MyD88-dependent TLR4 signaling is selectively impaired in alveolar macrophages from asymptomatic HIV+ persons

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Running head: HIV-1 targets TLR4-mediated MyD88-dependent signaling pathway

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ABSTRACT:

Alveolar macrophages (AM) are the predominant effector cell in the lungs and contribute to a critical first line of defense against bacterial pathogens via recognition by pattern recognition receptor (PRR) such TLR4. Recognizing that TLR4 can signal through MyD88-dependent and MyD88-independent pathways, whether HIV impairs one or both TLR4 signaling pathways in human macrophages is not known. Comparing human U937 macrophages with human HIV+ U1 macrophages (HIV-infected U937 subclone), the current study demonstrates that HIV infection is associated with impaired human macrophage TLR4-mediated signaling, specifically targeting the MyD88-dependent TLR4-mediated signaling pathway (reduced MyD88-IRAK interaction, reduced IRAK phosphorylation, reduced NF-kB nuclear translocation, and reduced TNFα release) while preserving the MyD88-independent TL4-mediated signaling pathway (preserved STAT1 phosphorylation, preserved IRF nuclear translocation, and preserved IL-10 and RANTES release). Extracellular TLR4 signaling complex was intact (similar levels of CD14 and MD2), and similar patterns of response were observed in clinically relevant alveolar macrophages from healthy and asymptomatic HIV+ persons at high clinical risk for pneumonia. Taken together, these data support the concept that chronic HIV infection is associated with specific and targeted disruption of critical macrophage innate immune TLR4 signaling, which in turn may contribute to susceptibility and disease pathogenesis of bacterial pneumonia.
INTRODUCTION:

Bacterial pneumonia remain a frequent and serious complication in asymptomatic HIV+ persons, despite relatively preserved peripheral blood CD4+ T-lymphocyte counts 1, use of HAART with undetectable plasma viral loads 2. These individuals have up to 25-fold greater risk of bacterial pneumonia compared to their healthy cohorts 3. However, the mechanism contributing to this increased risk is not well understood. Toll-like receptor 4 (TLR4) represents a critical pattern recognition receptor in the innate immune host cell response to bacterial infection. Functional deficiency or genetic deletion of TLR 4 increases susceptibility to H. Influenza, Streptococcus pneumoniae, and K. pneumoniae respiratory tract infection in murine models 4,5. Another indication of its importance is that TLR4 polymorphisms are associated with increased susceptibility to lung infection 6. Our lab recently reported impaired TLR4-mediated TNFα release in alveolar macrophages from asymptomatic HIV+ persons at increased clinical risk for bacterial pneumonia 7. Increased susceptibility may be in part related to reduced alveolar macrophage ERK1/2 MAP kinase phosphorylation attributed to elevated MKP-1 activity 7, and constitutive PI3K activation and heightened PI3K signaling in response to TLR4 activation 8. These data suggest that the host cell proinflammatory cytokine may be suboptimal in the lungs of HIV+ persons and may in part contribute to increased bacterial pneumonia susceptibility and pathogenesis.

TLR4 is the most studied of the TLR family of innate receptors 9. It is a unique member of the TLR family of mammalian receptors in that TLR4 is capable of both adaptor molecule MyD88-dependent and MyD88-independent signaling 10-12. TLR4-mediated
MyD88-dependent recognition of bacterial cell wall occurs at the cell surface\textsuperscript{11,13}, does not require receptor internalization\textsuperscript{11}, involves IRAK phosphorylation, and activation of TRAF6, NF-kB and MAP kinases (such as ERK1/2, p38 and JNK), with the subsequent release of proinflammatory cytokines such as TNF\textalpha\textsuperscript{14} that may contribute to an effective host defense response to bacteria. In contrast, TLR4-mediated MyD88-independent signaling requires receptor internalization and is mediated through TRIF\textsuperscript{11}, involves activation of IRF3 and STAT1\textsuperscript{15} with release of type-1 IFNs\textsuperscript{16}, IL-10\textsuperscript{15}, RANTES\textsuperscript{15} and may be involved in antiviral host defense\textsuperscript{17}. Recent work in our laboratory demonstrated impaired TLR4-mediated signaling response in alveolar macrophages from asymptomatic HIV+ persons at high clinical risk for bacterial pneumonia\textsuperscript{7,8}. However, whether both MyD88-dependent and MyD88-independent pathways are impaired by HIV infection is not known. Using human macrophage cell lines and clinically relevant human alveolar macrophages, the purpose of this study was to further examine the influence of HIV infection on TLR4-mediated intracellular signaling in greater detail, focusing on MyD88-dependent and MyD88-independent TLR4 signaling pathways.

**Materials and Methods:**

**Study Subjects.** Recruited healthy and asymptomatic HIV+ subjects showed no evidence of active pulmonary disease and with normal spirometry. Healthy individuals were confirmed to be HIV-negative by ELISA (Abbot Diagnostics, North Chicago, IL) and with no known risk factors for HIV infection. Demographic characteristics for all
participants were recorded on standardized forms and included age, gender, smoking status, HIV risk factor, medical history, and prescribed antiretroviral medications.

Reagents. Dynasore, lipid A (the biologically active component of LPS, and specific TLR4 ligand) from *E. coli* F583 Rd mutant, protease inhibitor cocktail, and phorbol myristic acid (PMA), were purchased from Sigma Chemical Company (St Louis, MO).

Antibodies. Anti-phospho-IRAK, anti-MyD88, anti-IkB, anti-IRF3, anti-phospho Stat1, anti-Stat1, anti-ERK1/2, anti-p65 antibodies were purchased from Cell Signaling (Danvers, MA) cytokine ELISA kits were from R & D (McKinley Place, Minneapolis, MN, anti-β-actin antibody from Sigma Chemical (St. Louis, MO). Neutralizing anti-human TLR4 antibody was purchased from eBioscience (San Diego, CA). Purified mouse IgG was used as isotype control (Pharmingen, Franklin Lakes, NJ).

