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Breast milk induces the differentiation of monocytes into macrophages, promoting human cytomegalovirus infection

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ABSTRACT Human cytomegalovirus (HCMV) is a ubiquitous herpesvirus found in human breast milk that is frequently transmitted from HCMV-seropositive mothers to their infants during the postnatal period. Despite extensive research, the mechanisms underlying HCMV transmission from breast milk and the anatomical location at which virus transfer takes place remain unclear. Breast milk contains many uniquely differentiated macrophages that undergo specific morphological and functional modifications in the mammary gland during lactation. Although the existence of permissive HCMV infection in differentiated macrophages has been well-described, the role of breast milk in this process remains unknown. Herein, we report that exposure of isolated peripheral blood monocytes to breast milk induces their differentiation into macrophages that exhibit an M2 phenotype (CD14^{high}CD163^{high}CD68^{high}CD206^{high}) and promotes a productive and sustained HCMV infection. We also found that breast milk triggers macrophage proliferation and thus sustains a unique population of proliferating, long-lived, and HCMV-susceptible macrophages that are capable of ongoing production of infectious virions. These results suggest a mechanism that explains chronic HCMV shedding into the breast milk of postpartum seropositive mothers. We also found that HCMV virions released from breast milk-induced macrophages generate a productive infection in primary infant tonsil epithelial cells. Collectively, our results suggest that breast milk may facilitate HCMV transmission from mother to infant via the oropharyngeal mucosa.

IMPORTANCE While human cytomegalovirus (HCMV) is frequently detected in the breast milk of HCMV-seropositive women and is often transmitted to infants via breastfeeding, the mechanisms by which this transmission occurs remain unclear. In this study, we modeled HCMV transmission at the oropharyngeal mucosa. We treated human monocytes with breast milk to mimic the lactating mammary gland microenvironment. We found that monocytes differentiated into macrophages with an M2 phenotype, which were highly permissive for HCMV. We also discovered that breast milk induces macrophage proliferation. Thus, exposure to breast milk increased the number of HCMV-susceptible macrophages and supported high levels of infectious HCMV. We found that HCMV virions released from breast milk-induced macrophages could infect primary infant tonsil epithelial cells. Collectively, these findings reveal the dual role of breast milk that induces the differentiation and proliferation of macrophages in the mammary gland and thus facilitates mother-to-child HCMV transmission at the oropharyngeal mucosa.

KEYWORDS breast milk, differentiation, macrophages, proliferation, HCMV

Human cytomegalovirus (HCMV) infection is highly common worldwide but typically remains asymptomatic in individuals who are not immunosuppressed (1). Mother-to-child transmission (MTCT) of HCMV can occur *in utero* via the placenta and

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during parturition when the newborn is exposed to infectious maternal cervicovaginal secretions (2). HCMV can also be transmitted by breast milk and oral mucosal secretions of HCMV-seropositive mothers during the postpartum period (3–6). HCMV DNA is detected in nearly half of the colostrum samples from HCMV-seropositive mothers, and HCMV virions are typically shed into the breast milk approximately 2 weeks after delivery and can be transmitted to the infant through the oropharyngeal and gastrointestinal mucosal epithelia (6–9). Numerous observational studies have demonstrated that HCMV can be transmitted postnatally from HCMV-seropositive mothers to their infants via breastfeeding. Some of these infants subsequently develop sepsis-like symptoms (3, 4, 6, 10). However, the mechanism by which HCMV undergoes MTCT via breast milk is not fully understood.

