduces ADE-induced inflammatory cytokine expression to levels induced by DENV-2 alone.

Additionally, Syk inhibition with BAY 61-3606 prevented DENV-2 complexed with mAb 5G22 from inducing elevated IL-1 β secretion compared with DENV-2 in the presence of isotype mAb 1F4 (Fig. 6*D*). This finding was verified using a second Syk inhibitor, R406 (Fig. 6*E*).

Syk Inhibition Ablates ADE-induced ERK1/2 Activation—We next sought to identify additional intracellular signaling events mediated by DENV-2 immune complexes. Fc γ R cross-linking by immune complexes activates ERK1/2 through

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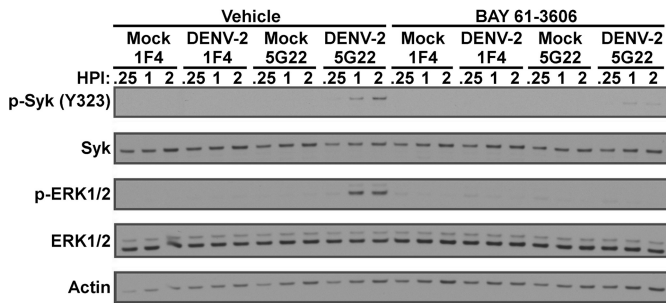


FIGURE 7. ADE-induced Syk activation mediates ERK activation. Monocytes were inoculated with DENV-2 after pretreatment with DMSO vehicle or 1 μ M BAY 61-3606 for 30 min. At 0.25, 1, or 2 hpi, cells were lysed and assayed via immunoblot for the indicated proteins. Blots are representative of three independent experiments.

activated Syk, leading to induction of cytokine expression (75–79).

Thus, we wanted to assess the relationship between Syk and ERK1/2 activation during a time course of DENV-2 inoculation with and without immune complexes. Monocytes were inoculated after pretreatment with BAY 61-3606 or DMSO vehicle, and cells were collected and lysed for immunoblot analysis at 0.25, 1, or 2 hpi (Fig. 7). As expected, DENV-2 complexed with mAb 5G22-induced Syk phosphorylation at tyrosine 323, which was detected by 1 hpi. DENV-2 inoculation in the presence of mAb 1F4 did not induce detectable Syk phosphorylation, confirming the need for immune complexes for strong Syk activation. BAY 61-3606 inhibited the Syk phosphorylation induced by DENV-2 immune complexes. Additionally, only DENV-2 immune complexes induced strong phosphorylation of ERK1/2, which was also ablated by Syk inhibition. These data suggest that Syk mediates activation of ERK1/2 after inoculation with DENV-2 immune complexes.

It has been reported that Syk inhibitors mediate their anti-inflammatory effects in mouse macrophages by nonspecific inhibition of JNK (80). Thus, we expanded our studies to include JNK activation while also assessing the activation of NF- κ B following inoculation with DENV-2 immune complexes (Fig. 8). As before, DENV-2 immune complexes induced the strongest Syk and ERK activation, and activation of both proteins was sensitive to inhibition by BAY 61-3606. In contrast, we could detect only faint phosphorylation of the p54 isoform of JNK, but this was not sensitive to inhibition by BAY 61-3606, suggesting no cross-reactivity of BAY 61-3606 with JNK in our system. Additionally, phosphorylation of NF- κ B subunit p65 was also less sensitive to inhibition with BAY 61-3606 than ERK. Of note, all conditions show baseline phosphorylation of p65 at time 0. This could be due to exposure to the small portion of cells that do not survive the thawing process. However, as shown in Fig. 2, *E* and *F*, increased resting time to reduce baseline activation is not possible as cells become refractory to activation with immune complexes with increased resting time. Regardless, as expected from gene expression studies, Syk inhibition reduced ADE-induced pro-IL-1 β expression to levels induced by DENV-2 in the presence of isotype mAb 1F4.