Human macrophage cell lines. U937 cells are human promonocytic cells (American Tissue and Cell Company, ATTC). U1 cells (HIV-infected subclone of U937 cells, AIDS Research and Reference Reagent Program; Bethesda, MD) contain two integrated copies of HIV-1 proviral DNA, and characterized by low levels of constitutive virus expression that can be modulated by cytokines and pharmacological agents. For experiments, U937 and U1 cells were harvested during exponential growth phase, washed and then incubated in complete medium (RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 100 U/ml Penicillin and 100µg/ml Streptomycin. To allow differentiation to macrophages, U937 and U1 cells were incubated with 100 nM PMA at 37°C in a humidified atmosphere containing 5% CO₂ for 5-Tachado
24 hours. Adherent cells were then washed 3x with PBS (to remove PMA), and incubated in complete media (without PMA) for an additional 24 hours prior to use in experiments.

**Human alveolar macrophages.** Select experiments using human alveolar macrophages were carried out to determine clinical relevance of the study. Recruited healthy and asymptomatic HIV+ individuals were without evidence for active pulmonary disease and had normal spirometry. Healthy individuals were confirmed to be HIV seronegative by ELISA and had no known risk factors for HIV infection. For the HIV+ subjects, peripheral blood CD4 lymphocyte counts were >200 cells/mm³, HIV risk factors included IVDU and homosexual exposures, all were prescribed highly active antiretroviral therapy (HAART), all had undetectable serum viral load (<50 HIV-1 RNA copies/ml) and none experienced a prior opportunistic pneumonia. Using standard techniques, bronchoalveolar lavage (BAL) was performed to obtain lung immune cells. All procedures were performed on consenting adults in accordance with the Declaration of Helsinki, following protocols approved by Beth Israel Deaconess Medical Center institutional review board and Committee for Clinical Investigations. The cells were separated from the pooled BAL fluid and AM isolated as described recently.

**Western blot analyses.** Human macrophages were allowed to adhere and treated with indicated dose of lipid A, washed 2x with ice-cold PBS (pH 7.4). Cells were lysed in lysis buffer containing 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail (Sigma Chemicals, St. Louis, MO), placed on ice for 20 mins. Cells were harvested by scraping, followed by
centrifugation at 4°C for 15 mins at 14,000 rpm. Equal amounts of cell lysates were subjected to SDS/PAGE and Western blot analysis with designated antibodies and detected by enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Piscataway, NJ). Resolved bands were quantified by densitometry (Amersham Biosciences, Piscataway, NJ).

**Immunoprecipitation:** Immunoprecipitation studies were carried out as described. Briefly, equivalent amount of proteins were precleared by incubation with protein A/G sepharose (Amersham Biosciences) for 1hr at 4°C. The supernatant was collected after a brief centrifugation. Antibody was then added to the samples and incubated overnight at 4°C. The immune complexes were precipitated with 50μl of protein A/G Sepharose (50% suspension) and incubated for 1hr at 4°C. Non-specific bound proteins were removed by washing the Sepharose beads three times with modified radioimmune precipitation assay buffer and once with 1 x PBS. The immune complexes bound to the beads were solubilized in 40μl of 2x Laemli loading buffer and further analyzed by western blotting as described above.

**Targeted gene silencing (RNAi) in macrophages.** To determine the functional relevance of MyD88-independent pathway in TLR4 signaling, targeted gene silencing of macrophage TRIF and IRF3 were performed using synthetic duplex RNA oligonucleotides. We used On-Target plus smart pool siRNA TRIF (catalog # L-012833-00, Dhharmacon, Inc. Boulder, CO). Target sequences were GGAGCCACAUGUCAUUUGG, CCAUAGACCACUCAGCUUU, GGACGAACACUCCAGAU, and CCACUGGCCUCCUGUA. In addition, we also
used On-Target plus smart pool siRNA IRF3 (catalog # L-006875-00-0010, Dharmacon). The target sequences were CGAGGCCACUGGUGCAUAU, CCAGACACCUCUCGGGACA, GGAGUGAUGAGCUACGUGA, and AGACAUUCUGGAUGAGUUA. On-target plus non-targeting siRNA were used as controls (catalog # D-001810-01-05, Dharmacon). The target sequence was UGGUUUAUCAUGUCGACUAA. Macrophages were electroporated with 100nM siRNA using Amaxa system following manufacturer’s protocol (Amaxa GmbH, Cologne, Germany). TRIF and IRF3 siRNA-mediated knockdown was determined by Western blot probed with anti-TRIF or anti-IRF3 antibody 24-48hrs after transfection.

For MyD88 targeted gene silencing undifferentiated human monocytic U937 cells were transfected with a shRNA vector specific for human MyD88 (Open Biosystems; Genebank accession number NM_002468). Plasmid vectors (pSM2) for catalogue numbers 1) RHS1764-9690114 and 2) RHS1764-9694563 corresponded to following shRNA sequences:

5'TGCTGTTGACAGTGAGCGGACCCTAAATCCAATAGAAATAGTGAAGCCACAGATGTATTTCATATTGGATTTAGGGTCCTTGCCTACTGCCTCGGA-3'
5'TGCTGTTGACAGTGAGCGACCTAACCATGTCCCTGAACAATAGTGAAGCCACAGATGTATTGTTCAGGGACATGGTTAGGCTGCCTACTGCCTCGGA-3'.