Human breast milk is a complex fluid with a unique composition. In addition to the nutrients required by infants, breast milk contains numerous biologically active components, including lipids, proteins, non-protein nitrogen, antibodies, carbohydrates, and oligosaccharides (11). Breast milk also contains cells derived from maternal blood and the mammary glands (12). Mammary gland-derived cells include lactocytes (secretory cells), myoepithelial cells, mammary stem cells, and squamous epithelial cells (13). Blood-derived cells include hematopoietic progenitor cells, hematopoietic stem cells, and immune cells such as macrophages, neutrophils, and lymphocytes (13). Breast milk contains leukocytes that originate from peripheral blood and migrate to the mammary glands through the systemic circulation and the lymphatic vessels (14). A unique lactation-associated population of macrophages was recently identified in both murine mammary tissue and human breast milk (15). These macrophages originated from bone marrow-derived monocytes, proliferated *in situ* during lactation, and were ultimately translocated into breast milk. This is particularly notable, given that tissue-resident macrophages typically do not migrate (15). CD14⁺ macrophages predominate among the leukocytes identified in freshly isolated colostrum and samples of early breast milk (14, 16–19). HCMV DNA was detected in CD14⁺ monocytes/macrophages isolated from breast milk (17, 20). While these cells may be responsible for viral transmission to newborns, their role in promoting and sustaining HCMV MTCT remains uncertain.

Circulating monocytes that are derived from the bone marrow CD34⁺ progenitors can migrate to nearly all tissues in the body and differentiate into innate immune cells that perform various homeostatic and immune functions. Numerous pathogens specifically target these cells and thus evade and manipulate systemic immune response. Although monocytes are short-lived cells that are not permissive for HCMV replication, they play a crucial role in disseminating HCMV to organs and tissues during primary infection and following reactivation from latency or during productive infection after their differentiation into macrophages (21–27). HCMV replication in monocytes/macrophages is tightly associated with the differentiation of monocytes into macrophages, which may contribute to the development of productive HCMV infection in immunocompetent individuals (26, 28–30). Monocytes can differentiate into “classically” activated (M1) and “alternatively” activated (M2) macrophages (31). While M1 macrophages have a pro-inflammatory phenotype and are capable of inhibiting and killing pathogens, M2 macrophages promote tissue repair and cell proliferation (32, 33). Compared with M1 macrophages, M2 macrophages are more permissive for HCMV and exhibit optimal infection susceptibility (32, 34, 35). Notably, monocytes within the bone marrow remain capable of proliferation, while monocytes circulating in the blood do not (36). Once monocytes have differentiated into macrophages, these cells can renew themselves by proliferating locally (36–38). Therefore, macrophage proliferation may also contribute to HCMV persistence by enlarging the population of cells that are carrying the virus.

HCMV infects fetal salivary gland epithelial cells. The abundant release of virions into the saliva of HCMV-seropositive individuals has been well documented (39, 40). This suggests that the oral mucosal epithelia may be a portal for viral entry and shedding. Results from our previous work revealed that infant tonsil epithelial cells are susceptible

to HCMV, and virion translocation through the oropharyngeal mucosa is a critical step in viral transmission (7).

In this study, we present a mechanism that explains how breastfeeding can promote HCMV MTCT through the oral mucosa. Our results revealed that breast milk induces the differentiation of monocytes into macrophages and triggers additional macrophage proliferation, thereby sustaining HCMV infection and the release of viral progeny. We also found that HCMV virions released from breast milk-induced macrophages can infect primary infant tonsil epithelial cells. These intriguing observations suggest that the MTCT of HCMV may be facilitated by the interactions of HCMV-infected breast milk macrophages with the infant oropharyngeal mucosal epithelia.

RESULTS

Breast milk induces the differentiation of monocytes into macrophages

While peripheral blood monocytes migrate into the mammary gland and differentiate into macrophages during lactation, the precise impact of this unique mammary gland microenvironment remains unclear. To imitate the lactating mammary gland microenvironment, we used human breast milk samples obtained from healthy donors in experiments designed to determine its impact on the phenotype of human monocytes. Monocytes were isolated from peripheral blood mononuclear cells (PBMCs) collected from three healthy donors using magnetic beads. To evaluate the purity of the isolated monocytes, cells were stained with antibodies to human CD14 (a marker of monocytes/macrophages); CD163, CD68, and CD206 (macrophage markers); and CD1a (a dendritic cell marker) (41–46) (Fig. S1A). We also evaluated the expression of endogenous HCMV protein in isolated monocytes by flow cytometry using an antibody that targets HCMV immediately early 1 (IE1) protein. Our results revealed that 94.9%–96% of the isolated cells were CD14⁺ positive. None of the isolated cells expressed CD163, CD68, CD206, CD1a, or HCMV IE1 (Fig. S1A). These results identified the purified cells as monocytes with no endogenous expression of HCMV IE1 protein.

