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Neisseria gonorrhoeae Enhances HIV-1 Infection of Primary Resting CD4+ T Cells through TLR2 Activation

Jian Ding,* Aprille Rapista,* Natalia Teleshova,*† Goar Mosoyan,* Gary A. Jarvis,‡§ Mary E. Klotman,* and Theresa L. Chang*

Sexually transmitted infections increase the likelihood of HIV-1 transmission. We investigated the effect of Neisseria gonorrhoeae (gonococcus [GC]) exposure on HIV replication in primary resting CD4+ T cells, a major HIV target cell during the early stage of sexual transmission of HIV. GC and TLR2 agonists, such as peptidylglycan (PGN), Pam3CSK4, and Pam3C-Lip, a GC-derived synthetic lipopeptide, but not TLR4 agonists including LPS or GC lipooligosaccharide enhanced HIV-1 infection of primary resting CD4+ T cells after viral entry. Pretreatment of CD4+ cells with PGN also promoted HIV infection. Anti-TLR2 Abs abolished the HIV enhancing effect of GC and Pam3C-Lip, indicating that GC-mediated enhancement of HIV infection of resting CD4+ T cells was through TLR2. IL-2 was required for TLR2–mediated HIV enhancement. PGN and GC induced cell surface expression of T cell activation markers and HIV coreceptors, CCR5 and CXCR4. The maximal postentry HIV enhancing effect was achieved when PGN was added immediately after viral exposure. Kinetic studies and analysis of HIV DNA products indicated that GC exposure and TLR2 activation enhanced HIV infection at the step of nuclear import. We conclude that GC enhanced HIV infection of primary resting CD4+ T cells through TLR2 activation, which both increased the susceptibility of primary CD4+ T cells to HIV infection as well as enhanced HIV-infected CD4+ T cells at the early stage of HIV life cycle after entry. This study provides a molecular mechanism by which nonulcerative sexually transmitted infections mediate enhancement of HIV infection and has implication for HIV prevention and therapeutics. The Journal of Immunology, 2010, 184: 2814–2824.

Sexual contact is the most common route of HIV-1 transmission. In addition to an estimated 33 million HIV-infected people worldwide in 2007, 2.7 million people have become newly infected (The Joint United Nations Programme on HIV/AIDS, report on the global HIV/AIDS epidemic 2008). In the United States, 56,300 adolescents and adults were newly infected with HIV-1 in 2006 (1). Sexually transmitted infections (STIs) are known to significantly increase the likelihood of HIV-1 transmission (2–5). STIs increase not only HIV-1 shedding in HIV-1–infected adults but also the susceptibility to HIV-1 infection in those who are HIV-1 negative (2–7). Although antibiotic chemoprophylaxis or treatment substantially decreases the incidence of STIs as well as HIV-1 shedding, it does not reduce the incidence of HIV-1 (6, 8). Understanding how STIs increase HIV transmission will provide insight into the development of new strategies to reduce the spread of HIV-1.

Gonorrhea is caused by Neisseria gonorrhoeae (gonococcus [GC]), a Gram-negative bacterium. The Centers for Disease Control and Prevention estimates that >700,000 persons in the United States contract new GC infections each year, with the highest reported rates of infection among sexually active teenagers, young adults, and African Americans. Several mechanisms of enhancement of HIV transmission by GC have been proposed. GC infection can enhance HIV transcription by activating HIV long-terminal repeat in transformed T cells (9). GC-infected women have more endocervical CD4+ T cells providing more targets for HIV (10). We have shown that antimicrobial peptides, such as human defensin 5 and 6, promote HIV infectivity in vitro and are induced by GC infection (11). In addition, GC infection enhances HIV infection in monocyte-derived dendritic cells (MDDCs) (12).

TLRs belong to the family of pattern recognition receptors (PRRs) and are important components of the innate immune system as the sensors of intruding pathogens (12, 13). TLR activation is known to modulate HIV infection or transmission in vitro and in vivo (15–23). Depending on specific TLR agonists and the target cells, TLR activation can either promote or inhibit HIV infection in vitro. Although TLR2 activation increases HIV infection in primary quiescent naive and memory CD4+ T cells and MDDC-mediated HIV transmission (12, 20, 21), TLR4 activation inhibits HIV infection in primary macrophages and MDDC-mediated HIV transmission (19, 20, 24). TLR4 ligands, such as LPS or lipooligosaccharide (LOS), inhibit HIV infection of primary macrophages through induction of type I IFN (19, 25), whereas LPS increases the susceptibility of monocytes and PBMCs to the HIV X4 virus (26, 27). Interestingly, TLR2, 7, and 9 agonists appear to promote HIV or SIV infection in vivo (18, 28), which may be due to immune activation (18, 23, 29). GC is known to induce proinflammatory cytokines through activation of TLR2 and TLR4 (30, 31), which may play a role in increased HIV transmission. Furthermore, TLR2 is involved in GC-mediated enhancement of HIV infection in MDDCs (12).

The earliest targets during heterosexual transmission of HIV/SIV are both activated and resting CD4+ T cells in the genital mucosa...
(32). However, the effect of GC exposure on HIV infection of primary CD4+ T cells has not been reported. In this study, we demonstrated that GC exposure increased HIV-1 infection of primary resting CD4+ T cells, and that TLR2 was involved in this HIV enhancing effect. Furthermore, a major component of TLR2-mediated enhancement of HIV infection of primary CD4+ T cells occurred at the step of nuclear import. These results offer an additional molecular mechanism of GC-mediated increased HIV transmission, which is important for the development of preventative strategies against HIV.