Bacteria transformed with each vector where grown in chloramphenicol-containing (25μg/ml) LB-broth and plasmids were isolated using PureLink HiPure Plasmid Maxiprep (Invitrogen) according to manufacturer’s protocol. In addition, a vector containing a scrambled shRNA sequence was also cloned and isolated. Plasmid purity was confirmed using restriction enzyme analysis as recommended by manufacturer. Plasmid vectors were
transfected into undifferentiated U937 cells using the Lipofectamine LTX and Plus reagents according to manufacturer’s protocols. Cells were allowed to grow for 72 hours after transfection and then selected for stable transfection using puromycin (0.4μg/ml) (Invivogen). Cells were differentiated with PMA (100nM) for 24 hours before assay for MyD88 expression and compared to cells transfected with a scrambled shRNA vector. U937 cells transfected with vector #1 showed best knockdown and were used for subsequent experiments

Flow cytometry analysis. Human AM CD14 receptor surface expression was analyzed in BAL cell suspension specimens with Epics XL flow cytometer (Beckman/Coulter, Miami, FL) with laser power of 5.76 mW. The instrument was calibrated before each measurement with standardized fluorescent particles (Immunocheck; AMAC, Inc. Westbrook, ME). Fluorescent signals of the cells were measured simultaneously by 3 photomultiplier tubes and optical filters and shown as the mean of the log fluorescence intensity of the cell population within gate. AM were labeled with primary phycoerythrin (PE)-conjugated antibody to CD14 receptor (Beckman-Coulter) for 1h at 4°C in the dark, washed twice with balanced salt solution with calcium and magnesium in 0.1% bovine serum albumin, fixed with 250 µl of Optilyse C buffer (Beckman/Coulter) diluted with PBS, and analyzed by flow cytometry. AM were first identified by the characteristic forward and side scatter parameters on unstained AM, and the population was confirmed by staining with PE-conjugated primary anti-human HLA-DR (Beckman/Coulter, Miami, FL). Results were expressed as a mean relative fluorescence units (RFU) and the percentage of cells staining positive. Isotype primary
conjugated antibodies served as a negative control. Samples were prepared and analyzed in duplicate, and a minimum of 5,000 cells was counted for each sample.

ELISA. Cells were stimulated for 24hrs and cultured supernatants were collected, centrifuged to remove cellular debris, and assayed immediately or stored at –80°C until assayed. Cytokine measurements were performed using commercially available ELISA (R&D Systems, Minneapolis, MN) following manufacturer’s instructions, and absorbance measured at 450 nm on a Bio kinetic Elisa reader (Bio-Tek Instruments; Winooski, VT). The detection limit for TNF-α was 4.4 pg/ml. All measurements were performed in duplicate, and mean values of the two measurements were used for statistical analysis.

Statistical Analysis. Group comparisons were performed using Student’s t-test (two sample test) or one-way ANOVA. Calculations were performed with StatView (SAS Institute, Inc; Cary, NC) and INSTAT2 (GraphPad Software, San Diego, CA) software package. Results are given as mean ± SEM. Statistical significance was accepted for p<0.05.

Results:

Reduced TLR4-mediated TNFα release associated with impaired MyD88-dependent signaling pathway in HIV+ human macrophages. In general, ligation of mammalian TLRs (with the exception of TLR3) promotes activation of a MyD88-dependent intracellular signal transduction pathway that includes IL-1R associated kinase-1 (IRAK-1), nuclear translocation of NF-κB, and release of cytokines such as
TNFα. Reduced TNFα release from LPS-tolerant cells is associated with impaired MyD88-IRAK interaction, reduced IRAK expression and reduced NF-κB activation. Previously we demonstrated reduced lipid A-mediated TNFα release in HIV+ human macrophages, although whether the MyD88-dependent TLR4 signaling pathway is intact was not determined. To further examine the influence of HIV on the MyD88-dependent TLR4 signaling pathway, human U937 macrophages demonstrated a robust release of TNFα in response to TLR4 ligand challenge (lipid A, 10 µg/ml), and macrophage TNFα release was significantly reduced in the presence of anti-TLR4 neutralizing antibody (Fig. 1A, left panel). Similarly, TNFα release by human HIV+ U1 macrophages was also significantly reduced in the presence of anti-TLR4 neutralizing antibody (Fig. 1A, right panel), although overall the levels of TNFα release were significantly lower compared to human U937 macrophages, consistent with our previous observations.

Next, to determine whether downstream MyD88-dependent TLR4-mediated signaling molecules were impaired, clinically relevant human alveolar macrophages from healthy and asymptomatic HIV+ persons at risk for bacterial pneumonia were incubated with TLR4 ligand (lipid A, 10 µg/ml), and then detergent soluble cellular extracts were immunoprecipitated with anti-MyD88 antibody and immunoblotted with anti-p-IRAK antibody. Experiments demonstrate evidence for MyD88-IRAK interaction in alveolar macrophages from both groups by Western blot (Fig. 1B). Although the pattern for IRAK phosphorylation was similar for both groups, quantitative densitometric analysis demonstrated marked reduction in the level of IRAK phosphorylation in alveolar macrophage from HIV+ persons at all time points examined (Fig. 1B, right panel).
compared to healthy individuals (Fig. 1B, left panel). This data suggest that the activity of IRAK in HIV+ macrophages is diminished in response to TLR4 activation and is in agreement with previous report\textsuperscript{24}. Recognizing the importance of NF-kB in TLR-mediated signaling pathway\textsuperscript{25}, human U937 and U1 macrophages were incubated with lipid A (10 μg/ml) up to 120 minutes, nuclear and cytoplasmic extract isolated and probed by Western blot with antibody directed against the NF-kB p65 component. Quantitative densitometric analysis demonstrated marked reduction of p65 nuclear translocation in HIV+ U1 macrophages compared to U937 macrophages (Fig. 1C). To formally demonstrate whether MyD88 mediates TLR4 driven TNF\textgreek{a} release, functional assays were carried out using targeted functional gene silencing of MyD88. A reduced MyD88 protein level was demonstrated in U937 macrophages after MyD88 silencing by 70-80% (Fig. 1D left panel). TLR4-mediated TNF\textgreek{a} release was robust in U937 with scrambled shRNA, whereas in MyD88 silenced cells, TLR4-mediated TNF\textgreek{a} release was markedly diminished (Fig. 1D, right panel), suggesting that TLR4-mediated TNF\textgreek{a} release in human macrophages is predominantly MyD88-dependent. Taken together, these data demonstrate that human macrophage TNF\textgreek{a} release is in part TLR4-dependent, MyD88-dependent and that reduced TLR4-mediated TNF\textgreek{a} release in HIV+ macrophages is associated with impaired MyD88-dependent signaling (as determined by reduced MyD88-IRAK interaction, reduced IRAK phosphorylation, and reduced NF-kB p65 nuclear translocation).

