Human Cytomegalovirus-Induced Interleukin-10 Production Promotes the Proliferation of Mycobacterium massiliense in Macrophages

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Human cytomegalovirus (HCMV) exploits the interleukin-10 (IL-10) pathway as a part of its infection cycle through the manipulation of the host IL-10 signaling cascade. Based on its immunomodulatory nature, HCMV attenuates the host immune response and facilitates the progression of co-infection with other pathogens in an immune-competent host. To investigate the impact of HCMV infection on the burden of non-tuberculous mycobacteria (NTM), whose prevalence is growing rapidly worldwide, macrophages were infected with HCMV and further challenged with Mycobacterium massiliense in vitro. The results showed that HCMV infection significantly increased host IL-10 synthesis and promoted the proliferation of M. massiliense in an IL-10-dependent manner. Transcriptomic analysis revealed that HCMV infection dampened the regulatory pathways of interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukin-1 (IL-1), consequently abrogating the immune responses to M. massiliense coinfection in macrophages. These findings provide a mechanistic basis of how HCMV infection may facilitate the development of pathogenic NTM co-infection by upregulating IL-10 expression.

Keywords: human cytomegalovirus, macrophage, Mycobacterium massiliense, interleukin-10, non-tuberculous mycobacteria

INTRODUCTION

Human cytomegalovirus (HCMV) is a species-specific beta-herpesvirus that infects the majority of the world's population (1). The general absence of HCMV disease imposes an extraordinarily large immunological burden on its infected host (2). The ability to establish and maintain a persistent infection in the presence of antiviral immunity requires the contribution of the capacity of HCMV to encode immune evasive proteins that alter cellular signaling and activation (3). The capacity of HCMV to successfully infect the host and cause disease is partially obtained from the cytomegalovirus-encoded human interleukin-10 (cmvIL-10) (4). The expression of cmvIL-10,
encoded by the UL111A gene, during productive HCMV infection is known to upregulate host IL-10 production in monocytes via phosphatidylinositol 3-kinase (PI3K)/signal transducer and activator of transcription 3 (STAT3) signaling pathway. Meanwhile, macrophages are important sites for HCMV replication (5), and act as notable producers of IL-10 in our body (6). Being the most potent anti-inflammatory cytokine (7, 8), this led us to consider that HCMV infection in macrophages might facilitate co-infection of Mycobacterium abscessus, which proliferates mostly inside the macrophages and is the most drug-resistant species among the non-tuberculous mycobacteria (NTM) (9). Recent reports support this idea in that they have noticed the convergent epidemiology of tuberculosis and HCMV infection (10, 11). Therefore, we assessed the effect of HCMV infection on the proliferation of Mycobacterium massiliense, a M. abscessus subspecies, in macrophages in order to understand the participation of HCMV-mediated immune modulation as a risk factor for coinfection with NTM and a high burden of NTM.

MATERIALS AND METHODS

Virus and Bacteria Strains

Human cytomegalovirus Towne (ATCC VR-977) and UL32-EGFP-HCMV-TB40E (ATCC VR-1578) strains were maintained as described previously (12, 13). M. massiliense CIP strain (ATCC 108297) was obtained from CIP (Collection of Institute Pasteur). M. massiliense was grown in Middlebrook 7H9 broth (BD Biosciences) supplemented with 0.2% glycerol (Sigma-Aldrich), 10% oleic acid-albumin-dextrose-catalase (OADC; Thermo Fisher Scientific), and 0.05% Tween 80 (Sigma-Aldrich). Cultures were incubated at 37°C with constant shaking (150 rpm) overnight to reach an optical density of 0.5–0.7 at 600 nm (OD600). Collected mycobacteria were homogenized and stored at −80°C.

Cell Culture

The human acute monocytic leukemia THP-1 (ATCC TIB-202) cell line was obtained from the ATCC and maintained in RPMI media containing 10% FBS (Gibco) and 1% penicillin-streptomycin (PS; Gibco) at 37°C in a humidified atmosphere with 5% CO₂. Differentiation of THP-1 cells into macrophages was performed by incubating the cells with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) for 2 days, and then, the media was changed to that without 1% PS for 1 day. PMA-differentiated THP-1 cells (THP-1 macrophages) were infected with HCMV Towne (otherwise indicated) or TB40E strains (in case of analyzing GFP expression) at a multiplicity of infection of 10 (MOI = 10) for 3 h, and the media was changed to establish HCMV infection. In case of obtaining HCMV culture supernatant, THP-1 macrophages were infected with HCMV Towne for 3 h, and the media was changed and further incubated for 24 h before collecting culture supernatant. M. massiliense was infected at (MOI = 2) 24 h post-HCMV infection. After 1 h of M. massiliense infection, cells were washed with PBS 2 times to remove extracellular bacteria and cultured with complete media without antibiotics for 1–3 days. For quantification of colony-forming units (CFUs), the infected cells were lysed in PBS containing 0.1% Triton X-100 (Sigma-Aldrich) and plated on LB agar.

Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors (Approved IRB No. C-1306-0210494) by Ficoll-Hypaque Plus (GE Healthcare) gradient centrifugation. Freshly isolated PBMCs were seeded and infected with HCMV at a MOI of 10 for 24 h, and the media was changed to establish HCMV infection. M. massiliense was infected at (MOI = 2) 24 h post-HCMV infection.

Recombinant Proteins and Neutralizing Antibodies

Recombinant cmvIL-10 was obtained from R&D systems. Purified viral HCMV IL-10 antibody (aClL-10, polyclonal goat IgG, R&D Systems) and human IL-10 antibody (shIL-10, clone 23738, R&D Systems) were used at 10 µg/mL to neutralize cIL-10 and hIL-10 protein. Human IL-10 receptor alpha neutralizing monoclonal antibody (shIL-10R, clone 37607, R&D Systems) was used at 10 µg/mL. Corresponding isotype controls for neutralization experiments were obtained from R&D Systems.

Immunofluorescence Staining

Cells were fixed in 4% paraformaldehyde (PFA) for 15 min and treated with 0.1% Triton X-100 for 5 min. After being blocked in PBS containing 5% FBS and 0.3% Triton X-100 for 1 h, the cells were incubated at 4°C overnight with the anti-IE antibody (a gift from E-S Huang) (14). The cells were rinsed and washed three times with PBS and incubated with the Alexa Fluor 594-conjugated anti-mouse IgG (Thermo Fisher Scientific) for 1.5 h. Finally, the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). To observe M. massiliense infection, M. massiliense cells were stained with a 5 µM of Vybrant CFDA-SE (CFSE) cell tracker kit (Thermo Fisher Scientific) for 30 min at 37°C, washed two times and suspended in complement media before infection. Fluorescence was observed under a Leica TCS SP8 confocal microscope (Leica Microsystems). For quantification of the infection burden, the number of bacteria per cell was counted in three individual images per sample.

Flow Cytometry

The HCMV TB40E-infected THP-1 cells and PBMCs were analyzed using an LSRFortessa™X-20 (BD Biosciences). PBMCs were stained with following antibodies; monoclonal antibodies to human CD45 (HI30), CD3 (HIT3a), CD19 (HIB19), CD11b (ICRF44), and CD14 (MfP9) all obtained from BD Biosciences. Data were analyzed using the FlowJo Software (version 7.6.2).

Quantitation of HCMV Copy Number

The copy number of HCMV was determined by real-time polymerase chain reaction (PCR). Sample DNA was prepared with Media kit (Qiagen) from HCMV-infected THP-1 cell lysates according to the manufacturer’s recommendation. Real-time PCR reactions were performed using a Taqman MasterMix (Applied Biosystems) with 10 pmol HCMV US17 primer pairs,
(5′-GGG TGT TTT GCA GCC TCT GCA-3′) and (5′-AGT TTG TGC CCC AAC GGT A-3′), 10 pmol probe, FAM-5′-TGA TCG GGC TTA TCG GTG TCT TGA TC-3′-TAMRA, and sample DNA in a 20 μL reaction with an ABI QuantStudio 5 sequence detection system (Applied Biosystems). Primers and probes were synthesized commercially at Bioneer (Daejeon, South Korea). Reactions were performed under standard universal reaction conditions; hot start cycle of 10 min at 95°C, and 40 cycles of denaturation for 10 s at 95°C and annealing and extension for 60 s at 60°C. Standard curve for the quantitation of HCMV was obtained with the serially diluted samples of the known amount of HCMV.