Syk-mediated ERK1/2 Activation Is Critical for ADE-induced IL-1 β Secretion—Because ERK has been shown to mediate IL-1 β secretion in THP-1 cells after Fc γ R cross-linking (79), we

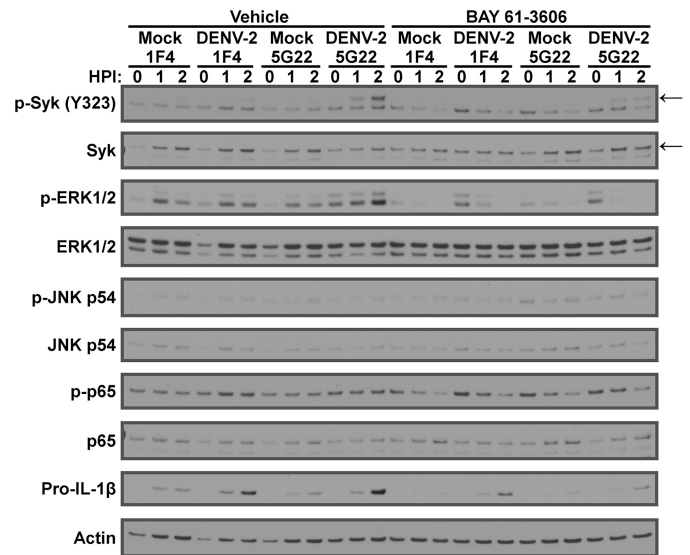


FIGURE 8. Syk inhibition reduces ADE-induced pro-IL-1 β expression to levels induced by non-ADE conditions. Monocytes were inoculated with DENV-2 after pretreatment with DMSO vehicle or 1 μ M BAY 61-3606 for 45 min. Cells were collected and lysed at the indicated time points (immediately after inoculation for 0 hpi), and the indicated intracellular proteins were assessed by immunoblot. Except for phospho-p65 and phospho-JNK, all phenotypes shown in the blots are representative of three or more independent observations. For phospho-p65, baseline phospho-p65 at 0 hpi was measured once but also detected several times at just 15 min postinoculation, even under mock conditions. For phospho-JNK, in some experiments, we were unable to detect phospho-JNK, whereas in other experiments, as shown here, very weak phospho-JNK is detected that did not correlate with DENV-2 inoculation.

sought to assess the involvement of ERK1/2 activation in ADE-induced IL-1 β secretion. Thus, we employed the use of the potent ERK1/2 inhibitor, PD98059. PD98059 blocks MEK1 and MEK2, the upstream kinases that specifically activate ERK1/2, and is highly selective at the concentrations used here (81, 82). Monocytes were inoculated with DENV-2 after pretreatment with PD98059 or DMSO vehicle. At 4 hpi, cells were lysed and assayed by immunoblot (Fig. 9A). ADE-induced Syk activation remained intact in the presence of PD98059, as expected if Syk activation is upstream of ERK activation. However, PD98059 ablated ERK activation and reduced pro-IL-1 β levels for all conditions. Correspondingly, we found that ERK inhibition completely ablated the presence of mature IL-1 β in the supernatants at 4 hpi (Fig. 9B). The data in Fig. 9, *A* and *B*, suggest that ERK is important in pro-IL-1 β expression as well as in IL-1 β maturation. However, until more sensitive assays are developed that allow us to accurately measure caspase-1 activation in the current system, we cannot directly measure the impact of ERK inhibition on caspase-1 activation.

Finally, we wanted to assess whether the other major MAPK signaling pathways, JNK and p38, must also remain intact for optimal ADE-induced IL-1 β induction. In addition to BAY 61-3606 and PD98059, primary monocytes were also incubated with p38 inhibitor SB 203580 and JNK inhibitor SP600125 prior to inoculation with DENV-2 immune complexes (Fig. 9C). Inhibition of p38 and JNK also inhibited IL-1 β secretion induced by DENV-2 immune complexes. These data suggest that intact MAPK signaling is important for ADE-induced IL-1 β secretion.