Materials and Methods

Reagents

Recombinant human IL-2 was purchased from R&D Systems (Minneapolis, MN). PHA, Escherichia coli O55:B5 LPS, and PGN were from Sigma-Aldrich (St. Louis, MO). The Pam3C-Lip (from EMC Microcollections, Tuebingen, Germany), based on the N-terminal sequence of the N. gonorrheae F62 Lip protein (sequence CGGKEAAEPAEAS), contains a tripalmitoyl-S-glyceryl-cystein in the N-terminus. The synthetic lipopeptide Pam3CPSK4 and muramyl peptides (MDP) were purchased from InvivoGen (San Diego, CA). L-73,198 was provided by Merck (Whitehouse Station, NJ); Zidovudine (AZT) was obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH. HIV-1Lai and HIV-1Bal were purchased from Advanced Biotechnologies (Columbia, MD).

Abs purchased from BD Biosciences (San Jose, CA) were PE-conjugated mouse anti-human CD25 (clone no. M-A251), FITC-conjugated mouse anti-human CCR5 (3A9), FITC-conjugated mouse IgG1, PE-conjugated mouse IgG1 (X40), and APC-conjugated mouse IgG2a (X59). Mouse anti-human TLR2 Ab (TL2.1) was purchased from eBioscience (San Diego, CA). FITC-conjugated mouse anti-human TLR2 Ab (TL2.1) was obtained from Imgenex (San Diego, CA).

Cell culture

PBMCs from normal healthy blood donors were isolated by Ficoll-Hypaque gradient centrifugation. CD4+ T cells were prepared from PBMCs by negative selection using CD4+ T cell isolation kit from Miltenyi Biotec (Auburn, CA). CD14+ Cells were positively selected from PBMCs by using CD14+ cell isolation kit (Miltenyi Biotec). The purity was >98% based on flow cytometry analysis. Freshly isolated CD4+ T cells were cultured in RPMI-1640 media with 10% FBS in the absence of IL-2 overnight at 37˚C before use. The cell surface levels of CD25 and CD69 decreased from ~33.8% and 13.9%, respectively, to 12.1% for CD25 and 1.1% for CD69 after overnight culture in the absence of IL-2. Cell cycle analysis by propidium iodide staining in the absence of IL-2. Cell cycle analysis by propidium iodide staining in the absence of IL-2

For HIV infection, primary CD4+ T cells were cultured in RPMI-1640 media supplemented with 10% FBS and 50 mM IL-2 unless otherwise described. To determine the effect of GC and various PRR ligands on HIV-1 infection after viral entry, primary CD4+ T cells (1 × 10⁶ per sample) were first infected with replication competent CXCR4 (X4)-using virus HIV-1Lai or CCR5 (R5)-using virus HIV-1Lai at multiplicity of infection (MOI) of 0.05 for 2 h. After washing off unbound viruses, infected cells were incubated with GC or TLR/ nucleotide-binding and oligomerization domain 2 (NOD2) ligands at 37˚C in the presence of IL-2. HIV production in culture supernatants was measured by p24 ELISA kit (SAIC-Frederick, Frederick, MD).

To examine HIV-1 infection of resting CD4+ T cells with pretreatment of PGN, CD4+ T cells were incubated with PGN (20 µg/ml) in the presence of IL-2 (200 IU/ml) at 37˚C for 3 d. Cells were washed with PBS and then exposed to HIV-1Lai or HIV-1Bal at MOI of 0.05 for 2 h. The infected cells were washed and then cultured at 37˚C in the presence of IL-2. HIV production in the culture supernatant was measured with ELISA for HIV-1p24.

For a single-cycle infection assay, replication-defective pseudotyped HIV-1LaiGFP luciferase reporter viruses were produced as described previously (34, 35). Briefly, HEK293T cells were cotransfected with a provirus plasmid encoding the envelope deficient HIV-1NL4-3 virus with the luciferase reporter gene inserted into nef (pNL4-3.Luc-R.E; gift of N. Landau, NIH AIDS Research and Reference Reagent Program, Germantown, MD) and a pSV plasmid expressing the HSV-1gp system (gift of D. Trono, University of Geneva, Geneva, Switzerland). The supernatant medium was collected 48 h after transfection, and filtered. HIV-1 p24 Ag concentration of viral stocks was determined by ELISA.

Primary CD4+ T cells were infected with replication-defective pseudotyped HIV-1LaiGFP luciferase reporter viruses at 37˚C for 2 h. After washing off unbound viruses, infected cells were treated with GC or TLR2 agonists in the presence of IL-2 at 37˚C for 4 d. The cells were lysed with passive cell lysis buffer (Promega, Madison, WI). Luciferase activity (in relative light units) was measured on an EG&G Berthold MiniLumat LB 9500 luminometer (Berthold Technologies, Bad Wildbad, Germany).

Reverse transcription-PCR analysis

Total RNA was isolated from resting CD4+ T cells, PHA-activated CD4+ T cells, or CD4+ cells with N-Rhesus isolation kit (Qiagen, Valencia, CA). To synthesize first-strand cDNA, total RNA (500 ng), oligo (dT)₁₆ (Invitrogen, Carlsbad, CA) at 25 µg/ml and 0.5 mM dNTP in a total volume of 12 µl were incubated at 65˚C for 5 min and quick chilled on ice. Reverse transcription (RT) was performed at 42˚C for 50 min using SuperScript II RT (Invitrogen) according to the manufacturer’s protocol. Primers used were listed as follows: TLR2 forward, 5′-AGGTGACCTATAGAAGTCTGGGACCCCTTATGGAAA-3′; TLR2 reverse, 5′-GATGCACCTATAGAAGTCTGGGACCCCTTATGGAAA-3′; TLR4 forward, 5′-AGGTGACCTATAGAAGTCTGGGACCCCTTATGGAAA-3′; TLR4 reverse, 5′-GATGCACCTATAGAAGTCTGGGACCCCTTATGGAAA-3′. The PCR reaction included Taq master mix, 0.2 µM each primer, and 3 µl RT reaction. After an initial incubation at 94˚C for 3 min, 35 cycles of amplification were performed as follows: denaturation for 30 s at 94˚C, annealing for 30 s at 56˚C, and 30 s at 72˚C, and a final extension cycle of 7 min at 72˚C in a DNA thermal cycler (Perkin Elmer 480). PCR products were separated by electrophoresis on a 2% agarose gel and analyzed with the FluoroChem 8800 imaging system (Alpha Innotech, San Leandro, CA).