**Gene Expression Analysis and RNA Sequencing**

Total RNA was solubilized in TRIzol reagent (Invitrogen) and extracted according to the manufacturer’s instructions. Messenger RNA was reverse transcribed into cDNA with reverse transcription kits (Enzymonics) and quantitative real-time PCR for UL111A and IL10 were performed using TaqMan PCR PreMix or SYBR Green PCR PreMix (Enzymonics) on an ABI PRISM 7900 (Applied Biosystems). The primer sequences used were as follows: UL111A (hcmvIL-10) forward: 5′-TGT TGA GGC GGT ATC TGG AGA-3′; UL111A reverse: 5′-CCG TCT TGA GTC CGG GAT AG-3′; IL10 probe: 5′-CCG GTT TCC CGC AGG CGA CC-3′; IL10 forward: 5′-GCC TAA CAT GCT TCG AGA TC-3′; IL10 reverse: 5′-TGA TGT CTG GGT CCT GTT TC-3′. For RNA sequencing analysis, total RNA was isolated, and the library preparation was performed using the NGS service provided by Ebiogen Inc. (Seoul, South Korea). In brief, each 500 ng total RNA were prepared and an oligo-dT primer containing an Illumina-compatible sequence at its 5′ end was hybridized to the RNA and reverse transcription was performed. After degradation of the RNA template, second strand synthesis was initiated by a random primer containing an Illumina-compatible linker sequence at its 5′ end. The double-stranded library was purified by using magnetic beads to remove all reaction components. The library was amplified to add the complete adapter sequences required for cluster generation. The finished library is purified from PCR components. High-throughput sequencing was performed as single-end 75 sequencing using NextSeq 500 (Illumina). QuantSeq 3′ mRNA-Seq reads were aligned using Bowtie2 (15). Bowtie2 indices were either generated from genome assembly sequence or the representative transcript sequences for aligning to the genome and transcriptome. The alignment file was used for assembling transcripts, estimating their abundances and detecting differential expression of genes. Differentially expressed gene transcripts, which encode the

**Gene classification** was based on searches done by DAVID1 method using EdgeR within R (17) using Bioconductor (18). Alignments using coverage in Bedtools (16). The RC (Read Count) data were processed based on quantile normalization method using EdgeR within R (17) using Bioconductor (18). Gene classification was based on searches done by DAVID1, and genes that changed at least fourfold (p < 0.01) were analyzed for enrichment of gene ontology (GO) biological processes. For the hierarchical clustering and GO analysis, we used the MeV software and applied Euclidean distance and average linkage clustering for obtaining hierarchical clustering. Enriched terms that passed FDR < 20% were reported. For the Gene Set Enrichment Analysis (GSEA) for gene expression data, Molecular Signatures Database (V7.0) was used based on computing overlaps with GO gene sets (C5), obtained from the Broad Institute. All sequencing data can be found at the Gene Expression Omnibus (GEO) database (GEO accession number: GSE141236).

**ELISA**

Culture supernatants were collected, centrifuged at 587 g for 5 min to remove particulates, and stored at −80°C until ELISA was performed. Human IL-10 was measured using the ELISA Duoset system (R&D Systems) according to the manufacturer’s instruction.

**Statistical Analysis**

The Student’s t-test, one-way ANOVA or two-way ANOVA test were performed to determine statistically significant differences between groups using the GraphPad Prism 6 (GraphPad Software). Bonferroni’s or Tukey’s multiple comparisons test between all possible combinations were performed as post-tests for one-way ANOVA or two-way ANOVA, respectively. A value of p < 0.05 was deemed to be statistically significant.

**RESULTS AND DISCUSSION**

To establish HCMV infection in macrophages, THP-1 cells differentiated using PMA for 3 days (THP-1 macrophages) were infected with the HCMV TB40E strain at a MOI of 10, which expresses enhanced green fluorescence protein (GFP) at the tegument. As a result, we detected spot punctuated IE expressions at the nucleus of THP-1 cells from 3 h post infection (hpi), and their further dissemination into the entire nucleus within 24 hpi (Figure 1A). HCMV efficiently replicated both in THP-1 macrophages (Figure 1B) and monocytes of human peripheral blood mononuclear cells (PBMCs) (Supplementary Figures S1A,B) from 24 to 96 hpi, as determined by analyzing the GFP intensity using flow cytometry and the viral copy numbers within THP-1 macrophages using real-time PCR (Figure 1C). Next, we examined the effect of HCMV infection in macrophages on the proliferation of *M. massiliense* infection. Uptake efficiency of *M. massiliense* infection (MOI = 2) for the initial 3 h of incubation were similar between the control and the HCMV Towne strain-infected macrophages (data not shown). However, we noticed that HCMV-infected macrophages had more intracellular bacteria from 24 hpi, and showed obviously increased bacterial burden at 72 hpi as determined by fluorescent particle numbers in the cells under a fluorescence microscope and by counting the CFUs of *M. massiliense* in the cell lysates (Figures 1D,E).

