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RESEARCH ARTICLE

Helicobacter pylori Reactivates Human Immunodeficiency Virus-1 in Latently Infected Monocytes with Increased Expression of IL-1β and CXCL8

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Abstract: ***Background*:** *Helicobacter pylori* are gram-negative bacteria, which colonize the human stomach. More than 50% of the world's population is infected by *H. pylori*. Based on the high prevalence of *H. pylori*, it is very likely that HIV and *H. pylori* infection may coexist. However, the molecular events that occur during HIV-*H. pylori* co-infection remain unclear. Latent HIV reservoirs are the major obstacle in HIV cure despite effective therapy. Here, we explored the effect of *H. pylori* stimulation on latently HIV-infected monocytic cell line U1.

***Methods*:** High throughput RNA-Seq using Illumina platform was performed to analyse the change in transcriptome between unstimulated and *H. pylori*-stimulated latently HIV-infected U1 cells. Transcriptome analysis identified potential genes and pathways involved in the reversal of HIV latency using bioinformatic tools that were validated by real-time PCR.

***Results*:** *H. pylori* stimulation increased the expression of HIV-1 Gag, both at transcription ($p<0.001$) and protein level. *H. pylori* stimulation also increased the expression of proinflammatory cytokines IL-1β, CXCL8 and CXCL10 ($p<0.0001$). Heat-killed *H. pylori* retained their ability to induce HIV transcription. RNA-Seq analysis revealed 197 significantly upregulated and 101 significantly downregulated genes in *H. pylori*-stimulated U1 cells. IL-1β and CXCL8 were found to be significantly upregulated using transcriptome analysis, which was consistent with real-time PCR data.

***Conclusion*:** *H. pylori* reactivate HIV-1 in latently infected monocytes with the upregulation of IL-1β and CXCL8, which are prominent cytokines involved in the majority of inflammatory pathways. Our results warrant future *in vivo* studies elucidating the effect of *H. pylori* in HIV latency and pathogenesis.

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1. INTRODUCTION

Combination antiretroviral therapy (cART) has emerged as an effective therapy for human immunodeficiency virus (HIV) infection resulting in the reduction of AIDS-related morbidity and mortality worldwide. HIV-infected subjects live longer with expanded life span. However, they are burdened with premature aging and non-AIDS morbidity [1-4]. While several factors including drug toxicity could contribute to this abnormal phenomenon, chronic inflammation and immune activation that persist despite suppressive therapy, seem to play an important role in non-AIDS morbidity. HIV-infected individuals continue to be at a higher risk of developing health issues despite cART [1, 5, 6]. This has diverted the entire focus of HIV research towards developing a cure. After the introduction of cART, it was estimated that HIV could be eliminated in 2-3 years with suppressive therapy [7]. However, despite the suppression of HIV viral load, the prospect for eradication of HIV diminished considerably and the persistence of a small but detectable pool of latently infected, resting CD4 T cells carrying replication-competent virus was documented in almost all study patients on cART [8]. Despite suppressive antiretroviral therapy, HIV remains as a latent reservoir with little or no viral expression, thereby rendering the virus undetectable to the host immune response. Latent cells are the major hurdle in curing HIV-1 infection. In latency, there is silencing of integrated proviral genome transcription through various pre and post-integration mechanisms. Though latency occurs rarely in 1 to 60 cells/million, it remains in individuals for years eluding immune response or antiretroviral

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therapy. HIV proviral transcription is activated in latently infected cells when they are stimulated by an antigen or are exposed to specific cytokines or chemokines. cART alone is unable to eradicate the virus, which resides in the reservoir and thought as a source for viral re-emergence after treatment interruption and also does not restore health [9, 10]. Therefore, it is paramount to develop an absolute cure for HIV infection to overcome the limitation of current therapy [11].

HIV reservoir serves as a major barrier to HIV cure, therefore the focus of HIV research has been shifted to finding tools for latent HIV reservoir eradication. Current strategies focus on breaking latency by inducing provirus to form viral particles and subsequently killing the infected cells by immune surveillance, while uninfected cells are prevented from infection by cART. This is known as shock-and-kill strategy [12]. There are certain therapeutic approaches to break HIV-1 latent reservoir such as chemokines, cytokines, HDAC inhibitors and apoptotic inducers, which specifically induce apoptosis in latently infected cells leading to cell death and immune clearance. Moreover, there are many classes of latency reversal agents (LRA) including protein kinase C agonist, HDAC inhibitors (Valproic acid, Suberoylanilide hydroxamic acid, Panobinostat), and bromodomain inhibitors, which induce proviral transcription reactivation through various signaling pathways [13, 14]. However, the introduction of LRA failed to show a significant decrease in reservoir size and also demonstrated a weak effect on HIV reactivation [15-19]. Therefore, there is a need for highly active LRAs with increased potential to reactivate latent HIV.

Helicobacter pylori (*H. pylori*) is a microaerophilic gram-negative bacterium with a global prevalence of 50%. It is able to grow and colonize in the human stomach at low pH. However, merely 10-15% of infected individuals develop symptomatic disease including gastric cancer [20]. Colonization of *H. pylori* leads to asymptomatic gastritis with increased infiltration of natural killer cells (NK cells), macrophage, dendritic cells (DC) and lymphocytes into gastric mucosa [21]. There are reports which show that urease secreted by *H. pylori* converts the helical form of *H. pylori* present in the stomach into coccoid form and this coccoid *H. pylori* through peyer's patches disrupts mucosal layer and infects T cells in gut [22]. These T cells differentiate into pro-inflammatory Th1 and Th17 cell subsets, as well as anti-inflammatory regulatory T cells (Tregs) [23-26]. Asymptomatic *H. pylori*-infected individuals have elevated levels of Tregs response, which is believed to be protective against allergy, asthma and inflammatory bowel diseases [27-29]. A study by Perry *et al.* showed that *H. pylori* infection is associated with the protection against tuberculosis [30]. These studies suggest that *H. pylori* infection can potentially modulate the immune system in a way that it could affect susceptibility of host for other infections or morbidities.