Flow cytometry

Cells were stained with fluorochrome-conjugated mAbs specific for T cell activation markers and HIV-1 coreceptors. Appropriate isotype-matched mAbs conjugated with PE, FITC, or APC were used as negative controls. Results were acquired with CellQuest software (BD Biosciences, San Jose, CA) on a FACScalibur (BD Biosciences) and analyzed with FlowJo (Tree Star, Ashland, OR).

Quantitative real-time PCR analysis of HIV-1 DNA

DNA was extracted from resting CD4+ T cells with infection by pseudotyped HIV-1LaiGFP luciferase reporter viruses using the DNeasy Blood and Tissue Kit (Qiagen). The level of HIV RT DNA products was determined by quantitative real-time PCR analysis. Each PCR reaction contained 100 ng genomic DNA, each primer at 0.2 µM, SYBR Green Master Mix (Qiagen). The primer sequences for the HIV-1 RT DNA products were as follows: 5′-GTGCTAGATTGTGTGCTGCTG-3′ for R/U5 forward, AA55 (5′-CGCTTCCTCAGGTACAGCTC-3′) for R/U5 reverse, AA55 (5′-CGCTTCCTCAGGTACAGCTC-3′). The PCR reaction included Taq master mix, 0.2 µM each primer set, and 3 µl RT reaction. After an initial incubation at 94˚C for 3 min, 35 cycles of amplification were performed as follows: denaturation for 30 s at 94˚C, annealing for 30 s at 56˚C, and 30 s at 72˚C, and a final extension cycle of 7 min at 72˚C in a DNA thermal cycler (Perkin Elmer 480). PCR products were separated by electrophoresis on a 2% agarose gel and analyzed with the FluoroChem 8800 imaging system (Alpha Innotech, San Leandro, CA).
the DNA input, the primer sequences were as follows: actin-forward (5’-TCGGGATCCTTCAAGAAGG-3’); actin-reverse (5’-GCTGTAGCTCCTCCTGCA-3’). Plasmids pNL4-3-Luc was used to generate standard curves for HIV RT DNA products. In every experiment, a standard curve for RT products derived from serial dilution of plasmids containing the target sequence and ranging from 10^3 to 10^6 copies was measured in triplicate. The level of HIV closed long terminal repeat (c2-LTR) circles was determined by using primers LR31: 5’-CGGCTCCCTGATGGCTCC-3’; and LR32: 5’-TTAGACGTCAAGGAAATGCC-3’ (37). Plasmid pTA2LTR (38) was used to generate a standard curve. The detection limit was 10 copies. PCR cycling conditions included a 95˚C denaturation for 10 min, followed by 40 cycles of 95˚C for 30 s, 55˚C for 30 s, and 72˚C for 30 s. Reactions were carried out and analyzed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems).

Results

GC exposure enhances HIV-1 replication in primary resting CD4+ T cells

To determine the effect of GC exposure on HIV-1 replication in primary CD4+ T cells, CD4+ T cells were infected with R5 HIV-1BaL at an MOI of 0.05 for 2 h, washed to remove unbound virus, and then exposed to live or fixed GC ATCC strain 43069 at various MOIs in the presence of IL-2. HIV-1 production was determined by measuring virus particles released into media with HIV-1 p24 ELISA. At day 14 postinfection, live GC at an MOI of 100 increased HIV-1 production in primary CD4+ T cells by 1.7-fold (Fig. 1A), whereas fixed GC at an MOI of 100 enhanced virus production by 5.8-fold (Fig. 1B). To determine whether GC exposure could affect proliferation of CD4+ T cells, primary resting CD4+ T cells were exposed to GC for 3 d in the presence of IL-2. Cell proliferation was determined by MTS assay. No significant difference in proliferation of primary resting CD4+ T cells was observed between cells with or without exposure to either live or fixed GC (Fig. 1C). These results demonstrated that GC exposure increased HIV-1 production after viral entry but did not induce proliferation in CD4+ T cells.

To determine whether the HIV enhancing effect of GC was strain specific, the effect of three clinical GC isolates (GC56, F62, and FA1090) on HIV infection was studied using a single-cycle infection assay, measuring the steps of the HIV life cycle from entry to gene expression. A replication-defective HIV-1 luciferase reporter virus pseudotyped with VSV-G protein, which is not dependent on HIV-1 coreceptors for virus entry, was used to maximize HIV infection efficiency. HIV-infected cells were exposed to fixed GCs at various MOIs in the presence of IL-2 for 4 d before measurement of luciferase activity. Similar to the strain in Fig. 1, three GC clinical isolates also enhanced HIV infection of primary resting CD4+ T cells (Fig. 2A). N. gonorrhoeae strains F62 and FA1090 at an MOI of 10 enhanced HIV infection of resting CD4+ T cells by 34.2- and 30.7-fold, respectively, whereas a higher MOI of 100 of N. gonorrhoeae strain GC56 was required to reach the similar enhancement of HIV (26-fold).

Pil and Opa are surface-exposed structures of GC that play a role in gonococcal adherence to and invasion of host cells. Both components are involved in GC-mediated activation and proliferation of CD4+ T cells in response to TCR signaling (39, 40). However, a recent report demonstrates that, in conjunction with anti-CD3 Abs and IL-2 treatment, both Opa+ and Opa− bacteria induce T cell proliferation to a similar extent (41). To determine the role of Pil and Opa in GC-mediated HIV enhancement, the effect of FA1090 Pil+/Opa+ and FA1090 Pil−/Opa− on HIV infection was examined using single-cycle infection assay. Both bacteria promoted HIV infection despite their distinct phenotypes (Fig. 2B); however, the degree of HIV enhancement by FA1090 Pil−/Opa− was significantly reduced, suggesting that Pil and Opa could modulate the extent of the HIV enhancing effect.