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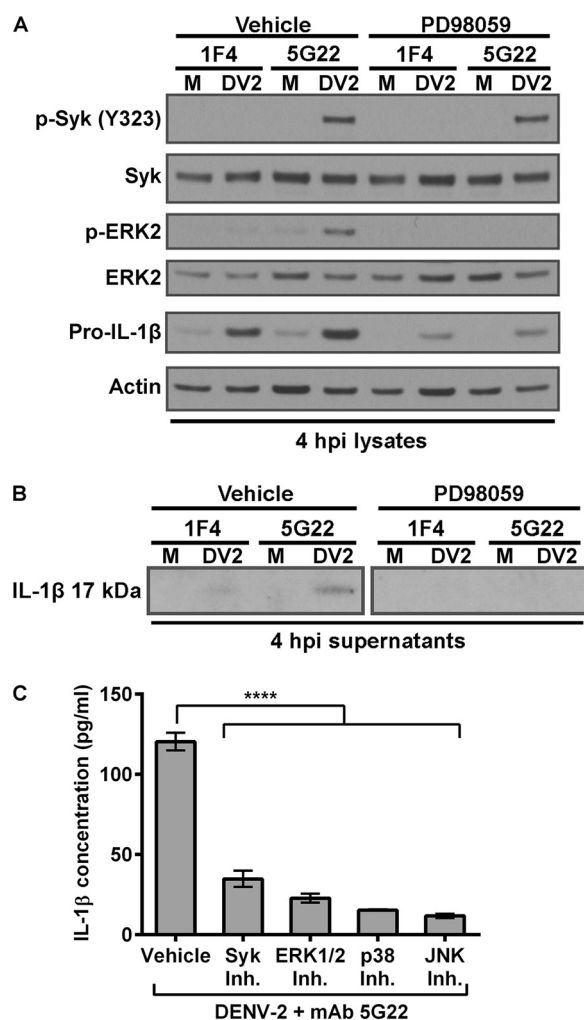


FIGURE 9. Syk activates ERK1/2 to mediate ADE-induced IL-1 β secretion. *A*, monocytes were inoculated with DENV-2 after pretreatment for 1 h with DMSO vehicle or 25 μ M PD98059 ERK1/2 inhibitor. At 4 hpi, cells were lysed, and the indicated proteins were measured via immunoblot. *B*, cells were inoculated with DENV-2 after a 1-h pretreatment with DMSO vehicle or 25 μ M PD98059. At 4 hpi, supernatants were collected and assayed by immunoblot for mature IL-1 β . Boxes indicate non-adjacent lanes taken from the same blot and exposure. *C*, monocytes were inoculated with DENV-2 immune complexes after pretreatment with 0.5 μ M BAY 61-3606 (*Syk Inh.*), 25 μ M PD98059 (*ERK1/2 Inh.*), 0.5 μ M SB 203580 (*p38 Inh.*), or 25 μ M SP600125 (*JNK Inh.*) for 45 min. At 4 hpi, supernatants were collected and assessed for IL-1 β by ELISA. The statistical test used (*C*) was ordinary one-way ANOVA (Dunnett's) comparing all inhibitor-treated samples with the vehicle control. *A* and *B* are representative of two independent experiments, and *C* is representative of three independent experiments. ****, $p < 0.0001$. Error bars, S.E.

Discussion

In this study, we confirm that ADE of DENV infection elevates secretion of IL-1 β by primary human monocytes and provide insight into the modulation of cytokine induction by ADE. DENV immune complexes activate a signaling axis containing Syk and ERK1/2 that is critical for cytokine induction but dispensable for DENV replication. ADE robustly induces pro-IL-1 β synthesis but not caspase-1 activation, suggesting that ADE primarily modulates IL-1 β secretion by increasing *IL1B* expression and pro-IL-1 β synthesis. Correspondingly, we find that ADE-induced expression of inflammatory cytokine genes *TNF* and *IL6* is sensitive to Syk inhibition as well, suggesting

that Syk activation may have broader implications for cytokine production during severe dengue.

Notably, the phenotype we describe here in primary monocytes differs from what is seen in a more differentiated myeloid cell. In inflammatory human macrophages, DENV triggers release of mature IL-1 β through induction of pro-IL-1 β synthesis and activation of the NLRP3 inflammasome (20). NLRP3 activation is induced by binding of DENV to the C-type lectin receptor CLEC5A on the macrophage cell surface, requiring signaling through DAP12 and Syk. Lack of strong NLRP3 inflammasome activation in the current system suggests either divergent roles or differential expression of CLEC5A in monocytes versus inflammatory macrophages. Correspondingly, macrophage differentiation is associated with increased expression of CLEC5A (83).