We then explored the possibility that breast milk was cytotoxic for human monocytes using CellTiter-Glo 2.0 assays. Breast milk supernatants from three different donors were diluted 1:2, 1:4, or 1:8 in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS). Human monocytes were incubated with diluted breast milk for 6 days; the culture media were refreshed on day 3. Monocytes incubated with RPMI 1640 medium supplemented with 10% FBS without breast milk served as the control. On day 6, we performed luminescence measurements to determine the number of viable cells in the culture. The results revealed that none of the breast milk supernatant samples tested in this study were cytotoxic to human monocytes (Fig. S1B). Based on these results, we selected the 1:4 dilution of breast milk supernatant for all subsequent experiments. Additionally, we determined the HCMV DNA levels in breast milk samples from all the donors by quantitative reverse transcription-PCR (qRT-PCR). HCMV DNA was detected only in breast milk samples from donor E (Fig. S2A). We also assessed the infectivity of the breast milk samples using HCMV-susceptible human fibroblasts. No infectious HCMV was detected in any of the breast milk samples used in our study (Fig. S2B). Notably, we have no information on anti-HCMV serology for any of the breast milk donors.

We noticed that a small portion of the untreated monocytes had increased in size after 6 days in culture (Fig. 1A, upper panel). This result suggests that monocytes may tend to differentiate into macrophages or dendritic cells when cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS *in vitro*. Moreover, we found that most of the monocytes cultured with breast milk increased in size and developed numerous protrusions (Fig. 1A, lower panel). These morphological changes were also clearly revealed by Giemsa staining (Fig. S3).

To explore these phenomena in greater depth, we treated human monocytes with breast milk for 5 days and then assessed their expression of monocyte, macrophage,