To delineate whether GC exposure affected T cell proliferation, which in turn facilitated HIV infection, primary resting CD4+ T cells were exposed to various fixed GC strains at MOIs of 1, 10, and 100 for 3 d in the presence or absence of IL-2. Cell proliferation was determined by MTS assay. We did not observe significant differences in proliferation of resting CD4+ T cells between cells with or without GC exposure (Fig. 2C). In addition, IL-2 did not affect cell proliferation of GC-exposed CD4+ T cells (Fig. 2C). These results indicated that GC exposure did not affect resting CD4+ T cell proliferation, even in the presence of IL-2 signaling.

TLR2 activation promotes HIV-1 infection of primary resting CD4+ T cells

GC expresses ligands for multiple PRRs, including TLR2, TLR4, and NOD2 (30, 42, 43). To investigate the role of PRR signaling in GC-induced enhancement of HIV-1 infection, we first examined the effect of TLR2, TLR4, and NOD2 agonists on HIV-1 infection of primary resting CD4+ T cells. CD4+ T cells were infected with X4 HIV-1IIIB (Fig. 3A) or R5 HIV-1BaL (Fig. 3B) for 2 h. After washing off unbound virus, infected cells were treated with various agonists for TLR2 (PGN), TLR4 (LPS and GC LOS), or NOD2 (MDP) and cultured in complete media with IL-2 for 7 d. HIV replication was determined by HIV p24 ELISA. The TLR2 agonist PGN at a concentration of 20 μg/ml significantly increased the replication of both X4 virus (12.8-fold) and R5 virus (13-fold) after viral entry. Although the degree of the HIV enhancing effect of PGN ranged from 2- to 46-fold depending on CD4+ T cell donor, TLR2 activation consistently increased HIV infection of primary CD4+ T cells. The NOD2 ligand MDP at 20 μg/ml exhibited a moderate effect on replication of X4 virus and no effect for that of R5 virus. In

FIGURE 1. GC exposure enhances HIV-1 infection of primary resting CD4+ T cells. Primary resting CD4+ T cells were infected with HIV-1BaL at an MOI of 0.05 at 37˚C for 2 h. After washing off unbound virus, infected cells were exposed to live (A) or fixed (B) GC (ATCC strain 43069) at various MOIs in the presence of IL-2. HIV production was determined by measuring the level of HIV capsid protein p24 at day 14 after viral infection using HIV p24 ELISA. C, Primary resting CD4+ T cells (1 × 10^6 cells per well) were exposed to live GC or fixed GC at 37˚C for 72 h in the presence of IL-2. Cell proliferation was examined by the MTS assay (Promega CellTiter96 aqueous one solution cell proliferation assay). Data are means ± SD of triplicate sample. Difference in HIV infection between samples without GC exposure and GC-exposed samples at an MOI of 100 is significant as calculated by the two-tailed, paired Student t test. *p < 0.05. There is no difference in cell proliferation between samples with or without GC exposure (p > 0.05). Data represent three independent experiments.
CD4+ T cells were exposed to pseudotyped HIV-1VSV luciferase reporter virus at 37°C for 2 h. After washing off unbound virus, infected cells were exposed to various strains of fixed GC in the presence of IL-2 for 4 d before measuring luciferase activity. There is a significant difference between samples with and without GC exposure. *p < 0.05. B, Resting CD4+ T cells were infected with pseudotyped HIV-1VSV-G luciferase reporter virus at 37°C for 2 h, washed, and then treated with FA1090 Pil/Òopa+ or FA1090 Pil/Òopa- in the presence of IL-2. Luciferase activity was measured at 4 d postinfection. The difference between cells treated with FA1090 Pil/Òopa+ and FA1090 Pil/Òopa- is significant. *p < 0.05. C, Resting CD4+ T cells (1 × 10^6 cells per well) were exposed to various strains of fixed GC at MOIs of 1, 10, and 100 at 37°C for 72 h in the presence of IL-2. Cell proliferation was examined by the MTS assay. Data are means ± SD of triplicate sample. Data represent two independent experiments.

FIGURE 2. Multiple GC strains enhanced HIV-1 infection of primary resting CD4+ T cells. A, Primary resting CD4+ T cells were infected with pseudotyped HIV-1VSV-G luciferase reporter virus at 37°C for 2 h. After washing off unbound virus, infected cells were exposed to various strains of fixed GC in the presence of IL-2 for 4 d before measuring luciferase activity. There is a significant difference between samples with and without GC exposure. *p < 0.05. B, Resting CD4+ T cells were infected with pseudotyped HIV-1VSV-G luciferase reporter virus at 37°C for 2 h, washed, and then treated with FA1090 Pil/Òopa+ or FA1090 Pil/Òopa- in the presence of IL-2. Luciferase activity was measured at 4 d postinfection. The difference between cells treated with FA1090 Pil/Òopa+ and FA1090 Pil/Òopa- is significant. *p < 0.05. C, Resting CD4+ T cells (1 × 10^6 cells per well) were exposed to various strains of fixed GC at MOIs of 1, 10, and 100 at 37°C for 72 h in the presence of IL-2. Cell proliferation was examined by the MTS assay. Data are means ± SD of triplicate sample. Data represent two independent experiments.

In contrast, TLR4 ligands, such as LPS (1 μg/ml) and GC LOS (0.1 μg/ml), did not exhibit any effect on HIV-1 replication in CD4+ T cells from any donor.

To further investigate the effect of other TLR2 agonists in addition to PGN on HIV infection of primary resting CD4+ T cells, CD4+ T cells were exposed to pseudotyped HIV-1VSV-G luciferase reporter virus for 2 h, washed, and then treated with synthetic TLR2 agonists Pam3CSK4 and Pam3C-Lip, a lipopeptide derived from the GC Lip protein (44). Both synthetic TLR2 agonists promoted HIV infection of resting CD4+ T cells after viral entry (Fig. 3C, 3D). Taken together, these results indicated that TLR2 signaling but not TLR4 or NOD2 played a role in GC-mediated enhancement of HIV-1 infection after viral entry.