Recently, a higher prevalence of *H. pylori* was shown in HIV-1- infected patients in developing countries [31]. Moreover, there are reports which show that eradication of *H. pylori* facilitates immune reconstitution in HIV-1 infected patients [32] in contrast to a recent report, which shows that *H. pylori* increases CD4 cell count in HIV-1 infected patients and its association with decreased T cell activation [33]. However, there are no studies describing the mechanisms behind molecular events that take place during HIV-*H. pylori* co-infection. Therefore, we aim to study the impact of *H. pylori* infection on the latent HIV reservoir, using U1 monocytic cells line as a model of HIV latency. In our study, we have shown differential gene expression in *H. pylori*- stimulated latently HIV-infected U1 cells using RNA seq analysis. Our data suggest that *H. pylori* can modulate host innate immune response leading to reactivation of latent HIV.

2. MATERIALS AND METHODS

2.1. Cell Line and Cell Culture

Human monocytic cell line U937 and latently HIV-1 integrated monocytic cell line U1 were used for the study. U1 cells are derived from parental cell line U937 and show minimal consecutive expression of HIV-1 [34]. These cells were cultured in RPMI 1640 (Himedia) containing 10% Fetal Bovine Serum (FBS), 5mM L-glutamine, 500units/ml (2%) penicillin and 10µL/ml (1%) streptomycin containing complete media. Before infection, the cells were seeded at 1.5 x10^6 cells/ml in RPMI-1640 containing 10% FBS. The culture was incubated in 5% CO_2 at 37°C overnight.

2.2. *H. pylori* Culture

H. pylori strain used in this study is S62295. The *H. pylori* was spread on the surface of agar that contained Brain Heart Infusion (BHI) supplemented with 7% Fetal bovine serum and IsoVitaleX (4ul/ml). Antibiotics 10 mg/mL vancomycin, 6 mg/mL trimethoprim and 8 mg/mL amphotericin b were added and incubated under microaerophilic conditions (10% CO_2, 5% O_2, and 85% N_2) at 37°C. *H. pylori* was harvested and inoculated into brucella broth that included 7% heat inactivated Fetal Bovine Serum (FBS) containing 500 units/ml (2%) penicillin and 10µL/ml (1%) streptomycin, and was incubated at 37°C with agitation at 200 rpm for 48 h under microaerophilic conditions.

2.3. *H. pylori* Stimulation

Human monocytic cell line U937 and latently HIV-1 infected monocytic cell line U1 were cultured in RPMI 1640 medium that included 10% heat-inactivated FBS in a 5% CO_2 incubator at 37°C. In co-culturing experiment with bacteria, the cells were washed and resuspended to a density of 10^6 cells/ml in 6 well plate. The culture medium was supplemented with 10% FBS and cells were infected with *H. pylori* with MOI of 30 and incubated for 24hrs. In one of the experiments, the cells were infected with heat-killed *H. pylori* and water extract of *H. pylori.* The heat killed *H. pylori* was prepared by incubating *H. pylori culture at* 56°C water bath for 30min, followed by chilling on ice. The extract was then further incubated at 80°C water bath for 10min [35]. The bacteria were plated at MOI 30 on the BHI plate for seven days to check the viability of bacteria. The water extract was prepared from *H. pylori* culture plate as described [36]. Briefly, the *H. pylori* was harvested using a cotton swab and suspended in sterile distilled water. The suspension was centrifuged for 15 min at 12,000 rpm and the supernatant was stored at -20°C until further use. Water extract was brought to room temperature before use and centrifuged at

15,000 rpm for 20 min. The supernatant was filtered through a 0.2 μm syringe filter (Axiva sichem biotech). This protocol removes most of the high molecular weight factors such as membrane vesicles and intact flagella.

2.4. RNA Extraction and cDNA Synthesis

U937 and U1 cells were collected at 24h after co-culture with *H. pylori* strains. Total RNA was isolated using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. RNA quality and quantity were assessed by agarose gel electrophoresis and Nanodrop (Total RNA Nano; Agilent), respectively. The cDNA was generated from the total RNA using the Affinityscript cDNA synthesis kit (Agilent bioscience).

2.5. Quantitative Real-time PCR

Quantitative real-time PCR was used for the relative quantification of gene expression. The cDNA was used to check the expression of proinflammatory and anti-inflammatory cytokines. The 1μL template cDNA was amplified in a total of 20 μl PCR reaction that contained 10μl of 2x SYBR Green, forward and reverse primers (each at a concentration of 250nM), and RNase-free water. The thermal cycler conditions included 40 cycles with an initial denaturation at 95°C for 10 s and annealing at 60°C for 1 min. Primers used for HIV Gag gene were as follows: (forward: TCTGAAGGGATGGTTGTAGC; reverse: ACGATTCG-CAGTTAATCCTG). For M1/M2 polarization, primers were used for IL-1β [37], iNOS [38], IL-12p40 [39], CXCL10 [40], NLRP3 [41], Caspase1 [42], RELM1 [43], CCL17 [44], STAB1 [45], CD209 [46], CCL1 [47], IL-10 [48], TGF-β [49], SPHK1 [50], IL-8 [51], and IL-6 [52]. Amplification of the reference gene, β-actin [53] was measured for each sample as an internal control for normalization. The comparative △△CT method was used for the calculation of fold change in the respective gene expressions in the sample [54]. The samples were run in duplicates and independently repeated three times.

2.6. Western Blot Analysis

U1 cells were stimulated with *H. pylori* for 24 hours and lysed with NP40 lysis buffer containing a protease inhibitor cocktail (Sigma Aldrich, USA). Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad Laboratories, Hercules, CA) and transferred to nitrocellulose membranes (MDI). The primary antibodies were a mouse anti-human HIV-1 p24 Gag monoclonal antibody (NIH, USA), rabbit anti-human β actin polyclonal antibody (Cell Signaling Technologies, Beverly, MA). β-Actin was used as a loading control. The secondary antibody used was anti-mouse IgG (Sigma Aldrich, USA) and goat anti-rabbit IgG (Sigma Aldrich, USA) to detect HIV Gag and β-Actin, respectively. The experiment was independently performed three times.