**Preserved TLR4-mediated MyD88-independent pathway in HIV+ human macrophages.** Next, to examine human macrophage TLR4-mediated MyD88-
independent signaling pathway, studies focused on prototypic markers of the MyD88-independent signaling pathway, RANTES and IL-10 \(^{26-29}\). Similar to the pattern of TNF\(\alpha\) release in human macrophages described above, macrophage IL-10 release was significantly reduced in the presence of anti-TLR4 neutralizing antibody in both human U937 and HIV+ U1 macrophages in response to the TLR4 ligand lipid A suggesting that IL-10 release is mediated by TLR4 signaling pathway (Fig. 2A). However, in contrast to TLR4-mediated TNF\(\alpha\) release, release of IL-10 (Fig 2B) and RANTES (Fig. 2C) were similar in magnitude in both U937 and HIV+ U1 human macrophages as measured by ELISA suggesting that TLR4-mediated MyD88-independent pathway is preserved in HIV+ macrophages. Furthermore, targeted gene silencing of MyD88 did not influence IL-10 or RANTES release in response to lipid A (Fig. 2D-E), suggesting that in contrast to TLR4-mediated TNF\(\alpha\) release (which is MyD88-dependent as above), TLR4-mediated IL-10 and RANTES release is predominantly MyD88-independent in human macrophages. The pattern of IL-10 release was confirmed in clinically relevant human alveolar macrophages, demonstrating similar levels of lipid A-mediated IL-10 release comparing alveolar macrophages from healthy individuals to alveolar macrophages from asymptomatic HIV+ persons (Fig. 2F). The observed differences in TLR4-mediated TNF\(\alpha\) and IL-10 release were not related to differences in macrophage expression of essential TLR4 modulator molecule MD2 \(^{30}\) (Fig. 2G) or surface expression of TLR4 co-receptor CD14 (Fig. 2H), consistent with our prior studies demonstrating similar TLR4 surface expression comparing 937 and HIV+ U1 macrophages \(^{7,31}\) and comparing alveolar macrophages from healthy and HIV+ persons \(^{8}\). In contrast to impaired TLR4-mediated MyD88-dependent release of TNF\(\alpha\),
collectively these data demonstrate that TLR4-mediated IL-10 and RANTES release in human macrophages is predominantly MyD88-independent, and suggest that HIV may specifically target the MyD88-dependent TLR4 signaling pathway, while preserving the MyD88-independent TLR4 signaling pathway.

**TLR4-mediated IRF3 nuclear translocation and Stat-1 phosphorylation is preserved in HIV+ macrophages.** To further examine the influence of HIV on TLR4-mediated MyD88-independent signaling pathway, studies focused on the activation of IRF3 and Stat1, important signaling molecules of the MyD88-independent pathway \(^{26,27}\). Human U937 and HIV+ U1 macrophages were incubated with lipid A (10μg/ml) up to 240 minutes, and then nuclear and cytoplasmic extracts isolated and probed with specific anti-IRF3 antibody by Western blot. Experiments revealed limited constitutive IRF3 nuclear translocation in unstimulated U937 macrophages, followed by nuclear translocation within 15 minutes in response to Lipid A, peaking over 30-120 minutes followed by gradual decline by 240 minutes (Fig. 3A). In comparison, HIV+ U1 macrophages demonstrated elevated constitutive IRF3 nuclear translocation in unstimulated macrophages, with further increased levels of IRF3 nuclear translocation in response to lipid A that continued to increase up to 240 minutes (Fig. 3B). Recognizing that TLR4-mediated MyD88-independent signaling through TRIF and IRF3 requires STAT-1 transcription factors \(^{32}\), we studied STAT-1 phosphorylation in untreated or lipid A treated U937 and HIV+ U1 cells. As shown in Fig 3C, time kinetics for Stat1 phosphorylation demonstrated low constitutive STAT-1 phosphorylation in unstimulated human U937 macrophages, but increased by 15 minutes in response to
lipid A. In comparison, HIV+ U1 macrophages demonstrated elevated constitutive STAT-1 phosphorylation, with further marked increased phosphorylation in response to lipid A (Fig. 3C). Taken together, in contrast to impaired TLR4-mediated MyD88-dependent signaling, these data suggest a preserved TLR4-mediated MyD88-independent signaling pathway in HIV+ macrophages. In the context of HIV infection, divergent regulation of MyD88-dependent and MyD88–independent TLR4 signaling pathways could underlie the differential gene expression and host cell responses in macrophages.