Pretreatment of quiescent naive and memory CD4+ T cells with the synthetic TLR2 agonist Pam3CSK4 has been reported to increase the susceptibility of cells to infection of both X4 virus and R5 viruses (21). Pam3CSK4 and GC activate TLR2 in association with TLR1 and TLR6, respectively (45-47), although heterodimerization of TLR2 with TLR1 or TLR6 does not lead to differential signaling (48, 49). We examined whether pretreatment of resting CD4+ T cells with PGN could enhance HIV infection. Primary resting CD4+ T cells were pretreated with PGN at 20 μg/ml for 3 d in the presence of IL-2. Cells were washed and then infected with HIV-1HXB or HIV-1NL4-3. Similar to the previous report using a TZM-bl cell reporter system (21), we found that pretreatment of primary CD4+ T cells with PGN increased viral infection for both X4 virus (by 2.4-fold) and R5 virus (by 2.1-fold) (Fig. 3E). However, the enhancing effect on HIV production was moderate when cells were pretreated with PGN compared with viral production from cells that were treated with PGN after HIV entry (Fig. 3A, 3B). These results indicated that TLR2 activation could enhance HIV-1 infection of primary CD4+ T cells through multiple mechanisms.

**HIV enhancing effect of GC is mediated through TLR2 signaling**

We observed differential effects of TLR2, TLR4, and NOD2 agonists on HIV infection of primary resting CD4+ T cells (Fig. 3). To investigate whether the levels of these PRRs contributed to their differential HIV enhancing effect, we examined gene expression of TLR2, TLR4, and NOD2 in primary resting CD4+ T cells by RT-PCR analysis. In agreement with previous reports (50, 51), gene expression of TLR2 and NOD2 was detectable in primary resting CD4+ T cells (Fig. 4A). After PHA activation, expression of TLR2 and NOD2 was elevated. In contrast, gene expression of TLR4 was not detectable in both primary resting and PHA-activated CD4+ T cells, whereas it was expressed in monocyctic CD14+ cells that are known to express TLR4 (Fig. 4A).

To establish the involvement of TLR2 in GC-mediated enhancement of HIV infection, we used Abs against TLR2 to block the HIV enhancing effect of GC- and GC-derived lipopeptide Pam3C-Lip using a single-cycle infection assay. HIV-infected resting CD4+ T cells were treated with anti-TLR2 Abs for 1 h before GC exposure. An isotype control Ab was included as a negative control. After 4 d incubation, cells were lysed, and the luciferase activity was measured. Fixed GC promoted HIV-1 infection by 8.2-fold in a single-cycle infection assay (Fig. 4B). Anti-TLR2 or isotype control Abs did not have any effect on HIV infection in the absence of GC exposure (data not shown). Anti-TLR2 Abs completely abolished the HIV enhancing effect of GC, whereas isotype control Abs did not exhibit any significant effect (Fig. 4B). Similarly, Pam3C-Lip at 0.2 and 1 μg/ml enhanced HIV infection by 1.8-fold and 5.6-fold, respectively (Fig. 4C). This enhancing effect was blocked by anti-TLR2 Ab but not isotype control Ab (Fig. 4C). These results demonstrated that TLR2 was involved in GC-mediated enhancement of HIV infection of primary resting CD4+ T cells after viral entry.

**TLR2 activation does not enhance postentry HIV-1 infection in activated CD4+ T cells and the HIV enhancing effect is dependent on IL-2**

To examine whether TLR2 activation exerted any effect on HIV infection of activated CD4+ T cells, PHA-activated CD4+ T cells were exposed to pseudotyped HIV-1VSV-G luciferase reporter virus for 2 h before treatment with PGN. Primary resting CD4+ T cells from the same donor were included as a comparison. We observed enhancement of HIV-1 infection by TLR2 activation in resting but not PHA-activated CD4+ T cells, despite our finding that activated CD4+ T cells, expressing TLR2, were highly susceptible to HIV-1 infection (Fig. 5A).

IL-2 is an important factor for T cell proliferation and survival as well as HIV replication in primary CD4+ T cells (52, 53). In addition, IL-2 is required for the TLR2-mediated induction of IFN-γ by Th1 effector cells (54). To investigate the role of IL-2 in TLR2-mediated enhancement of HIV infection, the HIV enhancing effect of PGN and Pam3CSK4 was determined in the presence or absence of IL-2. IL-2 treatment alone did not increase HIV infection. In the absence of IL-2, PGN or Pam3CSK4 did not enhance HIV infection of primary resting CD4+ T cells in both multiple-round infection assays and single-cycle infection assays (Fig. 5B). Enhancement of HIV infection was observed only in the presence of both IL-2 and TLR2 agonists. This result indicated that IL-2 signaling was required to achieve enhancement of HIV infection in response to TLR2.
GC and TLR2 agonists induce expression of T cell activation markers and HIV-1 coreceptors CCR5 and CXCR4 on CD4+ T cells

The synthetic TLR2 agonist Pam3CSK4 has been shown to induce T cell activation markers including CD69, CD25, ICAM-1, and HLA-DR on both quiescent naive and memory CD4+ T cells (21). Plant et al. demonstrated that piliated live GC exposure increased the TCR signaling-mediated activation and proliferation of primary CD4+ T cells (39). Because activation of T cells facilitates productive HIV-1 infection, we examined the effect of GC or PGN on expression of T cell activation markers. Primary resting CD4+