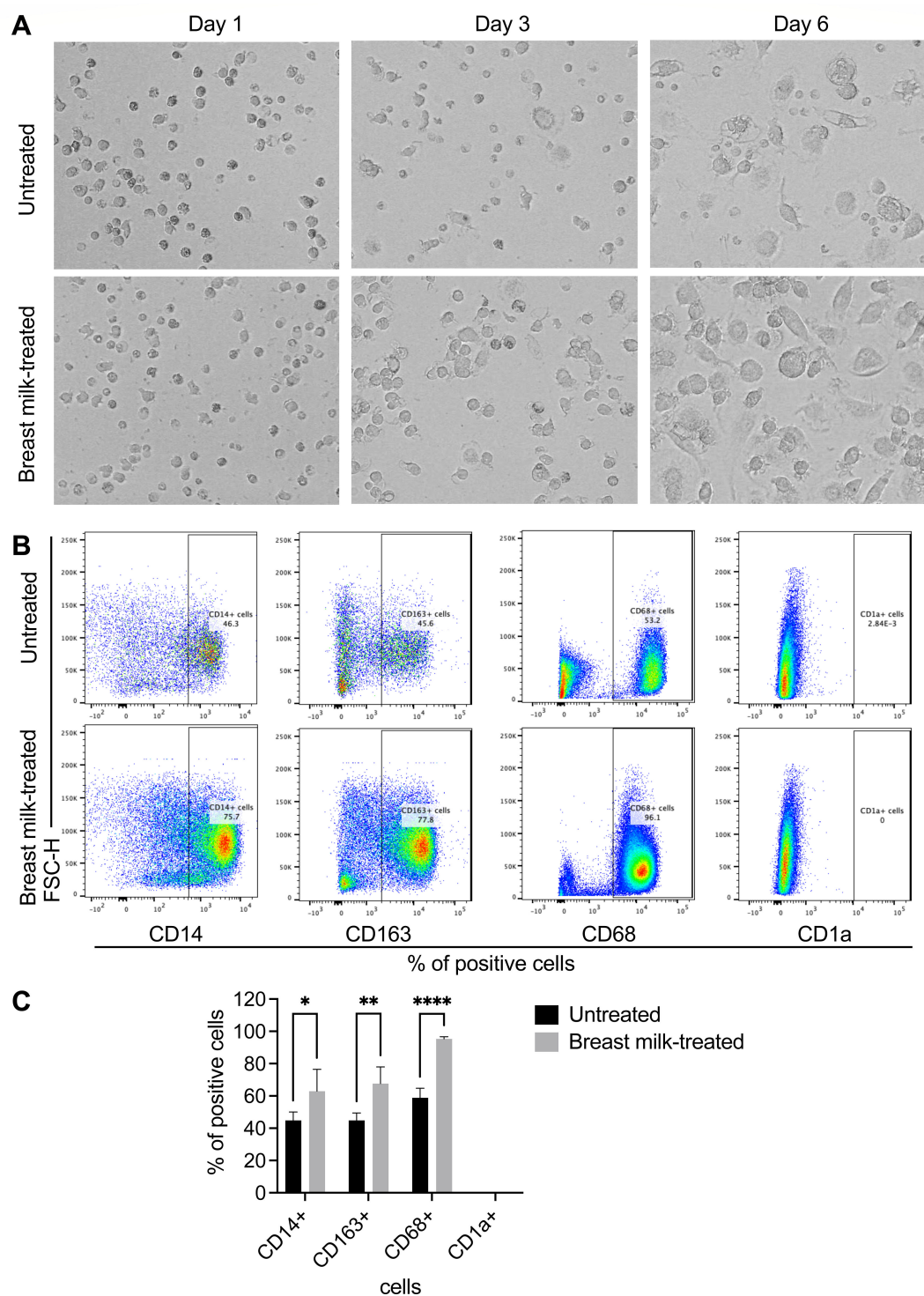


FIG 1 Breast milk induces the differentiation of monocytes into macrophages. (A) Phase-contrast images of human CD14⁺ cells (donor #1) left untreated or treated with breast milk (donor B). Original magnification: $\times 200$. (B and C) Human CD14⁺ cells (donor #2) were treated with a 1:4 dilution of breast milk (donor C) for 5 days with the media refreshed on day 3. Untreated cells served as the control. (B) The phenotype of human CD14⁺ cells after breast milk treatment was determined by flow cytometry. (C) Percentages of positive cells expressing CD14, CD163, CD68, and CD1a determined by flow cytometry. Results are presented as mean $\pm$ SD and are representative of three independent experiments; * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$.

and dendritic cell markers by flow cytometry with antibodies targeting CD14, CD163, CD68, CD206, and CD1a, respectively (41–46). This analysis revealed that the untreated

cultured cells were primarily $CD14^{+}CD163^{+}CD68^{+}CD206^{+}CD1a^{-}$, while those in the breast milk-treated cultures were $CD14^{high}CD163^{high}CD68^{high}CD206^{high}CD1a^{-}$ (Fig. 1B; Fig. S4 and S5). None of the untreated or breast milk-treated cells expressed CD1a, which confirmed that they had not differentiated into dendritic cells. Positive controls for these experiments included cells cultured with macrophage colony-stimulating factor (M-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF) to generate monocyte-derived macrophages. Other cultures were treated with GM-CSF and interleukin-4 (IL-4) to generate monocyte-derived dendritic cells (47, 48). The M-CSF-induced monocyte-derived macrophages were $CD14^{+}CD163^{+}CD68^{high}CD206^{+}CD1a^{-}$ (Fig. S4 to S6), which was similar to the phenotype of the breast milk-treated cells. By contrast, the GM-CSF-induced monocyte-derived macrophages were $CD14^{+}CD163^{low}CD1a^{-}$, while the GM-CSF/IL-4-induced monocyte-derived dendritic cells were $CD14^{low}CD163^{-}CD1a^{high}$ (Fig. S6). Both these results are consistent with those reported in previous studies (47, 48). As shown, significantly higher percentages of breast milk-treated cells expressed CD14, CD163, CD68, and CD206 compared to untreated control cells that were cultured in RPMI 1640 medium containing 10% FBS (Fig. 1B; Fig. S4) or in X-VIVO 15 serum-free medium (Fig. S5). Interestingly, 41.0%–68.9% of the untreated control cells cultured in RPMI 1640 with 10% FBS expressed these macrophage markers (Fig. S4); this decreased to 1.9%–13.5% in untreated cells maintained in X-VIVO medium (Fig. S5). By contrast, although we observed only a small increase (to 78.7%–95.9%) in the percentage of cells cultured in RPMI 1640 with 10% FBS in response to treatment with breast milk (Fig. S4), macrophage markers were expressed in 76.7%–85.6% of breast milk-treated cells maintained in X-VIVO, representing a sixfold increase over untreated control cells maintained in this medium (Fig. S5). Furthermore, a significantly higher percentage of M-CSF-stimulated cells expressed CD68 compared to untreated control cells (Fig. S4 and S5) in both culture conditions. Taken together, these data indicate that breast milk can induce the differentiation of monocytes into macrophages and that the phenotype of these macrophages is similar to that observed in response to M-CSF.