2.7. High Throughput RNA Seq

Total RNA was sent for RNA-Seq using Illumina HiSeq 2500 system. The reference assembly and differential expression for the samples were analysed using the TopHat version 2.1.1 and Cufflinks version 2.2.1 software. The differentially expressed gene (DEG) list with top 100 significantly up-regulated and significantly down-regulated genes with $p<0.05$ and $log2FC>2$ for up-regulated and $log2FC<-2$ for down-regulated genes was used to plot heat map using pheatmap package in R. To visualize the total number of differentially expressed genes, Volcano plot was constructed as scatter plot using a p-value from t-test statistics on y-axis against log2FC on x-axis using Tmisc package in R. The samples were sent for sequencing in duplicate.

2.8. Pathway and Gene Ontology Analysis

The list of DEGs derived from the pairwise comparison between *H. pylori* stimulated U1 cells and unstimulated U1 cells was submitted to the Panther software (http://www.pantherdb.org) for GO category analysis based on biological process. The DEGs were selected based on significance by t-test ($p<0.05$). KOBAS (KEGG Orthology Based Annotation System) (http://kobas.cbi.pku.edu.cn) is an online tool for the prediction of gene or protein functional set enrichment and functional annotation. KOBAS was used to get relevant biological pathways using subcategory as KEGG (Kyoto Encyclopedia of Genes and Genomes) [55] pathway between *H. pylori* stimulated U1 cells and unstimulated U1 cells. The gene set enriched was used to analyse the interaction between the DEGs, which were significantly up-regulated and significantly down-regulated using the KEGG pathway. The significantly upregulated and downregulated genes were sorted, keeping the p-value threshold less than or equal to 0.05 and log2 Fold change more than 2 and less than 2, respectively.

2.9. Statistical Analysis

Statistical analysis were carried out in GraphPad Prism v.6. Values in the figures are expressed as means ± SE. The one-way ANOVA test was used to evaluate all parameters. Statistical significance level was set at $p< 0.05$ and FDR <0.005.

3. RESULTS

3.1. *H. pylori* Infection Increases HIV Transcription in U1 Cells

Given the fact that eradication of *H. pylori* infection results in the reconstitution of CD4 count in HIV-infected subjects [32], we hypothesized that *H. pylori* infection might be responsible for increased HIV replication. To determine the effect of coinfection on HIV-1transcription in latently infected U1 cells, we stimulated U1 cells with *H. pylori* at MOI 30 and RNA was isolated after 24hrs and assayed for HIV-1 Gag expression using qPCR. Coculture of *H. pylori* with U1 cells at MOI 30 induced HIV-1 transcription post 24h. Interestingly, *H. pylori* stimulation significantly increased HIV transcription in U1 cells up to 7 folds compared to the unstimulated U1 cells. ($p=0.001$, Fig. **1A**). This suggests that *H. pylori* could stimulate HIV replication in latently HIV-infected monocytes.

3.2. Increased Levels of IL-8, IL-1β, CXCL-10 and CCL1 in *H. pylori* Infected U1 Cells

To further de-mystify *H. pylori*-mediated upregulation of HIV transcription, transcriptional study of signature genes of