FIGURE 3. TLR2 activation promotes HIV replication in primary resting CD4+ T cells. A and B, Primary resting CD4+ T cells were infected with X4 HIV-1NL4-3 or R5 HIV-1BaL at an MOI of 0.05 at 37°C for 2 h. After washing off unbound virus, infected cells were then treated with LPS (1 μg/ml), MDP (20 μg/ml), PGN (20 μg/ml), or GC DOV LOS (0.1 μg/ml) in the presence of IL-2 at 37°C. Infected cells without treatment were included as a control. The p24 level in the culture supernatant was determined on day 7 postinfection. C, Resting CD4+ T cells were infected with pseudotyped HIV-1VSV-G luciferase reporter virus at 37°C for 2 h and then treated with Pam3CSK4 at 5 μg/ml with IL-2 for 4 d before measuring luciferase activity. D, Resting CD4+ T cells were infected with pseudotyped HIV-1VSV-G luciferase reporter virus and then exposed to Pam3C-Lip at various concentrations with IL-2 for 4 d before measuring luciferase activity. E, Resting CD4+ T cells were treated with PGN (20 μg/ml) for 3 d, followed by washing. PGN-treated primary CD4+ cells were infected with HIV-1NL4-3 or HIV-1BaL. The p24 level in the culture supernatant was determined on day 7 postinfection. Data are means ± SD of triplicate sample and represent seven independent experiments. The difference in HIV infection between samples with and without TLR2 agonists treatment is significant as calculated by the two-tailed, paired Student t test. *p < 0.05.

FIGURE 4. Enhancement of HIV infection of primary resting CD4+ T cells by GC is mediated through TLR2 signaling pathway. A, Gene expression of NOD2, TLR2, and TLR4 in resting CD4+ T cells and PHA-activated CD4+ T cells was determined by RT-PCR analysis. Total RNA from CD4+ cells was included as a control for analysis of TLR4 gene expression. B, To determine the role of TLR2 in GC-mediated enhancement of HIV infection, resting CD4+ T cells were infected with pseudotyped HIV-1VSV-G luciferase reporter virus at 37°C for 2 h. After washing off unbound virus, infected cells were treated with anti-TLR2 Ab or isotype control Ab at 10 μg/ml for 1 h. Cells were exposed to fixed GC (ATCC strain 43069) at an MOI of 50 with IL-2 and cultured for 4 d before measurement of luciferase activity. C, Resting CD4+ T cells were infected with pseudotyped HIV-1VSV-G luciferase reporter virus and treated with anti-TLR2 Ab or isotype control Ab as described previously. Cells were then exposed to Pam3C-Lip at various concentrations in the presence of IL-2 for 4 d. Data are means ± SD of triplicate sample and represent two independent experiments. There is a significant difference between samples with and without GC or Pam3C-Lip treatment (*p < 0.05) as well as between stimulated cells in the presence or absence of anti-TLR2 Ab (**p < 0.05). There is no difference between stimulated samples in the presence or absence of isotype control Ab (***p > 0.05).
T cells were incubated with fixed GC (ATCC strain 43069) at an MOI of 50 or PGN at 20 μg/ml for 3 d in the presence of IL-2 before measuring cell surface expression of CD25 and CD69 by FACS analysis. PHA-activated CD4+ T cells were included as a positive control. As expected, PHA upregulated CD25 and CD69 expression significantly (Table I). In agreement with the previous reports using a synthetic TLR2 agonist (21) or GC (39), PGN and GC increased the level of cell surface CD25 and CD69 on primary resting CD4+ T cells (Table I).

Pretreatment of CD4+ T cells with PGN led to enhancement of HIV-1 infection using both X4 and R5 viruses (Fig. 3). We hypothesized that GC upregulates CXCR4 and CCR5 expression that in turn results in increased HIV-1 susceptibility of target cells. The effect of GC or PGN on expression of cell surface CXCR4 and CCR5 on CD4+ T cells was determined by FACS analysis. PHA-activated CD4+ T cells were included as a comparison. GC and PGN treatments increased the number of CCR5+ cells and the level of cell surface CXCR4 (Table I). These results suggest that pretreatment of cells with GC and PGN may promote HIV infection through induction of HIV coreceptors. It is noted that Pam3CSK4 also induces cell surface expression of CCR5 on naive and memory T cells (21).

The maximal HIV enhancing effect is achieved when TLR2 agonist was added at an early stage of the HIV life cycle

To dissect the stages of HIV infection that were enhanced by TLR2 activation after viral entry, we first studied the kinetics of the HIV life cycle in CD4+ T cells in the presence of PGN using a single-cycle infection assay. HIV-infected resting CD4+ T cells were cultured in presence of IL-2 and treated with PGN at 2, 24, 48, and 72 h after viral infection. The luciferase activity was determined at day 4 after viral infection. The maximal HIV-1 enhancing effect was observed when PGN was added at 2 h postinfection (Fig. 6A). The degree of the HIV enhancing effect decreased when PGN was added at later time points of the HIV life cycle. PGN did not affect HIV infection when added at 72 h postinfection, possibly because of degradation of viral DNA. To determine whether prolonged incubation could increase the level of HIV infection in samples with PGN treatment at later time points, infected cells were treated with PGN at 48 and 72 h postinfection and luciferase activity was determined on day 6 and day 7 postinfection, respectively. There was an increase in the level of HIV enhancement when infected cells with PGN treatment at 48 h postinfection were cultured for an additional 2 d, although this HIV enhancing effect was not as pronounced compared with that observed in cells with PGN treatment at 2 h postinfection. Prolonged culture of cells with PGN treatment at 72 h postinfection did not restore the level of enhancement of HIV infection. To ensure that resting CD4+ T cells at later time points were still viable in the presence of IL-2, the viability of resting CD4+ T cells at different time points postinfection was determined by MTS assay. There was no significant difference among CD4+ T cells at 2, 24, 48, or 72 h after HIV exposure in the presence of IL-2 (Fig. 6B). This result indicated that the lack of HIV enhancement by TLR2 at 72 h postinfection was not due to decreased cell viability.

We further investigated whether the lack of responsiveness to TLR2 stimulation at 72 h after viral infection was due to down-regulation of TLR2 expression by HIV. The mRNA level of TLR2 expression from HIV-infected CD4+ T cells at 2, 24, and 72 h after viral infection was first determined by RT-PCR analysis. There was no change in the level of TLR2 gene expression in the presence of IL-2 (Fig. 6C). We then examined cell surface expression of TLR2 on HIV-infected resting CD4+ T cells by FACS analysis. HIV infection did not alter the expression level of cell surface TLR2.