Breast milk induces macrophage proliferation

As described above, breast milk toxicity experiments were performed using a CellTiter-Glo assay to determine the number of viable cells in the culture (Fig. S1B). The results revealed that there were more viable cells in breast milk-treated monocyte/macrophage cultures than were found in cultures of untreated cells. These results suggest that breast milk may support cell growth and induce macrophage proliferation. To explore this possibility, a Click-iT EdU Proliferation assay was performed. Human monocytes were left untreated or treated with breast milk for 6 days. EdU (5-ethynyl 2'-deoxyuridine) was added to the culture media on day 5. On day 6, EdU incorporation into newly synthesized DNA was determined. The results from three independent experiments with monocytes and breast milk samples from three healthy donors revealed that breast milk-treated cells incorporated more EdU (and were thus proliferating more rapidly) than untreated control cells (Fig. 2A). Previous studies reported that M-CSF can induce macrophage proliferation (49–51); thus, we used M-CSF-treated cells as a positive control. In contrast to the untreated cells, M-CSF-treated cells exhibited significantly greater cell proliferation. The cell proliferation induced by M-CSF also significantly exceeded that observed in response to breast milk (Fig. 2A).

We used several additional assays to confirm the cell proliferation results, including the Click-iT Plus EdU Cell Proliferation imaging assay as well as an immunofluorescence staining assay that targets the cell proliferation marker, Ki67 (52, 53). Flow cytometric studies revealed that 0.61% of the newly isolated monocytes expressed Ki67 (Fig. S1A). Human monocytes were left untreated or treated with breast milk or M-CSF; EdU was added to the culture media on day 3 (Fig. S7) or day 5 (Fig. 2B and C). On the following day, the incorporation of EdU into newly synthesized DNA was detected with Alexa Fluor 488 dye; cells were also immunostained with an anti-Ki67 antibody and examined by fluorescence microscopy. Cells in culture media without EdU and had not been