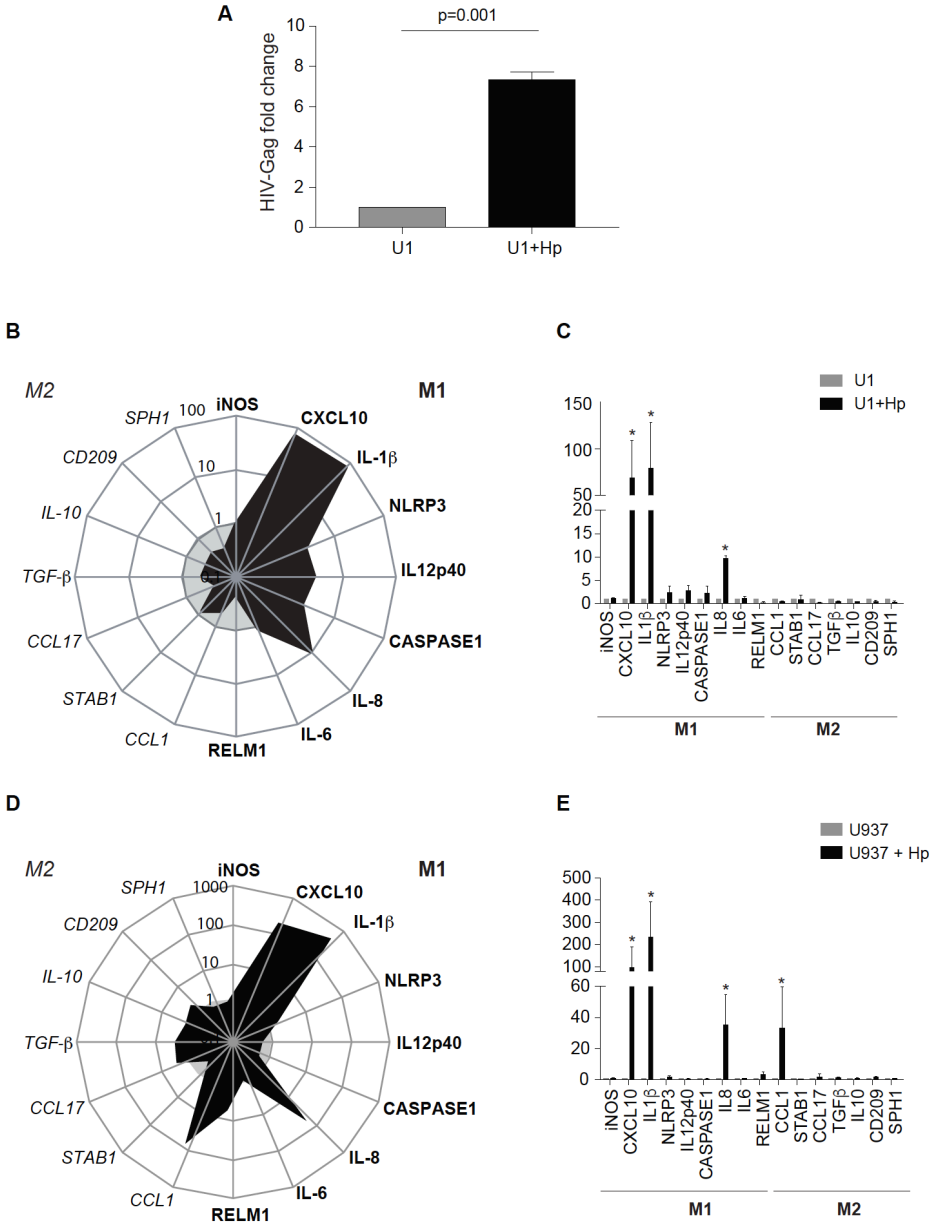


Fig. (1). qPCR analysis to study the gene expression profile during pre and post *H. pylori* stimulation. Data shows (**A**) Induction of HIV-1 Gag expression in *H. pylori*-stimulated U1 cells compared to unstimulated U1 cells. (**B**) Radar diagram and (**C**) bar diagram represent M1 polarization of *H. pylori*-stimulated U1 cells compared to control. (**D**) Radar diagram and (**E**) bar diagram represent mixed polarization (M1 and M2) of *H. pylori*-stimulated U937 cells compared to the unstimulated cells. Data are representative of three independent experiments. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

M1 and M2 macrophage in U1 cells -pre and post *H. pylori* stimulation was conducted to find out if the increase in HIV transcription following *H. pylori* stimulation affects macrophage polarisation. The induction of cytokine mRNA post *H. pylori* stimulation was examined using qPCR. The qPCR results clearly showed that *H. pylori* stimulation of U1 cells induced M1 polarisation as evident from the upregulation of M1-specific genes CXCL10 (68.6-fold change), IL-1β (79.2-fold change) and IL-8 (9.6-fold change) (Fig. **1B, C**). Further, the intent was to find out if the presence of HIV provirus or HIV replication contributes to *H. pylori*-mediated M1 polarisation of U1 cells. We stimulated U937 cells, a parent monocytic cell line of U1 without latent HIV, with *H. pylori* for 24 h and assessed the level of M1 and M2-specific gene transcripts. Interestingly, *H. pylori* stimulation of U937 resulted in mixed M1 and M2 phenotypes as shown by the increased levels of M2-specific gene transcripts: CCL1 (33.2-fold change) and RELM-1 (3.5-fold change), in addition to M1-specific gene transcripts: CXCL10 (98.4-fold change), IL-1β (235.4-fold change) and IL-8 (35.5-fold change) (Fig. **1D, E**).

Further, in order to investigate whether *H. pylori*-driven upregulation of HIV expression is restricted to transcription or there is upregulation at protein level too. Western blot analysis confirmed high protein expression of HIV-1 p24 Gag (24 kDa) post *H. pylori* stimulation compared to unstimulated cells (Fig. **2A**).