### Table I. Effect of TLR2 activation on cell activation and coreceptor expression

<table>
<thead>
<tr>
<th>Isotypes</th>
<th>CD25</th>
<th>CD69</th>
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<tbody>
<tr>
<td>Medium</td>
<td>1.6 ± 0.7</td>
<td>11.7 ± 0.6</td>
</tr>
<tr>
<td>PGN</td>
<td>1.1 ± 0.8</td>
<td>16.6 ± 2.8</td>
</tr>
<tr>
<td>Fixed GC</td>
<td>3.0 ± 1.0</td>
<td>19.6 ± 3.0</td>
</tr>
<tr>
<td>PHA</td>
<td>2.7 ± 1.3</td>
<td>97.6 ± 0.6</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Coreceptors (%)</th>
<th>CXCR4</th>
<th>CCR5</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>91.0 ± 0.2</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>PGN</td>
<td>90.4 ± 1.0</td>
<td>9.1 ± 5.2</td>
</tr>
<tr>
<td>Fixed GC</td>
<td>92.9 ± 0.5</td>
<td>9.5 ± 2.3</td>
</tr>
<tr>
<td>PHA</td>
<td>96.5 ± 3.1</td>
<td>2.0 ± 1.4</td>
</tr>
</tbody>
</table>

Primary resting CD4+ T cells were incubated with PGN or fixed GC for 3 d in the presence of IL-2. PHA-activated cells and mock-treated cells were included as controls. The percentage of cells expressing activation markers or HIV coreceptors is determined by FACS analysis. The data shown are mean ± SD of three independent experiments.

*There is a significant difference between ligand-treated and mock-treated samples, p < 0.05.
The maximal HIV enhancing effect of the TLR2 activation is achieved when PGN is added at an early stage of the HIV life cycle. A, Resting CD4+ T cells were infected with pseudotyped HIV-1VSV-G luciferase reporter virus for 2 h. Infected cells were cultured with IL-2 and then treated with PGN at 20 μg/ml at 2, 24, 48, or 72 h postinfection before measurement of luciferase activity at day 4, 6, or 7 postinfection. There is a significant difference between samples without PGN and with PGN at 2, 24, or 48 h postinfection (⁎p < 0.05). When samples were harvested at day 6 postinfection the difference between cells with PGN at 48 h postinfection and without treatment is significant (⁎⁎p < 0.05). B, The viability of resting CD4+ T cells postinfection with pseudotyped HIV-1VSV-G luciferase reporter virus was assessed by MTS assay at 2, 24, 48, and 72 h after viral infection. C, Total RNA from resting CD4+ T cells with infection by pseudotyped HIV-1VSV-G reporter virus for 2, 24, or 72 h was prepared. TLR2 gene expression of TLR2 was analyzed by RT-PCR. Samples from mock-infected resting CD4+ T cells were also included as a control. D, Cell surface expression of TLR2 on HIV-infected resting CD4+ T cells was determined by FACS analysis. Data are means ± SD of triplicate sample and represent three independent experiments.

To examine whether TLR2 activation promoted HIV replication at the step of nuclear import, we analyzed the level of c2-LTR circles, a marker of nuclear import (55). Primary resting CD4+ T cells are known to have low copy numbers of c2-LTR circles (56). At 12 h after viral infection, there were no detectable c2-LTR circles in samples with or without exposure to GC or TLR2 agonists, such as Pam3CSK4 and PGN (Fig. 8B, data not shown for PGN). At 24 h postinfection, the level of c2-LTR circles in HIV-exposed cells was below the detection limit (10 copies), whereas ~27 and 21 copies of c2-LTR circles per 100 ng total DNA was detected in primary resting CD4+ T cells with treatment of Pam3CSK4 and GC, respectively (Fig. 8C). At 48 h after viral infection, the level of c2-LTR circles in HIV-infected cells with exposure to Pam3CSK4 and GC was increased by 4.3- and 3.1-fold.

To further delineate the specific stages of the HIV-1 life cycle altered by TLR2 activation, we analyzed HIV DNA products representing HIV RT and nuclear import during HIV infection. Samples with GC exposure were also included as a comparison. DNA was prepared from HIV-infected primary resting CD4+ T cells with or without exposure to fixed GC or TLR2 agonists, Pam3CSK4 and PGN, at various time points postinfection. The levels of HIV early and late RT products were determined by quantitative real-time PCR analysis. Results in Fig. 8A revealed that there was no significant difference in the level of HIV early or late RT products from cells with or without exposure to GC or TLR2 agonists (data not shown for PGN).
performed to measure c2-LTR circles in infected CD4+ T cells with or without Pam3CSK4 or fixed GC treatment for 12, 24, and 48 h. Plasmid pTA2LTR at different copy numbers was used to generate a standard for c2-LTR circles detection. The detection limit of the PCR analysis was 10 copies. There is a significant difference between samples with or without exposure to TLR2 agonists or GC (p < 0.05) at 24 and 48 h postinfection.