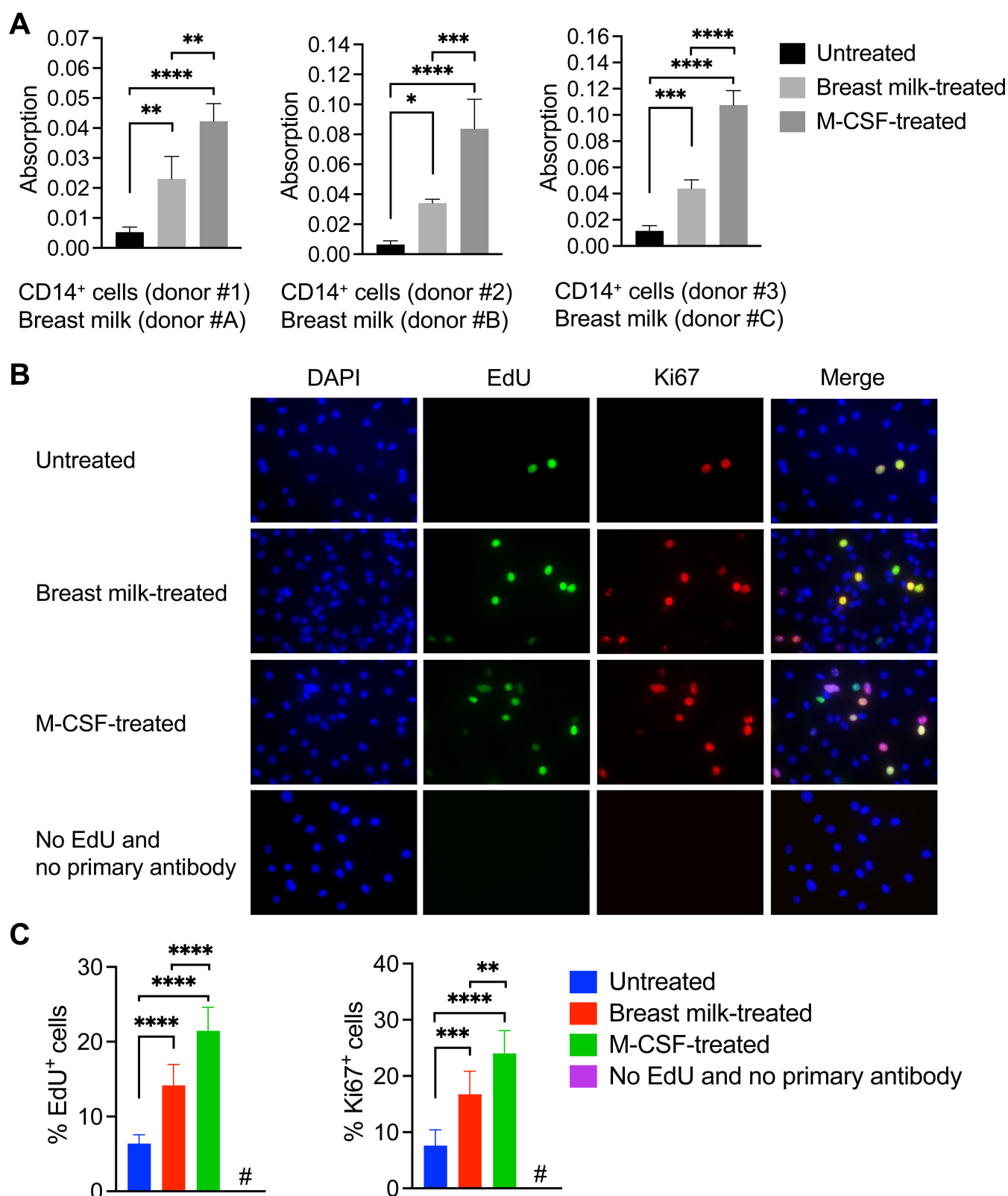


FIG 2 Breast milk induces macrophage proliferation. (A) Cell proliferation was examined by Click-iT EdU Proliferation assay for microplates. Human CD14⁺ cells (donor #2) were left untreated or treated with breast milk or M-CSF for 6 days. EdU was added to the media on day 5. On day 6, EdU incorporation into newly synthesized DNA was determined by absorption as measured by a plate reader. (B) Cell proliferation was evaluated by Click-iT Plus EdU Cell Proliferation imaging assay and immunofluorescence staining for Ki67. Human CD14⁺ cells were left untreated or treated with breast milk (donor B) or M-CSF for 6 days. EdU was (Continued on next page)

