Hemolysin Induces Toll-like Receptor (TLR)-independent Apoptosis and Multiple TLR-associated Parallel Activation of Macrophages

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Vibrio cholerae hemolysin (HlyA) displays bipartite property while supervising macrophages (Mφ). The pore-forming toxin causes profound apoptosis within 3 h of exposure and in parallel supports activation of the defying Mφ. HlyA-induced apoptosis of Mφ remains steady for 24 h, is Toll-like receptor (TLR)-independent, and is driven by caspase-9 and caspase-7, thus involving the mitochondrial or intrinsic pathway. Cell activation is carried forward by time dependent up-regulation of varying TLRs. The promiscuous TLR association of HlyA prompted investigation, which revealed the β-prism lectin domain of HlyA simulated TLR4 up-regulation by jacalin, a plant lectin homologue besides expressing CD86 and type I cytokines TNF-α and IL-12. However, HlyA cytolytic protein domain up-regulated TLR2, which controlled CD40 for continuity of cell activation. Expression of TOLLIP before TLR2 and TLR6 abrogated TLR4, CD40, and CD86. We show that the transient expression of TOLLIP leading to curbing of activation-associated capabilities is a plausible feedback mechanism of Mφ to deploy TLR2 and prolong activation involving CD40 to encounter the HlyA cytolytic domain.

Vibrio cholerae El Tor O1 and non-O1 strains produce a water-soluble cytolytic exotoxin that has been designated HlyA or El Tor hemolysin (1). HlyA, an extracellular membrane-damaging protein with a native molecular mass of 65 kDa (2) belongs to a subgroup of bacterial virulence factors called pore-forming toxins (PFTs). The PFTs lyse target eukaryotic cells and can exist in two stable states, a water-soluble monomer and an oligomeric integral membrane protein (3). Apart from lysing erythrocytes and other mammalian cells, HlyA exhibits enterotoxicity in experimental diarrhea models (1) and has been implicated in causing diarrhea during cholera epidemic (4). The fully active 65-kDa toxin consists of a central cytolsin domain followed sequentially by two contiguous lectin domains, a β-trefoil domain homologous to the galactose-binding site of ricin and a β-prism domain homologous to the carbohydrate-binding site of the plant lectin jacalin (5). The C terminus β-prism lectin domain, suggested as the only functional sugar-binding site of HlyA, is susceptible to proteolytic deletion (6).

Macrophages (Mφ) reside in almost all tissues and are involved as important effector cells at all stages of the innate and adaptive immune response. Mφ are vigorously involved in host defense against microbial infection (7, 8). Mφ produce chemokines, cytokines, and other inflammatory mediators upon recognizing pathogen-associated molecular patterns (PAMPs) (9) or molecules released by damaged host cells referred to as “danger signals” (10), which alert the remainder of the immune system of infection and injury. Several distinct classes of receptor proteins participate in PAMP sensing and triggering of signaling cascades, which culminate in expression of immune effector genes. Among such receptors, Toll-like receptors (TLRs) are an important group extensively studied in both vertebrates and invertebrates (11, 12). TLRs, found on cells of the immune system, are sensors of invading pathogens (13). PFTs such as pneumolysin from Streptococcus pneumoniae (14) and listeriolysin O from Listeria monocytogenes (15) have been reported to be recognized by TLR4. Here, we identify the TLRs that recognize HlyA and initiate signaling, thus enabling peritoneal cavity (PerC) Mφ to execute the first line of innate defense. Engagement of TLR by PAMP triggers the signaling pathway that drives the innate immune effector functions and leads to initiate and strengthen adaptive immunity (16, 17). TLRs mediate downstream signaling by recruitment of MyD88 (myeloid differentiation factor 88) (18), leading to activation of IL-1 receptor-associated kinases, TNF receptor-associated factor 6 (TRAF6), followed by proinflammatory transcription factor NF-κB and stress-activated protein kinases (19). On the contrary, TLR4 and TLR2 signaling are inhibited by TOLLIP to check overzealous TLR influence (20).