Similar to DENV binding CLEC5A in macrophages, Syk activation downstream of C-type lectin receptors licenses inflammasome activation in response to fungal pathogens and helminthes (70, 84, 85). Additionally, malarial hemozoin is engulfed by phagocytes, inducing inflammasome activation through Syk (71). Thus, Syk-mediated inflammasome activation may be linked intimately to certain pathways or even certain cell types and may not be induced by Fc γ R cross-linking in primary human monocytes. Syk activation cannot be viewed as universally equivalent in its downstream effects. Even within members of the Fc γ R family, signaling pathways quickly diverge downstream of Syk (86).

Regardless, the absence of high levels of caspase-1 activation by ADE in the current study did not completely block mature IL-1 β secretion. Instead, a low level of IL-1 β processing after induction of pro-IL-1 β synthesis did occur, and this process was caspase-1-dependent, as has been observed for monocytes (39–42, 58, 59, 83, 87). Both pharmacologic and genetic interference of NLRP3 inhibited DENV-induced IL-1 β processing. This inefficient processing is supported by the experiment showing that processing efficiency of DENV-induced IL-1 β in primary monocytes was greatly enhanced by the addition of ATP, which provided the necessary second signal for optimal inflammasome activation. We further showed that IL-1 β maturation upon DENV-2 inoculation and ADE is dependent upon Syk, because Syk inhibition ablated secretion of mature IL-1 β . Thus, Syk can affect IL-1 β at the level of mRNA as well as protein maturation.

Although the unique regulation of IL-1 β makes it an interesting model to study, the implications of the current study may extend far beyond any individual cytokine. Our study provides the first evidence that Syk activation is critical for ADE-induced expression of several inflammatory cytokines in undifferentiated primary human monocytes. This identifies Syk as a potential therapeutic target to interfere with the pathogenic cytokine storm of severe dengue.

Interestingly, a recent report indicates that ADE itself inhibits early Syk activation in THP-1 cells and primary monocytes by co-ligating surface receptor LILRB1 (88). However, ADE-induced Syk inhibition in the Chan *et al.* study (88) was strongest at 10 and 30 min postinoculation and appeared to decrease afterward. Consistent with this, we detected much higher levels of phosphorylated Syk between 2 and 8 h after inoculation than

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at earlier time points. Additionally, the Chan *et al.* study (88) measured total tyrosine phosphorylation after immunoprecipitation of Syk, whereas the current study measured Syk phosphorylation specifically at Tyr-323. Because Syk contains many phosphorylation sites, this confounds direct comparisons (89).

Another recent study indicated that immune complexes inhibit inflammasome activation (90). However, in the study by Janczy *et al.* (90), immune complexes interfered with inflammasome assembly induced by a separate priming signal. In the current study, DENV alone or DENV immune complexes were the sole stimulus. It would be interesting to determine whether ADE of DENV can interfere with inflammasome activation during co-infection with other pathogens.

Our findings indicate that ADE induces inflammation by signaling through a Syk-ERK pathway described in other studies investigating the cross-linking of Fc receptors (75–79). A DENV immune complex must have important viral binding epitopes exposed to be infectious, thus leading to ADE (8, 91). This suggests that DENV complexed to enhancing antibodies may still ligate other signaling receptors. By activating a pathway common to immune complexes, ADE may provide an additional, as opposed to an alternate, pathway of inflammation that augments signaling induced by other receptors. This is reflected in the current study, because inhibiting ADE-induced Syk activation reduced inflammatory cytokine expression to levels induced by DENV alone. Interfering with this additional signaling axis activated by DENV immune complexes may be of therapeutic use in dengue patients. Because inhibitors of Syk and ERK have been considered as therapeutics for cancer and autoimmunity, these compounds could be repurposed in an attempt to interfere with the cytokine storm in severe dengue. An additional benefit is that such treatments may provide relatively rapid anti-inflammatory effects in a patient after illness has developed, unlike the pre-exposure immunity that must be induced by a vaccine.

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