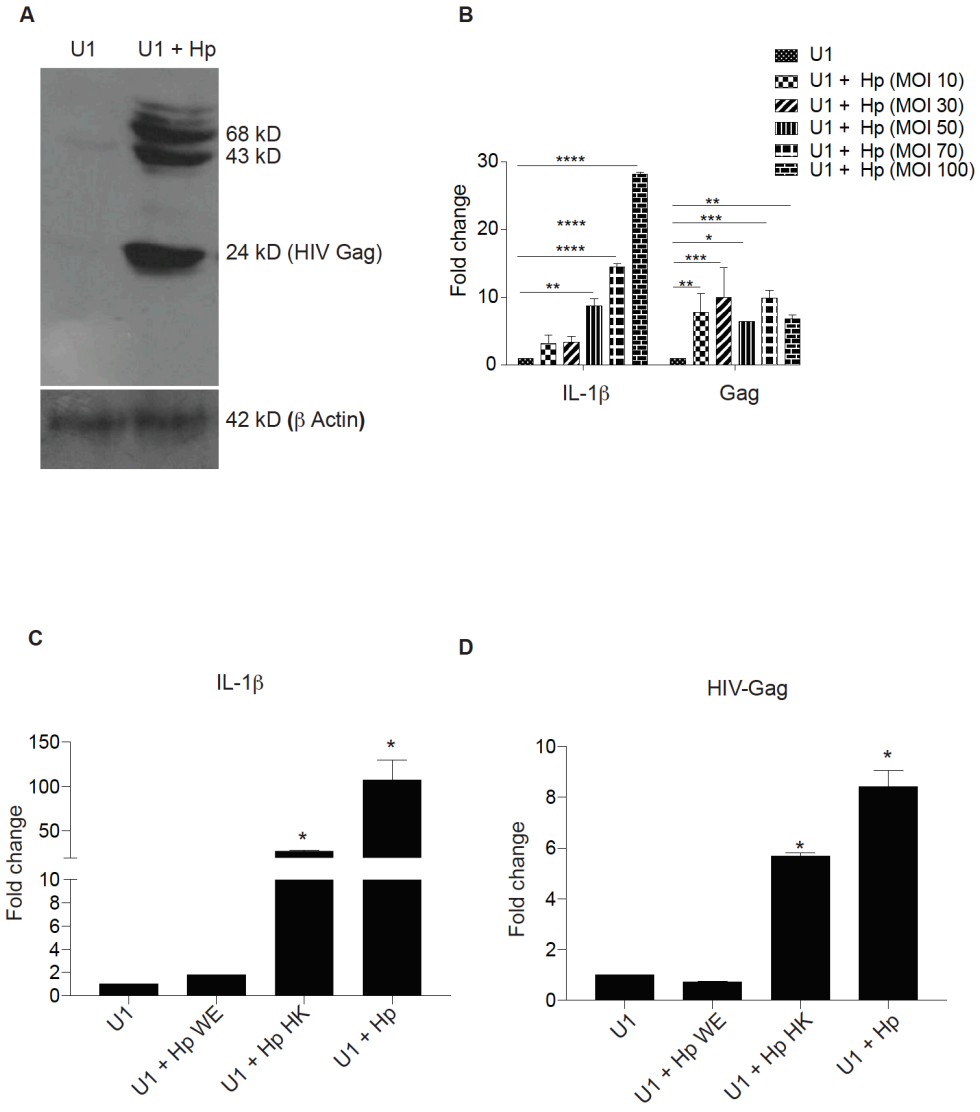


Fig. (2). Effect of different MOI and Heat killed *H. pylori* on HIV reactivation. The data shows (**A**) induction of HIV-1 p24 Gag expression (24kDa) in *H. pylori*-stimulated U1 cells compared to unstimulated. β-actin (42kDa) was used as a loading control. (**B**) Effect of different concentration of *H. pylori* from MOI 10 to MOI 100 on latently infected U1 cells. The data shows the expression of (**C**) IL-1β and (**D**) HIV Gag gene by heat killed (HK) or live bacteria (Hp) and water extract (WE) in *H. pylori*-stimulated latently HIV-infected U1 cells compared unstimulated U1 cells. Data are representative of three independent experiments. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.3. Effect of *H. pylori* Dose on IL-1β and HIV Transcription Levels

Among upregulated cytokines/chemokines in *H. pylori*-stimulated U1 cells, IL-1β was found to be highly upregulated. IL-1β has been previously known to induce HIV-1 expression in chronically infected U1 cells [56]. Therefore, the increase in IL-1β levels in *H. pylori*-stimulated U1 cells suggests that IL-1β could potentially play a major role in the reversal of HIV latency in *H. pylori* stimulated U1 cells. Having observed the increase in HIV transcription levels in U1 cells on *H. pylori* stimulation, we sought to check if *H. pylori* stimulation of U1 cells increases IL-1β and HIV transcription in a dose-dependent manner. We stimulated U1 cells with *H. pylori* at MOI 10, 30, 50, 70 and 100 for 24h and assessed the levels of IL-1β and HIV-gag transcription at different MOIs. We observed a dose-dependent increase in IL-1β transcription, however, HIV transcription level was independent of *H. pylori* dose (Fig. **2B**).

3.4. Exposure to Heat-killed *H. pylori* also Augments HIV Transcription and IL-1β Expression in U1 Cells

To further understand molecular interactions leading to the reactivation of HIV transcription in *H. pylori*-induced U1 cells, we assessed if cell-cell interaction is crucial for an increase in HIV transcription or latency reversal could also be achieved *via* soluble factors released by *H. pylori*. To address this question, we stimulated U1 cells with water extract prepared from *H. pylori* culture [36], heat-killed *H. pylori* [35] and live bacterium at MOI 30. Our results indicated that water extract failed to induce both IL-1β and HIV-Gag transcription, while heat-killed and live *H. pylori* significantly increased IL-1β and HIV gag transcription, suggesting that

cellular interaction between *H. pylori* and U1 cells is crucial for HIV transcription (Fig. **2C,D**).

3.5. RNA Seq Analysis Reveals Differential Gene Expression in *H. pylori*-stimulated U1 Cells Compared to Unstimulated Control

In order to gain insight into the mechanism and assess potential pathways that lead to *H. pylori*-mediated upregulation of HIV transcription, we performed high through-put RNA Seq using Illumina HiSeq 2500 system to study transcriptomics following *H. pylori* stimulation. Based on the RNA-Seq data performed on Illumina platform, total raw data obtained were 42 million and 57 million reads for unstimulated U1 and *H. pylori* stimulated U1 cell line respectively with an average of 78.00% reads from unstimulated U1 and 77.25% of reads from *H. pylori* stimulated U1 uniquely mapped to the human genome. The reference assembly and differential expression for both the samples were analysed using the Top Hat version 2.1.1 and Cufflinks version 2.2.1 software. Pairwise comparison was carried out between *H. pylori* stimulated U1 and unstimulated U1 cells; a total of 19,398 differentially expressed genes were identified and the expression was measured as fragment per kb of exon per million mapped reads (FPKM). To assess differentially expressed genes, the relative value of fold change (FC) >2 and p value< 0.05 were used as a factor to evaluate the significantly expressed gene. The transcriptome analysis revealed that 298 genes were significantly expressed in *H. pylori* stimulated U1 cells compared to unstimulated U1 with p value < 0.05. Within this group, 197 genes were significantly upregulated with fold change >2, p value< 0.05 and 101 genes were downregulated with fold change < -2, p value <0.05 in *H. pylori* stimulated U1 cells (Table **1**). The list of genes, their associated fold change and p-values are provided in Supplementary tables **S1** and **S2**. Using the list of total DEGs, the volcano plot was constructed to distinguish significantly expressed genes from non-significant genes using Tmisc package in R where Green dot represented significantly down-regulated genes, Red dot represented significantly up-regulated genes and black dot represented non-significant genes (Fig. **3A**). Global gene expression profile was analysed for the comparison of genes expressed in *H. pylori* stimulated U1 cells with the unstimulated U1 by constructing Heat map for top 100 significantly expressed gene with p-value <0.05 and Fold change ≥2/≤-2 using pheatmap package in R (Fig. **3B**). To gain insight into biological significance, the significant DEGs were identified (p value<0.05) and were submitted to panther software version14.0 for GO analysis. The functional classification of differentially expressed genes based on the biological process from gene ontology (GO) was enriched in multiple GO categories, mainly granulocyte chemotaxis, cellular responses to lipopolysaccharides, positive regulation of NFκB transcription factor and cytokine mediated signalling (Table **2**). The data show that the genes enriched in *H. pylori* stimulated U 1 cells compared to unstimulated U1 cells were predominantly involved in immune signalling, transcriptional regulation and metabolic process across the DEGs list. IL-1β, CXCL6, CXCL8 chemokines were the significantly upregulated genes expressed in the biological process of GO analysis among other expressed genes, which showed proinflammatory immune response. High level of IL-1β, IL-6 and IL-8 has been observed in viral infection and is involved in disease progression [57, 58] showing that IL-1β, CXCL6 and CXCL8 play a prominent role in latency reversal *via H. pylori* infection.