**Targeted gene silencing of MyD88-independent signaling pathway molecules**

**TRIF or IRF3 in human macrophages reduces TLR4-mediated RANTES release (but not TNFα release).** TLR4-mediated MyD88-independent signal transduction is mediated through TRIF. To determine whether TRIF regulates TLR4-mediated signaling in human macrophages, release of RANTES and TNFα was examined in the presence and absence of targeted functional gene silencing of TRIF using RNAi methodology. In human U937 and HIV+U1 macrophages, TRIF protein was constitutively expressed in the presence of non-silencing RNAi, but TRIF protein levels are markedly reduced with TRIF-targeted gene silencing (Fig. 4A). Furthermore, targeted gene silencing of TRIF was associated with reduction in lipid A-mediated RANTES release in both U937 macrophages (Fig. 4B) and HIV+ U1 macrophages (Fig. 4C). In contrast, lipid A-mediated TNFα release was not influenced by targeted gene silencing of TRIF, demonstrating specificity of MyD88-independent pathway (Fig. 4D).
To further investigate the MyD88-independent TLR4-mediated macrophage release of RANTES and TNFα, experiments were performed in the presence or absence of targeted gene silencing of IRF3, transcription factor downstream of TRIF signaling. In human U937 and HIV+ U1 macrophages, IRF3 protein was constitutively expressed in the presence of non-silencing RNAi, but IRF3 protein levels are markedly reduced following IRF3-targeted gene silencing (Fig. 4E). Furthermore, targeted gene silencing of IRF3 was associated with reduction in lipid A-mediated RANTES release in both U937 macrophages (Fig. 4F) and HIV+ U1 macrophages (Fig. 4G). In contrast, lipid A-mediated TNFα release was not influenced by targeted gene silencing of IRF3 (Fig. 4H), suggesting that TLR4-mediated RANTES and IL-10 release by human macrophages is predominantly MyD88-independent. Taken together, these data suggest that TLR4-mediated release of RANTES and IL-10 in human macrophages is mediated through a MyD88-independent process involving TRIF and IRF3, whereas TLR4-mediated TNFα release in human macrophages is mediated through MyD88-dependent process and does not involve TRIF and IRF3 signaling molecules.

**TLR4-mediated macrophage release of IL-10 and RANTES (but not TNFα) and IRF3 phosphorylation (but not ERK1/2 phosphorylation) dependent on TLR4 endocytosis.** TLR4-mediated MyD88-independent signaling requires endocytosis, whereas TLR4-mediated MyD88-dependent signaling does not require endocytosis. We investigated the role of endocytosis in TLR4-mediated MyD88-dependent and MyD88-independent signaling pathways in human macrophages measuring macrophage IL-10, RANTES and TNFα release in response to lipid A in the presence
and absence of an inhibitor of GTPase dynamin (Dynasore). Inhibition of endocytosis (with Dynasore) was shown to inhibit TLR4-mediated MyD88-independent dynamin-mediated endocytosis. As a measure of TLR4-mediated MyD88-independent signaling, incubation of human U937 or HIV+ U1 macrophages with lipid A in the presence of Dynasore markedly reduced macrophage IL-10 release (Fig. 5A) and RANTES release (Fig. 5B). In contrast, as a measure of TLR4-mediated MyD88-dependent signaling, lipid A-mediated TNFα release was not influenced by dynasore (Fig. 5C). Furthermore, pretreatment of human U937 or HIV+ U1 macrophages with dynasore impaired lipid A -mediated IRF3 phosphorylation (MyD88-independent signaling molecule) but did not influence ERK1/2 phosphorylation (MyD88-dependent signaling molecule) (Fig. 5D). These data demonstrate that in human macrophages, TLR4-mediated MyD88-independent signaling requires endocytosis (in contrast to TLR4-mediated MyD88-dependent signaling), and is not influenced by HIV infection.

Discussion:

This study demonstrates that HIV infection is associated with impaired human macrophage TLR4-mediated signaling, specifically targeting the MyD88-dependent TLR4-mediated signaling pathway while preserving the function of MyD88-independent TLR4-mediated signaling pathway. The impaired function of MyD88-dependent pathway may in part contribute to reduce TNFα release in AM from HIV+ persons in response to lipid A. Compared to human U937 macrophages, MyD88-dependent TLR4-mediated signaling in HIV+ U1 human macrophages demonstrated impaired
MyD88-IRAK protein interaction, reduced IRAK phosphorylation, reduced NF-kB nuclear translocation, and reduced TNFα release in response to the TLR4 ligand stimulation. In marked contrast, MyD88-independent TLR4-mediated signaling in HIV+ U1 human macrophages demonstrated preserved STAT1 phosphorylation, preserved IRF3 nuclear translocation, and preserved IL-10 and RANTES release in response to TLR4 agonist stimulation compared to human U937 macrophages. Similar patterns of response were observed in select experiments using clinically relevant human alveolar macrophages from healthy and asymptomatic HIV+ persons. Taken together, these data support the concept that chronic HIV infection is associated with specific and targeted disruption of critical macrophage innate immune TLR4 signaling, which in turn may contribute to susceptibility and disease pathogenesis of bacterial pneumonia.

The finding in the current study that TLR4-mediated impairment of TNFα release was significantly reduced in HIV+ macrophages is consistent with our prior observations and observations by other investigators. TLR4 is unique among the TLR family, supporting both MyD88-dependent and MyD88-independent signaling pathways, whereas TLR3 exhibits MyD88-independent signaling and all other TLR exhibit MyD88-dependent signaling. Activation of MyD88-dependent signaling pathway promotes pro-inflammatory cytokine release such as TNFα and IL-12, while activation of MyD88-independent pathway promotes release of IFN-inducible genes such as type-1 interferon, IL-10 and RANTES. In the current study, demonstration that macrophage release of IL-10 and RANTES was significantly reduced following targeted gene silencing of macrophage IRF3 and TRIF (signaling components of MyD88-independent...
TLR4 signaling), and demonstration of intact IRF3 nuclear translocation and STAT1 phosphorylation suggest that the MyD88-independent signaling pathway represents that principal mechanism for IL-10 and RANTES release in HIV+ macrophages. Also, the finding in the current study that MyD88-independent TLR4-mediated signaling requires endocytosis whereas MyD88-dependent TLR4-mediated signaling does not require endocytosis is consistent with data from other investigators \(^{11}\) and extends the concept of distinct molecular mechanisms for TLR4-mediated signaling to human macrophages.