The immune cells of a host, in response to a natural immunogen, can take either of two pathways, that of activation or apoptosis. The capacity of an immunogen to drive Mφ activation is reflected by its ability to induce the costimulatory molecules CD80–CD86, activation molecule CD40, MHC class II (21, 22), and production of chemokines and cytokines. The cytokines associated with polarized type I responses of activated Mφ, the M1 phenotypes, include production of proinflammatory IL-12 and TNF-α. In contrast, M2 cells typically produce IL-10 (23). On the other hand, the cells can get com.
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Immunogens—The HlyA monomer was purified from the culture supernatant of a non-O1* V. cholerae strain V₂ grown in brain heart infusion (Difco) broth by hydrophobic interaction chromatography on phenyl-Sepharose CL-4B, size-exclusion chromatography on Sephadex G-50 and chromatofocusing on PBE-94 (Pharmacia) (28). Because the native HlyA is resistant to proteolysis, the 50-kDa variant (VCC50) truncated at the C terminus β-prism lectin domain was prepared by restricted proteolysis with trypsin in 1.7 M urea, at an enzyme:substrate ratio of 1:100. Urea was removed by dialysis, and the 50-kDa variant was freed from HlyA by chromatography on phenyl-Sepharose CL-4B.

Animals—C57BL/6 mice were obtained from National Centre for Laboratory Animal Sciences (National Institute of Nutrition, Jamai-Osmania, Hyderabad, India) and maintained in the animal care facility of the National Institute of Cholera and Enteric Diseases (Kolkata, India). The C3H/HeJ mice were gifted by the Institute of Life Sciences (Bhubaneswar, Orissa, India). The mice were housed in groups of six and given food and water *ad libitum*. The experiments with animals were conducted in accordance with the Animal Ethical Committee guidelines of National Institute of Cholera and Enteric Diseases (Kolkata, India).

PerC MΦ Culture—PerC MΦ of 6-week-old C57BL/6 or C3H/HeJ mice were cultured as described elsewhere (7). Briefly, the peritoneal washing containing the MΦ was collected in sterile Petri dishes and incubated at 37 °C in 5% CO₂ for 1.5 h. The cells of monocyte-MΦ lineage adhered on the surface of the Petri dishes to form a monolayer. Adhered cells were collected by centrifugation at 400 × g for 8 min and suspended in RPMI 1640 (Invitrogen). Cells (200 µl) were seeded per well at a concentration of 1 × 10⁶ cells/ml in 96-well flat-bottomed plates (Corning). MΦ were cultured at 37 °C for designated periods in humidified atmosphere of 5% CO₂ with absence and presence of 0.5 µg/well HlyA, 0.5 µg/ml FSL-1 (Pam₃CGDPKHPSK) (InvivoGen), 5 µg/ml Pam₃CSK₄, 100 ng/ml LPS (Escherichia coli 0111:B4) (Sigma-Aldrich) or 1 µg/ml jacalin (Artocarpus integrifolia) (Sigma-Aldrich) in RPMI 1640 containing 5 units/ml penicillin, 5 µg/ml streptomycin, 0.1% gentamicin, 2% fetal bovine serum (Hyclone Lab-oratories), and 0.1% insulin-transferrin-selenium (Invitrogen). For inhibition studies, MΦ were incubated with either 10 µg/ml purified anti-mouse TLR2 mAb (Functional Grade; eBioscience), 5 µg/ml purified anti-mouse TLR4 Ab (Santa Cruz Biotechnology), or both for 30 min prior to the addition of HlyA.

Flow Cytometry—Cells were cultured with and without HlyA and stained at 4 °C for 20 min with phycoerythrin-conjugated anti-mouse CD11b mAb (BD Pharmingen) followed by any one of FITC-conjugated anti-mouse TLR1 (eBioscience), TLR2, CD40, CD80 or CD86, or biotin-conjugated anti-mouse TLR4 (eBioscience) or MHC class II (I-Ab). Biotinylated Abs were recognized by streptavidin-FITC. To detect surface expression of TLR6, cells were stained with polyclonal anti-TLR6 (Santa Cruz Biotechnology) and protein A-FITC (Sigma-Aldrich). Parallel sets of cells were incubated with monoclonal Ig isotypes. Stained cells were analyzed on a FACSCalibur using CellQuest software (Becton Dickinson).

Cytokine ELISA—Release of IL-12 p70 (eBioscience), IL-10, and TNF-α (R&D Systems) in 24- and 48-h-old culture supernatants was measured in sandwich ELISA with pairs of Abs for binding and detection using ELISA kits according to the manufacturer’s instructions.