Table 1. Number of differentially expressed genes (DEGs) and differentially regulated KEGG pathways among in *H. pylori*-HIV co-infected cells compared to the control. The DEGs included had p<0.05 and Fold change >2. The KEGG pathways were enriched using KOBAS 3.0. The HH represents HIV-*H. pylori* co-infected U1 cells and H represents U1 cells with latent HIV only.

-	-	HHvsH
DEGs	Up-regulated	197
	Down-regulated	101
	Total	298
KEGG pathways	Up-regulated	152
	Down-regulated	104
	Total	256

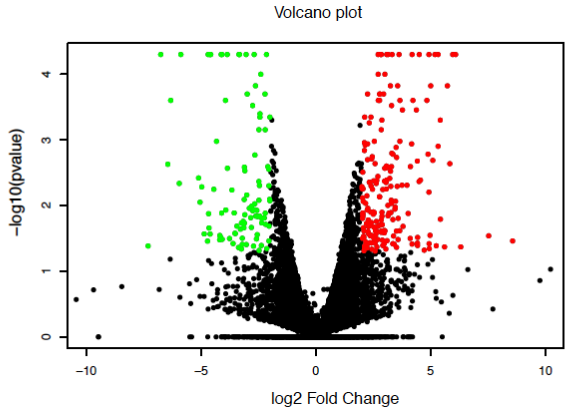
3.6. Pathway Analysis of Differentially Expressed Genes

DEGs were subjected to pathway analysis using KOBAS version 3.0 to get enriched KEGG pathways. A list of up-regulated genes (P<0.05 and log2FC>2) and down-regulated genes (p<0.05 and log2FC<-2) was separately uploaded to the KOBAS software. The molecular interaction revealed that for pairwise comparison of *H. pylori*-stimulated U1 cells compared to unstimulated cells, 152 and 104 KEGG pathways were enriched in significantly upregulated genes (p<0.05 and FC>2) and downregulated genes (p<0.05 and FC<-2), respectively (Table **1**). Top 10 pathways were obtained on the basis of significantly up-regulated and down-regulated genes, respectively (Tables **3** and **4**). The topmost network from pathway analysis of up-regulated genes showed the upregulation of NF-kB signalling pathway, which showed significantly elevated levels of IL-1β and IL-8 induced through NFκB transcription regulators (Fig. **4**). These cytokines are involved in inflammation and may promote HIV-1 replication in latently infected U1 cells. Further, we have also shown the topmost network for down-regulated genes, which play a role in the regulation of complement and coagulation cascade (Fig. **S1**). We found IL-1β as a predominately regulated molecule in all top pathways derived from RNASeq analysis (data not shown). This is consistent with our qPCR data where IL-1β is found to be upregulated on *H. pylori* stimulation (Fig. **1**). Our data suggest that cytokines like IL-1β and IL-8 that are induced as a result of *H. pylori* stimulation, may play a very crucial role in HIV reactivation.

4. DISCUSSION

In this study, we showed that *H. pylori* stimulation profoundly activates HIV in latently HIV-infected myeloid cell

A.



B.

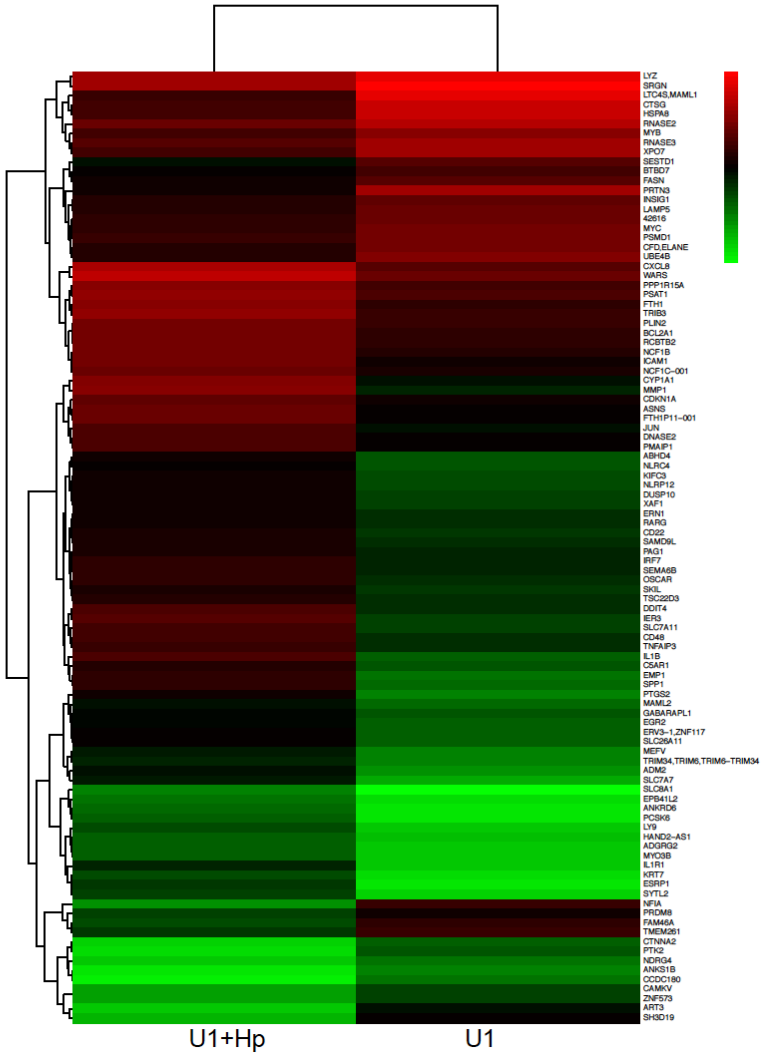


Fig. (3). Differential regulation of pathways discriminating between HIV and HIV-*H. pylori* co-infection identified by transcriptome analysis of U1 cells and pathway analysis among DEGs. Data compares list of differentially expressed genes (DEGs) in U1 and *H. pylori*-stimulated U1 cells. (**A**) Volcano plot of statistical significance against fold change between U1 and *H. pylori*-stimulated U1 cells. Red dots represent DEGs significantly upregulated, Green dots represents DEGs significantly downregulated and Black dots represents non-significant genes with p value>0.05. (**B**) Heat map for differentially expressed gene are compared between control and HIV-*H. pylori* coinfected cells line plotted using R ggplot2. Each row represents genes and each column represents samples. Red indicates higher expression and green indicates lower expression genes with Fc>2 and <-2 and p-value<0.05. Data are representative of two independent experiments. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 2. Enrichment of GO biological process categories. The enriched genes were significantly overrepresented in the list of DEGs in *H. pylori* co-infected HIV cells compared to the control (p<0.05 and FDR<0.05). GO biological process were analysed using panther version 14.0.