The current study provides an additional mechanism for the observed reduction in TLR4-mediated TNF\(\alpha\) release in HIV+ macrophages \(^{7,8,20}\). Our prior work demonstrated that the observed reduction in macrophage TLR4-mediated TNF\(\alpha\) release in HIV+ macrophages may in part be attributed to activation of macrophage cellular phosphatase MKP-1 \(^{7}\), and may in part be attributed to constitutive activation of PI3K pathway \(^{8}\). The current study extends the mechanisms to include impaired TLR4-mediated MyD88-dependent signaling pathway, suggesting that chronic HIV infection may influence macrophage innate immune signaling pathways at several molecular levels. Importantly, our prior observations that pharmacological agents can partially restore macrophage TLR4-mediated immune functions in alveolar macrophages from HIV+ persons \(^{7}\) suggest the potential for immunomodulation of macrophage innate immune function to restore host cell response bacterial challenge.

Recognizing that HIV infection of macrophages is generally non-productive\(^{36}\), the preserved or elevated levels of IRF3 and pSTAT1 in HIV+ macrophages may in part
represent antiviral mechanism to limit or control HIV replication in macrophages. IRF3 signaling in general is MyD88-independent and may involve TRAF3 self ubiquitination. A consequence of preserved or elevated IRF3 signaling to limit viral replication may be impaired MyD88-dependent signaling, although was not specifically established in the current study and represents an area of active investigation.

The mechanism for the select impairment of MyD88-dependent TLR4 signaling (while preserving MyD88-independent signaling) was not established in the current study. In the current study, the observed differences in TLR4-mediated signaling comparing healthy and HIV+ human macrophages was not related to differences in macrophage expression of the TLR4 signaling complex, as MD2 levels and surface expression of CD14 were similar. Our prior publications demonstrated similar levels of macrophage TLR4 surface expression and TLR4 mRNA, confirmed by other independent investigators. Taken together, these data suggest that the TLR4-MD2-CD14 signaling complex is intact in both alveolar macrophages from healthy individuals and alveolar macrophages from asymptomatic HIV+ persons, and suggests that impairment of signaling in HIV+ macrophages represents events downstream of the TLR4 receptor complex. Our prior investigations demonstrated that HIV nef protein is sufficient to induce macrophage MKP-1, activate PI3K, and impair TLR4-mediated TNFα release suggests that HIV nef protein may selectively influence MyD88-dependent TLR4-mediated signaling, although this mechanism was not specifically investigated in the current study. Our prior observation that HIV nef was sufficient to impair TLR4-mediated TNFα release while simultaneously promoting IL-10 release is
consistent with findings in the current study, suggesting targeted effects of MyD88-dependent TLR4-mediated signaling.

Other limitations of the current study include whether the findings are specific to TLR4 or whether MyD88-dependent signaling was affected in other macrophage TLRs was not investigated. Also, whether these findings apply in general to the other family members of the TIR-containing domains \(^{38}\) was not established. Finally, the \textit{in vitro} observations may not accurately reflect \textit{in vivo} function, but the use of clinically relevant human AM may allow for more direct translation of observed results to human disease.

This is the first study to report that MyD88-dependent signaling pathway is specifically targeted by HIV that resulted in diminished TLR4-mediated TNF\(\alpha\) release in response to lipid A. Importantly, the TLR4-mediated MyD88-independent pathway is preserved in HIV+ human macrophages leading to robust IL-10 and RANTES release in response to lipid A. Furthermore, it has been recently shown that HIV induces downregulation of IRAK-4 and inhibits TNF\(\alpha\) in response to TLR4 activation and supports the present study \(^{24}\). Taken together, these observations support the concept that HIV infection is associated with targeted and specific impairment of macrophage innate immune function rather than a global impairment of macrophage function.

In conclusion, this study demonstrates that the impaired TLR4-mediated macrophage response demonstrated in HIV-infected human macrophages is targeted to the MyD88-dependent signaling pathway, whereas the TLR4-mediated MyD88
independent signaling pathway is preserved. Recognizing that the MyD88-dependent signaling pathway is critical to the acute inflammatory response that promotes clearance of various pathogens \(^{39-42}\), impaired MyD88-dependent TLR4-mediated in alveolar macrophages from HIV+ persons may contribute to bacterial pneumonia pathogenesis. In general, this study supports the concept that chronic HIV infection is associated with altered macrophage innate immune function. Moreover, the influence of chronic HIV infection on macrophage innate immune function is targeted and specific, with evidence of impaired innate immune pathways (ex. MyD88-dependent TLR4 signaling) while other innate immune pathways remain preserved (ex. MyD88-independent signaling). Identifying specific abnormalities in lung macrophages from HIV+ persons may provide potential novel therapeutic targets aimed to restore or rescue innate immune function against potential pathogens and augment current antimicrobial therapy in HIV+ persons with pneumonia.

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Conflict of Interest Disclosure: The authors declare no competing financial interests.