RT-PCR Analysis—RNA was isolated from 1 × 10⁶ MΦ using an RNAqueous™-PCR kit (Ambion). Equal amounts of RNA from cells treated with and without HlyA were reverse-transcribed using a RETROscript™ kit (Ambion). cDNA served as template for the amplification of MyD88 and GAPDH transcripts by PCR in an automated thermal cycler (Applied Biosystems GeneAmp PCR System 2700) using SuperTaq™ polymerase (Promega). The primers for amplification of MyD88 and GAPDH were used as described elsewhere (30). The images were captured using a gel documentation system (Ultra-Violet Products, U.K.). Amplified RT-PCR product of MyD88 was normalized for the relative quantity of amplified product of GAPDH using Ultra-Violet Products Vision Works LS software. Change in mRNA expression was presented as mean fold induction by HlyA relative to the untreated control.

Phosphatidyserine Exposure on MΦ and Intracellular Detection of Caspases—Cells cultured with and without HlyA monomer for 3 and 24 h were incubated with Fc block (anti-CD16/32) followed by phycoerythrin-conjugated anti-mouse CD11b mAb (BD Pharmingen). Phosphatidyserine exposure was measured in these cells by annexin V-FITC and 7-amino acti-
nomycin D (eBioscience) using an annexin V-FITC apoptosis detection kit according to the manufacturer’s instructions (BD Pharmingen). To study whether HlyA-induced apoptosis is TLR4-dependent, cells were incubated with anti-mouse TLR4 Ab prior to the addition of HlyA and cultured for 3 h for detection of apoptosis. Intracellular caspase-8 and caspase-9 were detected in the cells by fixing and permeabilizing with Cytofix/Cytoperm kit (BD Pharmingen) and staining with rabbit anti-mouse caspase-8 Ab (Santa Cruz Biotechnology) or caspase-9 Ab (Cell Signaling Technology), respectively, followed by FITC-conjugated goat polyclonal anti-rabbit Ig. Intracellular staining of caspase-3 was done with FITC-conjugated rabbit anti-mouse active caspase-3 mAb (BD Pharmingen).

**Immunoprecipitation and Immunoblot**—Treated cells were lysed in cold lysis buffer (Cell Signaling Technology) centrifuged to clear the debris and incubated overnight with rabbit anti-mouse TLR4 Ab (Santa Cruz Biotechnology) or anti-mouse TLR2 mAb-bound Protein A Sepharose™ CL-4B (Pharmacia). The Ag-Ab-bound beads were centrifuged, washed, boiled in Laemmli sample buffer for 5 min, and the immunoprecipitates were separated by 12.5% SDS-PAGE. The electrophoresed proteins were transferred to nitrocellulose membrane and probed with rabbit anti-mouse MyD88 Ab (Santa Cruz Biotechnology) and anti-mouse TLR2 Ab for detection of the respective molecules in the MyD88-TLR4/MyD88-TLR2 complex. Immunoblotting was performed to detect the presence of TOLLIP, anti-apoptotic proteins, and caspases. The cell lysates were electrophoresed, Western-blotted, and incubated overnight with rabbit anti-mouse TOLLIP mAb (Santa Cruz Biotechnology), anti-mouse Bcl-xL, caspase-6, or caspase-7 Ab (Cell Signaling Technology). The Western blots were imaged by incubation with HRP conjugated anti-rabbit IgG and Immobilon™ Western Chemiluminescent HRP substrate (Millipore).

**Statistical Analysis**—Results were expressed as the means ± S.E where applicable, of three independent experiments. The data were analyzed by using one-way analysis of variance. A p value of < 0.05 was considered significant, and a p value of < 0.005 was considered highly significant.