Discussion

In this study, we demonstrated that GC enhanced HIV-1 infection of primary resting CD4+ T cells through TLR2 activation. Pre-treatment of uninfected CD4+ T cells as well as stimulation of HIV-infected primary CD4+ cells after entry with the TLR2 agonist PGN increased HIV replication, suggesting that multiple mechanisms were involved in TLR2-mediated enhancement of HIV infection of primary resting CD4+ T cells. GC exposure and TLR2 stimulation activated resting CD4+ T cells and induced cell surface expression of HIV coreceptors, resulting in an increase in the susceptibility of primary CD4+ T cells to HIV infection. STIs, such as GC and Chlamydia trachomatis, are known to activate TLR2 and TLR4 (30, 57, 58). Although there is a discrepancy in the literature with respect to gene expression of TLR4 in primary CD4+ T cells (51, 59, 60), we did not detect TLR4 mRNA in primary CD4+ T cells by RT-PCR nor did we find an HIV enhancing effect of TLR4 agonists LPS and GC LOS. Importantly, anti-TLR2 Abs completely abolished the enhancement of HIV infection induced by GC or GC-derived lipopeptide Pam3C-Lip. These results suggest that TLR2 may play a major role in GC-mediated enhancement of HIV transmission, particularly if bacterial products come in contact with intraepithelial resting T cells. Indeed, TLR2 activation is also involved in GC-mediated enhancement of HIV transmission by dendritic cells to T cells (20) as well as induction of HIV LTR gene expression (9) and cytokine production by cervicovaginal fluid from women with bacterial vaginosis (61). Interestingly, bacterial vaginosis is also associated with increased HIV acquisition and HIV shedding (15, 62), suggesting common pathways for HIV enhancement. Our results also show that components on GC surface may affect the efficiency of TLR2-activation. Although both Pil+/Opa+ as well as Pil−/Opa− GC enhanced HIV infection, the Pil+/Opa− variant was more active presumably due to the known interactions of Pil and Opa with T cells that may bring the bacterial membrane into closer or more prolonged proximity of TLR2 receptors (40, 63).

Our studies presented a novel mechanism demonstrating that TLR2 activation promoted HIV infection of primary resting CD4+ T cells after viral entry. The maximal effect of PGN was achieved when PGN was added to HIV-infected cells immediately after viral infection, indicating that TLR2 activation acted on an early stage of the HIV life cycle. Analysis of HIV DNA products revealed that TLR2 activation significantly enhanced the step of nuclear import. Several viral proteins, including Vpr, intgrase, and Gag, are reported to play a role in nuclear import (64, 65). Some viral proteins, particularly Vpr, influence cell cycle progression, which modulates nuclear import (66). Our results showed that GC and TLR2 agonists promoted HIV infection in a single-cycle infection assay using pseudotyped HIV-1 luciferase reporter virus that does not contain the Vpr gene, suggesting that TLR2-mediated enhancement of HIV infection of resting CD4+ T cells is not dependent on HIV Vpr. Nuclear import of SIV and HIV requires signaling pathways induced by both TCRs and CD28 receptors in resting T cells (67, 68). IL-2 can replace CD28 ligation to enhance the level of HIV 2-LTR circles, a marker of nuclear import, in CD3-activated T cells (67). Although the synthetic TLR2 agonist Pam3CSK4 has been shown to enhance HIV infection of quiescent naive and memory T cells in the absence of TCR cross-linking or IL-2, HIV infection was significantly increased in the presence of IL-2 or OKT3 Ab (21). Our studies indicated that IL-2 signaling was essential for TLR2-mediated enhancement of HIV infection of primary resting CD4+ T cells using both PGN and Pam3CSK4. It is not clear whether purification of specific subsets of T cells or the use of TZM-bl reporter gene system contributes to the different outcome with respect to IL-2 dependency. Nevertheless, we are currently investigating the effect of GC and TLR2 agonists using subsets of T cells in the presence or absence of IL-2.

HIV 2-LTR circle formation is blocked at G0/G1 transition (55, 69, 70). In agreement with the previous report using naive and memory CD4 T cells in response to Pam3CSK4 (21), we found that GC and PGN induced T cell activation, although the effect on cell cycle in primary resting CD4+ T cells remains to be determined. It is possible that, in conjunction with TLR2 signaling pathways, IL-2-dependent T cell activation and G0/G1 transition promote nuclear import of HIV DNA. After nuclear import and integration, TLR2 signaling may further enhance HIV transcription because TLR2 activation is known to activate HIVLTR-driven gene expression through NF-kB activation and induction of TNF-α and IL-8 (15, 61).

In vitro, HIV enters primary resting CD4+ T cells and remains latent in the absence of T cell stimulation (71–73), although HIV infection has been reported in resting CD4+ T cells in HIV-infected individuals and SIV-infected macaque monkeys (32, 74, 75). Barriers blocking completion of HIV infection in resting CD4+ T cells include host restriction factors, such as Trim5α (76), TLR2-activation. Although both Pil+/Opa+ as well as Pil−/Opa− GC enhanced HIV infection, the Pil−/Opa+ variant was more active presumably due to the known interactions of Pil and Opa with T cells that may bring the bacterial membrane into closer or more prolonged proximity of TLR2 receptors (40, 63).
the blockage of nuclear import of preintegration complex (77), nuclear retention of multiply spliced HIV RNA in resting CD4+ T cells (78), inhibition of NF-kB activation by Murr1 (79), and cellular microRNA that inhibits HIV gene expression (80). Although it remains to be determined the effect of TLR2 activation on other HIV inhibitory factors in resting CD4+ T cells, our results showed that TLR2 activation overcame a block at the level of nuclear retention of multiply spliced HIV RNA in primary CD4+ T cells in a single-cycle infection assay (Fig. 6). We excluded the possibility that the lack of responsiveness was due to decreased cell viability (Fig. 6B) or reduced TLR2 expression (Fig. 6C, 6D). This lack of responsiveness to TLR2 activation could be due to degradation of HIV DNA in resting CD4+ T cells at 72 h postinfection. We demonstrated that TLR2 signaling also induced HIV infection of primary resting CD4+ T cells when added 48 h postinfection (Fig. 6A). The specific stage of the HIV life cycle affected by TLR2 signaling at 48 h postinfection and the effect of TLR2 activation of endocervical T cells from women is dominated by activated effector memory T cells with activation (81, 82). In resting CD4+ T cells, HIV-1 DNA in preintegration latency decays rapidly but HIV infection can be rescued when T cells are activated at early stages of HIV infection (77, 83). When PGN was added at 72 h after viral infection, there was no enhancement of HIV infection of primary CD4+ T cells in resting CD4+ T cells (77, 83). When PGN was added at 72 h after viral infection, there was no enhancement of HIV infection of primary CD4+ T cells in resting CD4+ T cells when added 48 h postinfection (Fig. 6A). Our results suggest that induction of adaptive immunity. The authors have no financial conflicts of interest. Disclosures.

References
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