Biological Process	Gene Ontology	Fold Enrichment	P-Value	FDR	Core Enriched Genes
Granulocyte chemotaxis	GO:0071621	5.94	2.15E-05	2.96E-03	CXCL6, CXCL8, CCL2, CXCL3, IL1B, CCL26, CCL3, CCL20, CCL1, CXCL2
Cellular response to lipopoly-saccharide	GO:0071222	5.73	4.51E-04	3.23E-02	CXCL6, CXCL8, CXCL3, CD14, IL1B, CD86, CXCL2
Positive regulation of NF-kappaB transcription factor activity	GO:0051092	4.85	4.88E-04	3.35E-02	CD14, NFKB2, TLR7, RELB, IL1B, TNFRSF19, IRAK2, NLRC4
Cell chemotaxis	GO:0060326	4.83	1.99E-06	5.94E-04	CXCL6, CXCL8, CCL2, CXCL3, CCRL2, CCR1, VEGFA, IL1B, CCL26, CCL3, CXCR3, CCR2, CCL20, CCL1, CXCL2
Cytokine-mediated signaling pathway	GO:0019221	3.4	4.32E-04	3.22E-02	CXCL6, CXCL8, CCL2, CXCL3, F3, IL1B, CCL26, CCL3, IRAK2, CCL20, CCL1, CXCL2

Table 3. Pathway analysis using significantly up-regulated (p<0.05 and FC>2) differentially expressed genes. The KEGG pathways were analysed using KOBAS version 3.0. with P<0.05 and FDR<0.05 using Fisher's exact test. The top 10 pathways are listed based on p value.

Pathways	ID	Genes	P-Value	FDR
Pathways in cancer	hsa05200	13	0.00038	0.005805
NF-kappa B signaling pathway	hsa04064	10	7.76E-08	1.18E-05
Metabolic pathways	hsa01100	10	0.821779	0.843989
TNF signaling pathway	hsa04668	9	2.03E-06	0.000121
Rheumatoid arthritis	hsa05323	8	9.35E-06	0.000355
Osteoclast differentiation	hsa04380	8	5.78E-05	0.001756
Apoptosis	hsa04210	8	0.000131	0.002926
Influenza A	hsa05164	8	0.000545	0.005805
Transcriptional misregulation in cancer	hsa05202	8	0.000573	0.005805
Herpes simplex infection	hsa05168	8	0.000761	0.007231

Table 4. Pathway analysis using significantly down-regulated (p<0.05 and FC>-2) differentially expressed genes. The KEGG pathways were analysed using KOBAS version 3.0. with P<0.05 and FDR<0.05 using Fisher's exact test. The top 10 pathways are listed based on p value.

Pathways	ID	Genes	P-Value	FDR
Metabolic pathways	hsa01100	8	0.30158	0.49478
PI3K-Akt signaling pathway	hsa04151	5	0.033428	0.447981
Hippo signaling pathway	hsa04390	4	0.007728	0.344734
Complement and coagulation cascades	hsa04610	3	0.006738	0.344734
Bacterial invasion of epithelial cells	hsa05100	3	0.009944	0.344734

(Table 4) contd….

Pathways	ID	Genes	P-Value	FDR
Amoebiasis	hsa05146	3	0.018338	0.447981
Transcriptional misregulation in cancer	hsa05202	3	0.063433	0.468753
Epstein-Barr virus infection	hsa05169	3	0.095542	0.468753
Focal adhesion	hsa04510	3	0.101789	0.468753
MAPK signaling pathway	hsa04010	3	0.142424	0.469822