**RESULTS**

**TLR4 Up-regulation of MΦ Gets Transiently Curbed by TOLLIP for Shaping TLR2 and -6 Induction**—The TLR4-specific ligand LPS, TLR2 and -1 ligand Pam3CSK4, and TLR2 and -6 ligand FSL-1 up-regulated the respective TLRs on Perc MΦ of C57BL/6 mice (data not shown). Hence, MΦ was cultured with HlyA for 48 h in a time course study of TLR expression. Selective up-regulation of TLR4 was traced from as early as 2 h
that continued until 48 h of cell incubation (Fig. 1A). Although not detected in the initial hours, TLR2 expression then of TLR6 was found after 20 and 24 h of culture with HlyA, respectively, which continued until 48 h. Analysis of relative fluorescence intensity showed a maximum of 3.6-fold (3.61 ± 0.21 S.E., p < 0.05) increase in TLR4 expression followed by a 1.9-fold (1.89 ± 0.84 S.E., p < 0.05) and 1.7-fold (1.69 ± 0.24 S.E., p < 0.005) increase in TLR2 and TLR6 expression, respectively. The expression of TLR1, known to be associated with TLR2 expression, remained unaffected all along the time-course study. Unexpectedly, none of the participating TLRs were found to be present between 8 to 12 h of cell culture, when expression of TOLLIP, known to cast its inhibitory effects on TLR4, was detected at 10 h of culture (Fig. 1B). The data demonstrate that both TLR4 and TLR2, known to recognize absolutely dissimilar PAMPs, are required by MФ to distinguish HlyA of V. cholerae in its entirety, but their temporal operations seemed to be monitored by TOLLIP. Next, we assessed whether TLR2 up-regulation of MФ was TLR4-dependent because TLR2 expression through TLR4 signaling has been seen in endothelial cells (31). The up-regulation of TLR2 recorded a drop of only 44% (44.01 ± 1.8 S.E., p < 0.05) and that of TLR6 of 33% in their expression when the cells were preincubated with neutralizing anti-TLR4 Ab before HlyA treatment (Fig. 1C). In TLR4 non-responder C3H/HeJ mice, HlyA treatment up-regulated TLR2 1.6-fold (1.63 ± 0.04 S.E., p < 0.005) over control confirming expression of TLR2 was an event by itself.

**TLR4-dependent Recruitment of MyD88 for Signaling through NF-κB—RT-PCR indicated an increase in the mRNA level of MyD88 by 1.7-fold (1.66 ± 0.04 S.E., p < 0.05) in response to HlyA (Fig. 2A).** Thus, we examined the time-dependent association of the adaptor molecule MyD88 with TLR4 and TLR2 by immunoprecipitation after exposure to HlyA (Fig. 2B). The MyD88:TLR4 complex was detected within 15 min of HlyA treatment that persisted until 60 min, whereas no such interaction was observed between MyD88 and TLR2, clearly showing that the PFT singularly depends on TLR4-mediated signaling.

Nuclei, prepared from MФ treated with and without HlyA for 40 min, were used for flow cytometric detection of nuclear translocation of the p65 (RelA) member of NF-κB family. HlyA induced the NF-κB translocation that was 1.8-fold (1.78 ± 0.09 S.E., p < 0.05) higher than the untreated control (Fig. 2C), showing that the transcription factor is responsible for the HlyA-induced MФ response. MФ, when pretreated with neutralizing anti-TLR4 Ab, showed 73% (73.15 ± 0.84 S.E., p < 0.05) inhibition of NF-κB:p65 translocation on the nuclei. The data indicate that nuclear translocation of NF-κB in response to HlyA treatment is dependent on TLR4 signaling.

**TLR-specific Temporal Induction of Activation and Co-stimulatory Molecules—**HlyA induced a 1.7-fold (1.75 ± 0.89 S.E., p < 0.05) increase of the activation molecule CD40 and 2-fold (2.01 ± 0.75 S.E., p < 0.005) increase of the co-stimulatory molecule CD86 as early as 2–4 h (Fig. 3A). However, there was no expression of the activation and the co-stimulatory molecules on MФ between 8 to 12 h of HlyA treatment, when none of the participating TLRs could be traced. Interestingly, after

**FIGURE 2. TLR4-dependent, HlyA-induced expression of MyD88 and NF-κB.** A, total RNA was extracted from the cells cultured with or without HlyA and subjected to RT-PCR using the corresponding primers of MyD88 and GAPDH. Ethidium bromide-stained PCR products were photographed, and then images were digitized and analyzed. PCR products were quantified and expressed as ratio of the product to GAPDH band density. B, MФ were cultured with HlyA, and cell lysate at each time point was normalized for the protein content and immunoprecipitated (IP) with anti-TLR4 Ab or anti-TLR2 Ab. The precipitates were separated by SDS-PAGE and immunoblotted (IB) with anti-MyD88 Ab to assess the association of MyD88 with either of the two TLRs. The data given were obtained in one of three representative experiments. C, cells were cultured in absence (thin black line), presence (thick black line) of HlyA and anti-TLR4 Ab plus HlyA (thin gray line), incubated in Hepes-Triton buffer (pH 7.4) to obtain pure nuclei and analyzed for NF-κB translocation. The shaded profile indicates isotype-matched control. The bar diagram represents mean fluorescence intensity (MFI) of the cells as mean ± S.E. of three independent experiments. *, p < 0.05.
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A