References

Figure 1. Reduced TLR4-mediated TNFα release associated with impaired MyD88-dependent signaling pathway in HIV+ human macrophages. (A) Human macrophage TNFα release is mediated by TLR4. Human macrophage U937 and U1 cells were incubated with lipid A (10 µg/ml) in the presence or absence of neutralizing anti-TLR4 antibody (10µg/ml) for 24hrs and cell-free supernatants analyzed for TNFα by ELISA. Data shown are mean + SEM of four independent experiments done in triplicate. (*p< 0.01 compared to U937 with lipid A alone; ** p<0.05 compared to U1 with lipid A alone). (B) Reduced TLR4-mediated MyD88/IRAK interaction and IRAK phosphorylation in HIV+ AM. Healthy AM (B, left panel) and HIV+ AM (B, right panel) were incubated with lipid A (10 µg/ml) up to 60 minutes, detergent soluble extracts were immunoprecipitated with anti-MyD88 antibody and immunoblotted with anti-p-IRAK antibody. Densitometric analysis for each p-IRAK band is displayed beneath Western blot. Western blot is a representative of three independent experiments with similar results (n=3). (*p<0.01 unstimulated control; **p<0.01 compared to healthy in the presence of lipid A with time). (C) Reduced TLR4-mediated NF-κB nuclear translocation in HIV+ macrophages. U937 and U1 cells were incubated with lipid A (10µg/ml) up to 120 minutes, nuclear and cytoplasmic extract isolated and probed with anti-p65 antibody. Densitometric analysis of p65 bands for each lane is displayed beneath Western blot. Western blot is a representative of four independent experiments with similar results. (*p<0.01 compared to unstimulated control; **p<0.01 compared to U937 cells in the presence of lipid A with time). (D) Functional silencing of human MyD88
leads to marked diminution of TLR4-mediated TNF\(\alpha\) release in U937. Western blot analysis of human MyD88 after gene silencing using MyD88 shRNA and scrambled shRNA. \(\beta\)-actin was used to monitor protein loading after stripping the membrane. A representative blot shows results from one experiment with similar results of three independent experiments (left panel). U937 cells were pretreated with shRNA MyD88 and scrambled shRNA. Cells were differentiated with phorbol ester, challenged with lipid A and incubated for 24hrs. Cell-free supernatant was assayed for TNF\(\alpha\) by ELISA. Results are representative of three independent experiments in triplicate.

**Figure 2. Preserved TLR4-mediated MyD88-independent pathway in HIV+ human macrophages.** (A) Human macrophage IL-10 release is mediated by TLR4. U937 and U1 cells were incubated with lipid A (10\(\mu\)g/ml) in the presence or absence of neutralizing anti-TLR4 antibody (10\(\mu\)g/ml) for 24hrs and cell-free supernatants analyzed for IL-10 by ELISA. Data shown are mean + SEM of four independent experiments done in triplicate. (*p < 0.01 compared to U937 with lipid A alone; ** p<0.05 compared to U1 with lipid A alone). (B-C) Lipid A-mediated release of IL-10 and RANTES is preserved in HIV+ macrophages. U937 and U1 cells were incubated with or without lipid A (10 \(\mu\)g/ml) for 24hrs and cell-free supernatants were analyzed for IL-10 (B) or RANTES (C) by ELISA. Data shown are mean + SEM of four independent experiments done in triplicate. (D-E) Functional silencing of human MyD88 did not affect TLR4-mediated release of RANTES (D) and IL-10 (E). Cells were challenged with lipid A and incubated for 24hrs. Cell-free supernatant was assayed for RANTES and IL-10 by ELISA. Results are representative of three independent experiments done in triplicate.
Healthy and HIV+ AM demonstrate similar levels of IL-10 release (F), similar levels of the TLR4 adaptor molecule MD2 (J) and surface expression of TLR4 co-receptor CD14 (H). (F) Healthy AM and HIV+ AM were incubated with or without lipid A (10 μg/ml) for 24hrs and cell-free supernatants were analyzed for IL-10 by ELISA. Data shown are mean ± SEM from four independent experiments in triplicate. (n= 4 subjects for each group). (J) Total cell lysates from healthy AM and HIV+ AM were probed with specific anti-MD2 antibody by Western blot, with β-actin probed for protein loading. (H) Healthy AM and HIV+ AM were incubated with PE-conjugated anti-CD14 antibody or isotype control antibody and surface expression determined by flow cytometry. Representative blot and profiles were similar of three independent experiments (n=3 subjects for each group).

**Figure 3. TLR4-mediated IRF3 nuclear translocation and Stat-1 phosphorylation is preserved in HIV+ macrophages.** Lipid A-mediated nuclear translocation of IRF3 is intact comparing U937 human macrophages and HIV+ U1 cells. (A) U937 and (B) U1 macrophages were incubated with lipid A (10μg/ml) over time, and then nuclear and cytoplasmic extract isolated and probed with anti-IRF3 antibody by Western blot. Western blot is a representative of four independent experiments with similar results. (C) Preserved lipid A-mediated phosphorylation of STAT1 in HIV+ human macrophages. U937 and U1 macrophages were incubated with lipid A (10μg/ml) over time, and detergent-soluble cell extracts were probed with anti-phosphorylated Stat1 antibody by Western blot. Membranes were stripped and probed with anti-β-actin for protein loading. Western blot is a representative experiment of three independent
experiments with similar results. Quantitative densitometric analysis of IRF3 nuclear extract bands (A-B) and phosphorylated-STAT1 bands (C) are displayed directly beneath the blots.