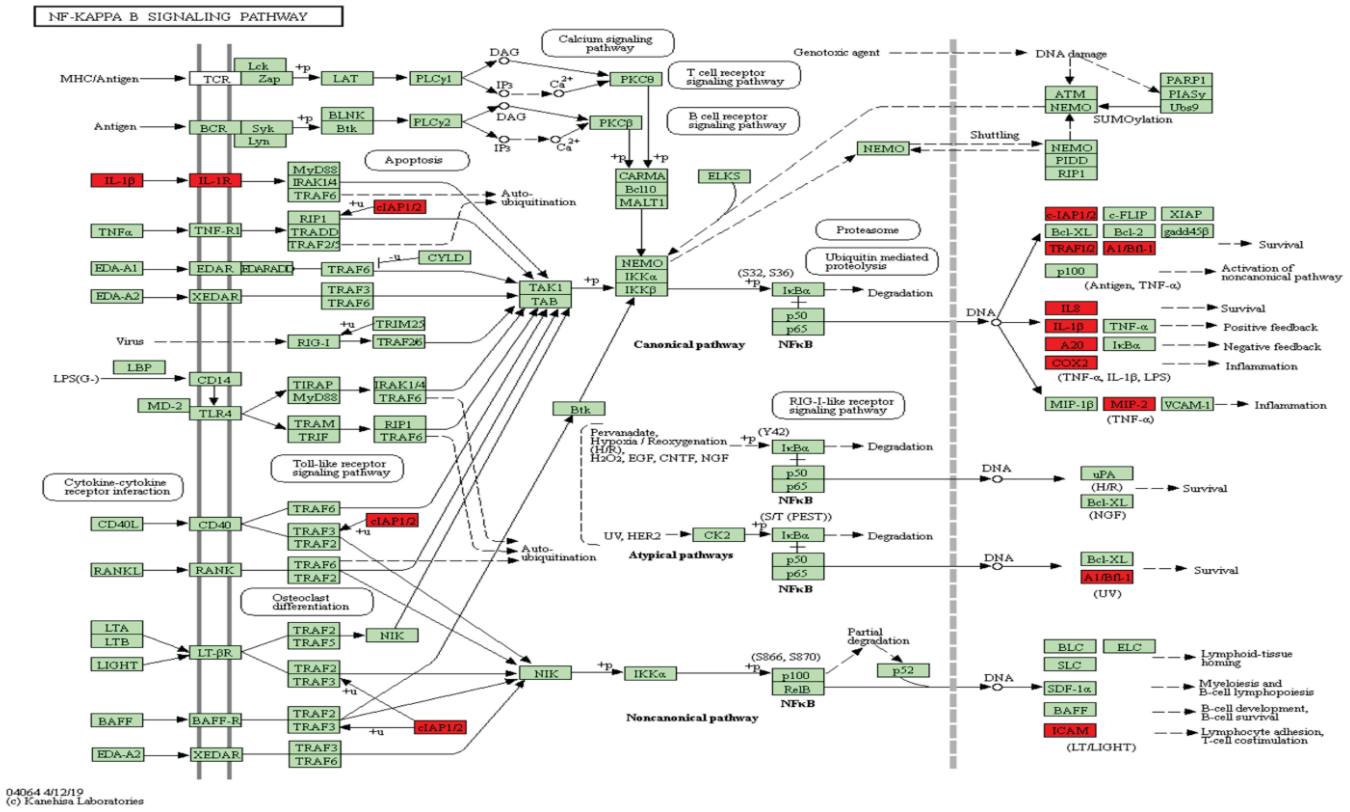


Fig. (4). Co-ordinately up-regulated genes of NF-Kappa B signalling pathway in HIV-*H. pylori* co-infected cells *versus* control cells. The pathway figure is adapted from Kyoto Encyclopedia of Gene and Genomes. Red highlighted are protein encoded by the co-ordinately upregulated genes in HIV-*H. pylori* co-infected cells *versus* control. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

line. Further, using RNA seq analysis, we showed that stimulation with *H. pylori* differentially expressed 298 genes in U1 cells compared to unstimulated cells, and KEGG pathway analysis revealed 152 and 104 KEGG pathways that were enriched from significantly upregulated and downregulated genes, respectively. Moreover, data revealed that most prominent cytokines like IL-1β, CXCL8 were involved in a majority of the regulated inflammatory pathways that have a potential role in reversing HIV latency in myeloid cells.

Latent HIV reservoir is a major hurdle to absolute HIV cure in the era of antiretroviral therapy. It is necessary to find some promising approach to reactivate the latent reservoir and kill the infected cells using antiretroviral therapy. There are many reports that demonstrate the role of LRA in reactivating latently infected cells but there is not much achievement in minimizing the reservoir size. Moreover, most of these previous studies on LRAs have focused on T cell reservoir [13], therefore, it is imperative to identify potent HIV latency reversal agents (LRA) that are effective in reversing latency in myeloid cells, which may be used with ART.

Several reports have described myeloid cells as an important latent reservoir [11, 59, 60]. Monocytes are early targets for HIV infection. Cells of myeloid lineage have been shown to be more resistant to HIV-induced cytopathic effects, hence they can harbor the virus for a long time [60]. These monocytes differentiate into macrophages when they enter into tissues such as microglial cells in the brain [60]. Macrophages are present in most organ systems, hence they can potentially disseminate the virus throughout the body as an important viral reservoir.

Macrophage polarisation in the presence of polarizing cytokines, TNF-α plus IFN-γ has been shown to regulate the HIV transcription in monocyte-derived macrophage (MDM)

with a reduced capacity to support productive CCR5-dependent (R5) HIV-1 infection [61]. Interestingly, we observed that *H. pylori* stimulation of U1 cells upregulates HIV transcription and replication in polarised M1 stage of U1 cells. It is now known that HIV induction can be mediated by several cytokines. A previous study showed that IL-β can induce the expression of HIV in chronically HIV infected U1 cells [62]. In addition, IL-8/CXCL8 and CXCL10 can also upregulate HIV production [63-65]. The cytokines IL-1β, CXCL8 and CXCL10 have been shown to play crucial roles in various other pathological conditions like cancer, inflammation and autoimmune disorders [66-73]. We observed elevated levels of IL-1β, CXCL8 and CXCL10 in *H. pylori*-stimulated U1 cells as compared to unstimulated cells that suggests that reactivation of HIV in U1 cells may potentially be driven by these chemokines. The transcriptome analysis revealed the presence of IL-1β and CXCL8 as central molecules in the reactivation of HIV transcription post *H. pylori* stimulation while no significant difference was observed in CXCL10 expression.

At the pathway level, a panel of significantly altered pathways was identified, which were found to involve proinflammatory cytokine IL-1β as a predominantly expressed cytokine. IL-1β activates the canonical NFκB signalling pathway, which was found to be the most significant pathway enriched in upregulated genes. NFκB has previously been shown to be central to signal-dependent transcription of HIV [74, 75]. It initiates transcription by recruiting Histone acetyltransferases to the HIV-LTR [76]. Consistent with increased transcription level, an induction of downstream proinflammatory cytokines, IL-1β, IL-6, and TNF-α was also observed during HIV-1 infection in several *in vitro* studies [77-79]. Our study also revealed that in addition to live/intact bacteria, heat-killed *H. pylori* can also reactivate HIV transcription. This suggests that bacterial cell components released by heat-inactivated bacteria are sufficient to activate HIV transcription.

CONCLUSION

H. pylori infection seems to reactivate HIV in latently HIV-infected monocytes *in vitro* with an increased level of proinflammatory cytokines IL-1β and CXCL8. Our findings establish the basis of *in vivo* research in the future to study the effect of *H. pylori* in HIV pathogenesis and explore the therapeutic potential of *H. pylori* to reduce the size of HIV reservoirs.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data supporting the findings of the article is available in the NCBI's Sequence Read Archive (SRA) at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA594294, reference number [SRR10665512, SRR10665513, SRR10665514, and SRR10665515].

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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