2-4 h 12 h 24 h
Counts

2-4 h 12 h 24 h
Counts

MHC II (I-A^d)

4 h 12 h 18 h 24 h
Counts

Fluorescence intensity

B

Fluorescence intensity

Anti-TLR4 Ab + HlyA

CD86

CD40

2 h

Counts

CD86

CD40

HlyA

Anti-TLR4 Ab

Fluorescence intensity

C

MFI

24 h

CD40

CD86

CD40

HlyA

Anti-TLR4 Ab

β-actin

D

Anti-TLR2 Ab

HlyA

24 h

MFI

CD40

HlyA

Anti-TLR4 Ab

Anti-TLR2 Ab
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FIGURE 3. HlyA-induced time-dependent up-regulation of activation, co-stimulatory and MHC class II (I-Ab) molecules are controlled by TLR4 and 2. A and B, cells were cultured with (black line) and without (shaded) HlyA for CD86–CD86, CD40, and MHC class II (I-Ab) expression at the indicated time points. C, cells were cultured with (gray line) and without (shaded) HlyA or with anti-TLR4 Ab plus HlyA (black line) for the expression of CD86 and CD40 at 2 h. The bar diagram represents mean fluorescence intensity (MF) of the cells as mean ± S.E. **, p < 0.005. D, cells were cultured with (gray line) and without (shaded) HlyA or with Abs as indicated plus HlyA (black line) for expression of CD86 at 24 h. The bar diagram represents mean fluorescence intensity of untreated (open), treated (solid), and anti-TLR2 Ab plus HlyA treated (hatched) cells as mean ± S.E. *, p < 0.05. G, cell lysate was prepared of MΦ treated with and without HlyA, which was immunoblotted for Bcl-xL and reprobed with anti-β-actin Ab to ensure equal loading of protein.

FIGURE 4. Quantification of cytokines by ELISA. MΦ obtained from C57BL/6 and C3H/HeJ mice were cultured in absence and presence of HlyA or with anti-TLR4 Ab/anti-TLR2 Ab plus HlyA. The supernatants were assayed for TNF-α after 24 h and IL-10/IL-12 p70 after 48 h of culture. Data represent mean ± S.E. of three independent experiments, each done in triplicate. *, p < 0.05; **, p < 0.005.

ppearance with regard to its role in cell activation was tested. It was found that at a later time (24 h), CD40 expression was strongly inhibited by 93% (92.5 ± 0.87 S.E., p < 0.005) with neutralizing anti-TLR2 Ab, 64% (64.15 ± 2.8 S.E., p < 0.005) with anti-TLR4 Ab, and 99.9% with both of the Abs together (Fig. 3D). The HlyA-induced expression of CD40 coupled to its inability to express CD86 in C3H/HeJ mice strongly supported the importance of TLR4-independent activity of CD40 but TLR4-dependent role of CD86 (Fig. 3E). Inhibition of CD40 by anti-TLR2 Ab in C3H/HeJ mice strongly supported the importance of TLR2 signaling in expression of the activation molecule (Fig. 3F), thus indicating that TLR2 played an independent role in cell activation. Preincubation of C57BL/6 MΦ with a pan-caspase inhibitor could neither increase nor inhibit HlyA-induced CD86 and CD40 expression, showing that TLR4 and -2-mediated cell activation is an apoptosis-independent event (data not shown) supported by the moderate presence of anti-apoptotic protein Bcl-xL even after strong depletion due to parallel apoptosis (Fig. 3G).

TLR4 Controls HlyA-mediated Release of IL-12 p70 and TNF-α—HlyA-treated MΦ (1 × 10⁶) released 33 pg of IL-12 p70, 60.75 pg of TNF-α, and no IL-10 over untreated controls (Fig. 4, left bars). Preincubation of MΦ with neutralizing anti-TLR4 Ab prior to antigen treatment released 9.25 pg of IL-12 p70 and 23.5 pg of TNF-α over untreated cells showing 71.5% (71.55 ± 0.97 S.E., p < 0.05) and 60.6% (60.57 ± 0.71 S.E., p < 0.005) inhibition of the cytokines, respectively. However, pre-treatment of MΦ with neutralizing anti-TLR2 Ab could not inhibit the release of the two cytokines. The HlyA-induced TLR4-dependent release of the two type I cytokines, IL-12 and TNF-α, was confirmed by the failure of HlyA to induce the release of both the cytokines by MΦ of C3H/HeJ mice (Fig. 4, right bars). The data show that unlike TLR2 and -4 control over the expression of the activation molecule CD40, TLR4 alone monitors the HlyA-induced cytokine release.