**Figure 4. Targeted gene silencing of MyD88-independent signaling pathway molecules TRIF or IRF3 in human macrophages reduces TLR4-mediated RANTES release (but not TNF release).** RNAi-based targeted gene silencing of TRIF (A-D) or IRF3 (E-H) in human macrophages was performed as detailed in Methods. (A) Western blot analysis of human U937 and HIV+ U1 macrophages following pretreatment with RNAi targeted to TRIF (TRIF siRNA) or non-silencing RNAi (N.S siRNA), and probed with anti-TRIF antibody. Anti-β-actin antibody was used to monitor protein loading. The blot is representative of three independent experiments with similar results. (B-D) Human macrophage U937 and U1 cells, pretreated with either TRIF siRNA or non-silencing (NS) siRNA, were then incubated with or without lipid A (10 μg/ml) for 24hrs and cell-free supernatants were analyzed for RANTES (B-C) or TNFα (D) by ELISA. Data shown are mean ± SEM of three independent experiments done in triplicate with similar results. (E) Western blot analysis of human U937 and U1 macrophages following pretreatment with RNAi targeted to IRF3 (IRF3 siRNA) or non-silencing RNAi (N.S siRNA), and probed with anti-IRF3 antibody. Anti-β-actin antibody was used to monitor protein loading. The blot is representative of three independent experiments with similar results. (F-H) U937 and U1 cells, pretreated with either IRF3 siRNA or non-silencing (NS) siRNA, were then incubated with or without lipid A (10 μg/ml) for 24hrs and cell-free supernatants were analyzed for RANTES (F-G) or TNF.
(H) by ELISA. Data shown are mean + SEM of three independent experiments done in triplicate with similar results. (I-J) Functional silencing of human MyD88 preserved lipid A-mediated RANTES and IL-10 release. U937 cells were pretreated with shRNA MyD88 and scrambled shRNA. Cells were differentiated with phorbol ester, challenged with lipid A and incubated for 24hrs. Cell-free supernatant was assayed for RANTES (I) and IL-10 (J) by ELISA. Results are representative of three independent experiments in triplicate. (*p<0.05, **p<0.01 compared to unsilenced control in the presence of lipid A.

Figure 5. TLR4-mediated macrophage release of IL-10 and RANTES (but not TNFα) and IRF3 phosphorylation (but not ERK phosphorylation) dependent on TLR4 endocytosis. MyD88-independent TLR4-mediated IL-10 and RANTES release requires endocytosis. (A-C) U937 and U1 macrophages were pre-treated with a highly specific inhibitor of the endocytosis regulator dynamin GTPase (dynasore 50μM) for 1h and then incubated in the presence or absence of lipid A (10 μg/ml) for 24hrs, and cell-free supernatants analyzed for IL-10 (A), RANTES (B), or TNFα (C) by ELISA. Data reflect representative experiments (performed in triplicate) of three independent experiments with similar results. (*p<0.01 compared to lipid A alone). (D) MyD88-independent TLR4-mediated IRF3 phosphorylation IRF3 requires endocytosis. U937 and U1 macrophages were pre-treated with a highly specific dynamin GTPase inhibitor (dynasore 50μM) for 1h and then incubated in the presence or absence of lipid A (10 μg/ml) for 15 mins, and cell lysates probed with specific antibodies to phosphorylated ERK1/2 and phosphorylated IRF3 antibody. Protein loading controls utilized antibody to
total ERK1/2 and total IRF3 protein. Representative Western blot from one of three independent experiments with similar results.
Fig. 1

A. TNF-α (pg/ml)

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<th>U1</th>
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<tr>
<td>unstim</td>
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<td>Lipid A</td>
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B. P-IRAK volume (RU)

Healthy

IP: MyD88

Lipid A

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HIV+

IP: MyD88

Lipid A

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C. p65 volume (RU)

Lipid A 10μg/ml

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D. Scrambled MyD88 shRNA

MyD88

β-actin

Scrambled shRNA

MyD88 shRNA

TNF-α (pg/ml)

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<td>Lipid A + anti- TLR4</td>
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* p < 0.05, ** p < 0.01
Fig. 3

A. U937

IRF3

Nuclear extract

Cytoplasmic extract

Lipid A 0' 15' 30' 60' 120' 240'

IRF3 volume (RU)

B. U1

IRF3

Nuclear extract

Cytoplasmic extract

Lipid A 0' 15' 30' 60' 120' 240'

IRF3 volume (RU)

C. U937

Lipid A 0' 15' 30' 60'

U1

Lipid A 0' 15' 30' 60'

pStat-1

β-actin

pStat-1 volume (RU)
Fig. 4

A. For personal use only.

B. C. D.

E. N.S. siRNA TRIF siRNA

F. G. H.

N.S. siRNA IRF3 siRNA

U937 U1

β-actin

RANTES (pg/ml)

unstim Lipid A

TNF-α (pg/ml)

unstim Lipid A

IRF3

RANTES (pg/ml)

unstim Lipid A

U937

unstim Lipid A

RANTES (pg/ml)

unstim Lipid A

U937

unstim Lipid A

N.S. siRNA TRIF siRNA

N.S. siRNA IRF3 siRNA

U937 U1

β-actin

RANTES (pg/ml)

unstim Lipid A

TNF-α (pg/ml)

unstim Lipid A

IRF3

RANTES (pg/ml)

unstim Lipid A

U937

unstim Lipid A

RANTES (pg/ml)

unstim Lipid A

U937

unstim Lipid A

N.S. siRNA TRIF siRNA

N.S. siRNA IRF3 siRNA

U937 U1

β-actin

RANTES (pg/ml)

unstim Lipid A

TNF-α (pg/ml)

unstim Lipid A

IRF3

RANTES (pg/ml)

unstim Lipid A

U937

unstim Lipid A

RANTES (pg/ml)

unstim Lipid A

U937

unstim Lipid A

N.S. siRNA TRIF siRNA

N.S. siRNA IRF3 siRNA
Fig. 5

A. IL-10 (pg/ml)

B. RANTES (pg/ml)

C. TNF-α (pg/ml)

D. Western Blot

From U937 and U1 with and without Lipid A and Dynasore.
MyD88-dependent TLR4 signaling is selectively impaired in alveolar macrophages from asymptomatic HIV+ persons

Souvenir D. Tachado, Xin Li, Medhavi Bole, Katharine Swan, Asha Anandaiah, Naimish R. Patel and Henry Koziel