To investigate whether HCMV uses IL-10 signaling to promote *M. massiliense* proliferation in macrophages, we first examined the UL111A gene transcripts, which encode the
cmvIL-10 in the HCMV-infected macrophages, using real-time PCR. As expected, HCMV infection induced UL111A transcription at 12 hpi (Supplementary Figure S2A). We next analyzed the expression of human IL-10 and found that HCMV infection significantly elicited host IL-10 synthesis both in THP-1 macrophages (Figures 1F,G) and PBMCs (Supplementary Figure S2B). Treatment of recombinant cmvIL-10 (Supplementary Figures S2C–E) or culture supernatant of HCMV-infected macrophages (Supplementary Figures S2F,G) directly induced human IL-10 synthesis, and the presence of neutralizing antibodies for the cmvIL-10 during HCMV infection significantly reduced the host IL-10 synthesis (Supplementary Figures S2H,I), suggesting that cmvIL-10 secreted from HCMV-infected macrophages elicited host IL-10 production. In particular, M. massiliense co-infection could not change markedly IL-10 production by HCMV-infected macrophages (Figure 1F), and we observed a clear distinction between IL-10 synthesized by HCMV-infected macrophages versus that by non-infected macrophages in the presence and absence of M. massiliense co-infection (Figure 1G), implicating the dominant immunomodulatory effects of HCMV infection, excluding the effects of challenges with other bacteria.

To evaluate the impact of IL-10 on the proliferation of M. massiliense, we neutralized the effect of endogenous human IL-10 by adding anti-IL-10 receptor neutralizing antibody (αIL-10R Ab) to the culture supernatant of M. massiliense-infected macrophages. As a result, bacterial burden decreased in an αIL-10R Ab dose-dependent manner in the HCMV-infected THP-1 macrophages (Figure 1H) as well as in PBMCs (Supplementary Figure S2J). Neutralization of IL-10R did not affect M. massiliense proliferation without a precedent HCMV infection. Adding neutralization antibodies for the cmvIL-10
Our data showed that HCMV infection blocks pro-inflammatory and anti-bacterial immune responses in macrophages. (A) Schematic figure for each experimental groups for RNA sequencing: Control, M. massiliense-infected (MOI = 2, 24 h), HCMV-infected (MOI = 10, 48 h), and HCMV/M. massiliense co-infected. Triplicates per group from a single experiment were used. (B) Hierarchical clustering analysis showed a high similarity between HCMV- and HCMV/M. massiliense-infected cells compared to M. massiliense-infected cells. (C) Biological processes enriched in the gene ontology analysis of M. massiliense- versus HCMV/M. massiliense- (Upper) and control versus HCMV-infected macrophages (Bottom) are shown as GO plots. (D) Heatmaps showing differentially expressed genes associated with pattern recognition, inflammatory cytokine, antigen presentation, and T cell activation in the control and HCMV-infected cells.
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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.518605/full#supplementary-material

FIGURE S1 | HCMV replicates in human monocytes. (A) Contour plots for the gating strategy. PBMCs isolated from healthy volunteers were infected with HCMV TB40E strain at a MOI of 10. CD45+CD4+CD11b+ monocytes, CD45+CD3+CD19+B cells and CD45+CD3+CD19−T cells were further analyzed for the expression of GFP at 72 h post infection. Representative from three different donors. (B) Quantitative graph indicating the GFP expression intensities in each population assessed by flow cytometry. N = 2. MFI, mean fluorescence intensity. One-way ANOVA was performed. *p < 0.05, **p < 0.001.

FIGURE S2 | HCMV promotes M. massiliense proliferation in THP-1 macrophages and PBMCs via increasing host IL-10 production. (A) UL111A gene expression was examined at 12 h post infection (hpi) with M. massiliense (MOI = 2) using real-time PCR in THP-1 macrophages. HCMV was infected (MOI = 10) at 24 h prior to M. massiliense infection. N = 6. (B) IL-10 mRNA expression was determined using real-time PCR in THP-1 macrophages treated with 200 ng/mL of cmvIL-10 for 12 h. N = 6. (C) IL-10 gene expression was determined using real-time PCR in THP-1 macrophages treated with 200 ng/mL of cmvIL-10 for 48 h. N = 6. (D) IL-10 in the culture supernatant of THP-1 macrophages was measured using ELISA. N = 2. (E) IL-10 in the culture supernatant of PBMCs treated with 200 ng/mL of cmvIL-10 for 48 h was measured using ELISA. N = 2. (F) IL-10 gene expression was determined using real-time PCR. Culture media of differentiated THP-1 macrophages was changed with HCMV culture supernatant at 24 h prior to M. massiliense infection. N = 3. (G) IL-10 in the culture supernatant of THP-1 macrophages at 48 hpi was determined using ELISA. N = 2. MFI, mean fluorescence intensity. One-way ANOVA was performed. *p < 0.05, **p < 0.001, ***p < 0.0001. N = 3–7 biological replicates.
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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