Expression of TLR4 by Jacalin and TLR2 by VCC⁶⁰ Reveals Dependence of β-Prism Lectin and Cytosol Domains of HlyA on Two TLRs for MΦ Activation—The HlyA β-prism lectin homologue jacalin solely up-regulated TLR4, 2-fold (2.1 ± 0.58 S.E., p < 0.005) over control on MΦ of C57BL/6 mice but failed to do so of C3H/HeJ MΦ, indicating that the lectin is a ligand with specificity for TLR4 (Fig. 5). Jacalin did not express TLR2, CD86, and CD40 in both of the mice strains. Truncated VCC⁶⁰ (lacking the β-prism lectin domain) treated MΦ isolated from both C57BL/6 and C3H/HeJ mice showed 3.2-fold (3.17 ± 0.27 S.E., p < 0.005) and 1.8-fold (1.82 ± 0.1 S.E., p < 0.005) up-regulation of TLR2, respectively. The 50-kDa truncated protein expressed CD86, 2.3-fold (2.31 ± 0.01 S.E., p < 0.005) and 1.7-fold (1.68 ± 0.05 S.E., p < 0.05) and CD40, 2.6-fold (2.61 ± 0.31 S.E., p < 0.005) and 1.9-fold (1.94 ± 0.15 S.E., p < 0.005), more over control in C57BL/6 and C3H/HeJ mice, respectively, indicating dependence of the cytosol domain of full-length HlyA on TLR2 for executing its role in cell activation.

HlyA-induced Apoptosis of MΦ—Annexin V, which determines the number of cells undergoing apoptosis, and nuclear dye 7-amino actinomycin D, which ensures integrity of the cells, were simultaneously used in presence of HlyA monomer. The MΦ treated with HlyA for 3 h showed apoptosis of 87% (87.39 ± 0.82 S.E., p < 0.005) of the cells compared with 23% (22.88 ± 0.78 S.E.) of the untreated, and cells treated for 24 h with the PFT resulted in 88% (88.44 ± 1.07 S.E., p < 0.005) apoptotic cells compared with 23% (23.3 ± 1.73 S.E.) as control (Fig. 6A, left bars). MΦ apoptosis remained unaltered both in presence of neutralizing anti-TLR4 Ab (Fig. 6A, left bars) and in TLR4-hyporesponsive C3H/HeJ mice (Fig. 6A, right bars), indicating that apoptosis was a TLR4-independent event. HlyA-treated MΦ up-regulated caspase-9, an initiator caspase that participates in the mitochondrial pathway of apoptosis by 1.7-fold (1.72 ± 0.37 S.E., p < 0.05) (Fig. 6B). Caspase-9, the other initiator caspase involved in death receptor-induced apoptosis, was found to remain unaffected. Among the effector caspases, caspase-7 alone was found to be involved in HlyA-mediated apoptosis.

DISCUSSION

HlyA is released as an exotoxin in the bacterial milieu and therefore can be postulated to directly influence and regulate the cells of the mucosal immune system. In this work, we evaluated the monitoring skill of HlyA in dictating the fate of PerC MΦ, which could be induced to follow any one of the three pathways: that of activation, apoptosis, or anergy. The decision

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about which pathway is to be followed by the cell is determined by the interaction of the immunogen with the host cell. Our present study demonstrates an intriguing phenomenon, where *V. cholerae* HlyA induces simultaneous activation and apoptosis of Mφ (Fig. 7).

TLRs, known to detect PAMPs, are crucial for activation of innate immunity as well as connection of the innate with acquired immunity. Like several PFTs that have been recognized by TLR4 (14, 32), HlyA up-regulated the TLR from as early as 2 h on Mφ. Surprisingly, TLR4 expression disappeared between 8–12 h of HlyA treatment accompanied with the abolishment of activation molecule CD40 and co-stimulatory molecule CD86. To evaluate the status of the cells during this hiatus, we tested the inhibitory role of TOLLIP on HlyA mediated cell activity because it imparts suppression of TLR-induced cell activation directly associated with TLR2 and TLR4 (20, 33). An interesting finding of this study has been the induction of the adaptor protein TOLLIP following TLR4 that went missing when the TLRs again showed up, a mechanism developed by the cell to tackle the toxin effects even more efficiently. Thus, we show feedback regulation of TLR signaling. Although the specificities of TLR2 and TLR4 for PAMPs are absolutely unrelated, TLR2 was unexpectedly expressed from 20 h of HlyA treatment along with the reappearance of TLR4. The expression of TLR6 and TLR1, sometimes associated with TLR2, was assessed. TLR6 alone was found to be up-regulated in concurrence with TLR2. It has been shown that TLR4 up-regulation can induce the recruitment of TLR2 as a part of the signaling cascade in certain cells (31). Because HlyA induced TLR2 at a later stage, we investigated whether it was expressed as a result of TLR4-mediated downstream signaling. Anti-TLR4 Ab treatment of Mφ prior to addition of HlyA mildly inhibited TLR2 expression compared with cells given only HlyA. When TLR2 expression was analyzed in C3H/HeJ mice, which are TLR4-insensitive, it was up-regulated by HlyA confirming that TLR2 was independent of TLR4 signaling. Thus, the data point toward Mφ reliability on both TLR4 and -2 to detect the HlyA molecule on its entirety.

Next, we checked the HlyA-induced signaling downstream of TLR recognition. After ligand binding, TLRs dimerize and undergo conformational change required for the recruitment of downstream signaling molecules (11). Up-regulation of the TLRs by HlyA-treated Mφ led us to study its effect on the signal transduction molecules that are activated by the receptors. Recognition of HlyA by the TLRs was expected to stimulate the recruitment of MyD88, an intracellular Toll/Interleukin-1 receptor domain-containing adaptor that activates inflammatory pathways (11). Because HlyA induced the sequential up-regulation of TLR4 and TLR2, it was imperative to study which MyD88-TLR association drives the downstream signaling. It was found that only the MyD88-TLR4 association had a role in MyD88-mediated downstream signaling, which was further established by the absence of MyD88-TLR2 association in C3H/HeJ mice. Moreover, inhibition of the nuclear translocation of the p65 subunit of NF-κB by anti-TLR4 Ab validated HlyA-induced TLR4-mediated cell activation. Expression of activation molecule CD40, co-stimulatory molecule CD86, and MHC class II (I-Ab) showed the ability of HlyA to activate Mφ. Interestingly, in the presence of TLR4, although HlyA-induced CD86 at 2 h postincubation prior to the MHC class II molecule, the co-stimulatory molecule could not be traced at later period. Preincubation of Mφ with anti-TLR4 Ab before HlyA addition revealed that CD86 expression was indeed TLR4-dependent, more so because it could not be detected on C3H/HeJ Mφ. In contrast, CD40 appeared twice, first with TLR4 at 4 h of culture and next along with TLR2, -4, and -6 at 24 h. The CD40 expression, which remained unaltered in the early stages, was substantially inhibited by anti-TLR4 Ab and strongly by anti-TLR2 mAb or both Abs together at later stages of culture. Unlike CD86, CD40 was up-regulated in C3H/HeJ, which could be strongly inhibited by anti-TLR2 mAb. These data reveal that HlyA-induced expression of CD86 and CD40 are two separate events independently regulated by TLR4 and TLR2, which together cater to Mφ activation. Because PFTs are known to cause pronounced cell damage, unaltered expression of CD40 and CD86 in presence of HlyA plus pan-caspase inhibitors.

**FIGURE 5.** Jacalin- and VCC50-induced expression of TLRs, co-stimulatory and activation molecules. Mφ obtained from C3H/HeJ and C57BL/6 mice were cultured with (black line) and without (shaded) jacalin or VCC50. Up-regulation of TLR4, TLR2, CD86, and CD40 were analyzed by flow cytometry.
shows cell activation is an event independent of apoptosis supported by the depleted presence of anti-apoptotic Bcl-xL. Besides expression of the two cell link molecules CD40 and CD86 on MΦ, HlyA triggered cells to release IL-12 and TNF-α instead of IL-10, thus pointing toward HlyA-induced M1 polarization. Interestingly, unlike expression of the activation molecules where both TLR2 and TLR4 had important roles to play, preincubation with anti-TLR2 or anti-TLR4 Ab showed that both IL-12 and TNF-α release was solely dependent on TLR4. Inability of HlyA to induce the release of the two cytokines by MΦ of C3H/HeJ mice conclusively shows that cytokine activity is TLR4-dependent.

The full-length 65-kDa monomeric HlyA can undergo proteolytic deletion giving rise to the 50-kDa truncated protein (VCC50) without the β-prism lectin domain. Thus, up-regulation of only TLR2 by VCC50 from early hours of culture, otherwise detected at later hours with the full-length HlyA, unequivocally demonstrates TLR2 expression is under the purview of the cytolytic protein domain, supported by VCC50-induced up-regulation of the TLR on C3H/HeJ MΦ. Induction of CD86 and CD40 by VCC50 on the cells of both C3H/HeJ and C57BL/6 mice shows the direct role of the domain in cell activation and likely parallels the reappearance of CD40 at later stages in the presence of TLR2 and -6 when cells are cultured with full-
length HlyA. In contrast, the plant lectin jacalin, which is homologous to the β-prism lectin domain of HlyA induced TLR4 on MΦ of C57BL/6 mice within 2 h of culture. The inability of jacalin to express TLR4 on MΦ of C3H/HeJ mice shows the lectin to be a definite TLR4 inducer and strongly suggests TLR4 up-regulation is the property linked to HlyA/β-prism lectin domain when the cells are cultured with full-length monomer. Thus, our work shows MΦ take a prudent approach to thwart HlyA of *V. cholerae* by harnessing TLRs that not only recognize the dissimilar domains of HlyA but precisely signal in tandem for the cells to respond.

Interestingly, this work demonstrates that apart from getting activated, MΦ were driven to simultaneous apoptosis by HlyA. The PFT-induced caspase-9- and caspase-7-mediated apoptosis of ~88% of the treated MΦ in as early as 3 h of culture, showing the involvement of the mitochondrial or intrinsic
pathway of cell death. These data fall in line with an earlier finding of our laboratory where 90% of the B-1a cells underwent apoptosis (27). Signaling through TLRs, besides activating gene expression employing NF-κB, can trigger an apoptotic response in eukaryotic cells as evidenced by TLR4-dependent apoptosis by pneumolysin (34). Preincubation of MΦ with anti-TLR4 Ab could not block apoptosis. In C3H/HeJ mice, the percentage of apoptotic MΦ remained similar to that of C57BL/6, confirming apoptosis as a TLR4-independent event. Moreover, TLR involvement in apoptosis requires blocking of NF-κB activation (35). Although blocking of NF-κB by anti–TLR4 Ab showed HlyA-induced TLR4-dependent expression of NF-κB (Fig. 2C), it failed to prevent apoptosis (Fig. 6A). These observations emphasized that HlyA-mediated apoptosis is TLR-independent. Apoptosis can be initiated through two pathways: the extrinsic pathway and the intrinsic pathway. The extrinsic pathway involves an extracellular receptor belonging to the TNF receptor family, which carries a death domain. The extrinsic transduction initiated by this death domain ultimately culminates in the activation of caspase-8. The TLR-mediated apoptosis takes place through this extrinsic pathway. HlyA is not only unable to up-regulate caspase-8 but also does not involve TLRs in its apoptogenic activity. It recruits caspase-9 as the executor enzyme of the apoptotic process. Thus, it can be said that HlyA-mediated cell death takes place through the mitochondrial or intrinsic pathway. Immunoblot analysis of caspase-6 and caspase-7 showed overexpression of caspase-7 in HlyA-treated MΦ. This refers to apoptosis, which is initiated by caspase-9 and terminates through caspase-7, leaving caspase-3 unaffected; therefore, a caspase-3-independent, caspase-7-mediated cellular apoptosis is observed (36).

This work sheds new light on how MΦ undergoing HlyA-induced apoptosis, a phenomenon carried out by most PFTs, tackle in parallel the situation for survival by selecting a panel of varying TLRs responsible toward two major PAMPs: a carbohydrate binding β-prism lectin with affinity for TLR4 and a cytolytic protein that engages TLR2 and -6. The promiscuous TLR association of MΦ is maneuvered by TOLLIP for continuance of cell activation with display of surface molecules and cytokines. In conclusion, the current study identifies the mechanism by which a single molecule/ligand stimulates multiple TLRs to turn some cells toward activation while handing out death to the rest.

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