FIG 2 (Continued)

added to the media on day 5. On day 6, cells were fixed and incubated with the Click-iT Plus reaction cocktail and then immunostained with an anti-Ki67 primary antibody. To generate a no EdU and no primary antibody control, CD14⁺ cells were cultured for 6 days without added EdU and then incubated with Click-iT Plus reaction cocktail and secondary antibody only. Original magnification: $\times 400$. (C) The percentages of EdU or Ki67 positive cells were quantified using images acquired from at least six randomly selected fields; [#] not detected. Results are presented as means $\pm$ SD and are representative of three independent experiments; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

incubated with anti-Ki67 primary antibody served as a control. Surprisingly, a small portion of the cells from the untreated cultures (2.8%) incorporated EdU and expressed Ki67 on day 4 (Fig. S7). By contrast, breast milk-treated cells showed higher levels of EdU incorporation and Ki67 expression on day 4 (both at 4.3%), although this difference did not achieve statistical significance (Fig. S7). The levels of EdU incorporation (8.3%) and Ki67 expression (8.3%) were also significantly higher among cells that had been treated with M-CSF than those of untreated cells and breast milk-treated cells (Fig. S7). On day 6, we found that 6.4% of untreated cells had incorporated EdU and 7.6% expressed Ki67 (Fig. 2B and C). By contrast, breast milk-treated cells exhibited significantly higher levels of EdU incorporation (14.2%) and Ki67 expression (16.8%; Fig. 2B and C). M-CSF-treated cells also exhibited significantly higher levels of both EdU incorporation (21.4%) and Ki67 expression (24.0%) than those detected in breast milk-treated and untreated cells (Fig. 2B and C). Of note, these results, which were obtained using CD14⁺ cells from donor #2 and breast milk from donor B (Fig. 2B and C; Fig. S7), were consistent with those obtained using CD14⁺ cells from donor #3 and breast milk from donor C (Fig. S8A through D).

We confirmed the cell proliferation results with a flow cytometry assay designed to detect intracellular expression of Ki67. We examined Ki67 expression in untreated, breast milk-treated, or M-CSF-stimulated human monocytes after 6 days in culture with RPMI 1640 medium containing 10% FBS (Fig. S4) or X-VIVO 15 serum-free medium (Fig. S5). While a higher percentage of breast milk-treated cells cultured in RPMI 1640 medium with 10% FBS expressed Ki67, the difference from levels observed in untreated cells did not achieve statistical significance (Fig. S4). However, we identified a significantly higher percentage of M-CSF-treated cells that expressed Ki67 or both CD68 and Ki67 compared to their untreated or breast milk-treated counterparts (Fig. S4). Furthermore, a significantly higher percentage of breast milk-treated cells cultured in X-VIVO 15 serum-free medium was Ki67⁺ compared to untreated cells; the percentages of CD14⁺/Ki67⁺, CD163⁺/Ki67⁺, CD206⁺/Ki67⁺, and CD68⁺/Ki67⁺ cells detected in breast milk-treated cultures were also significantly higher than those observed in their untreated counterparts (Fig. S5). Similarly, the percentages of Ki67⁺, CD206⁺/Ki67⁺, and CD68⁺/Ki67⁺ cells in M-CSF-treated cultures were significantly higher than those observed in untreated cultures (Fig. S5). The results of these different cell proliferation assays indicate that one or more components of breast milk are capable of inducing macrophage proliferation.

Breast milk-induced macrophages are permissive for HCMV infection

Numerous reports have documented the tight association between HCMV replication and the differentiation of monocytes to macrophages (26–28, 30, 54). The results presented thus far indicate that breast milk promotes the differentiation of monocytes into macrophages (Fig. 1; Fig. S3), which may promote HCMV infection. To determine the extent of HCMV infection in breast milk-induced macrophages, monocytes were left untreated or treated with breast milk for 6 days and were then inoculated with HCMV clinical strain VR1814 at a multiplicity of infection (MOI) of 1. Mock-infected cells served as a negative control. At 3 dpi, cells were harvested for flow cytometric detection of CD14, CD163, CD1a, and HCMV IE1. A significantly higher percentage of mock-infected breast milk-treated cells expressed CD14 (70.2%) and CD163 (67.9%) compared to mock-infected untreated cells; 37.1% of the mock-infected untreated cells expressed CD14, and 30.3% expressed CD163 (both $P < 0.05$ vs breast milk-treated cells). None of the mock-infected cells expressed CD1a or HCMV IE1 protein (Fig. 3A and B). These

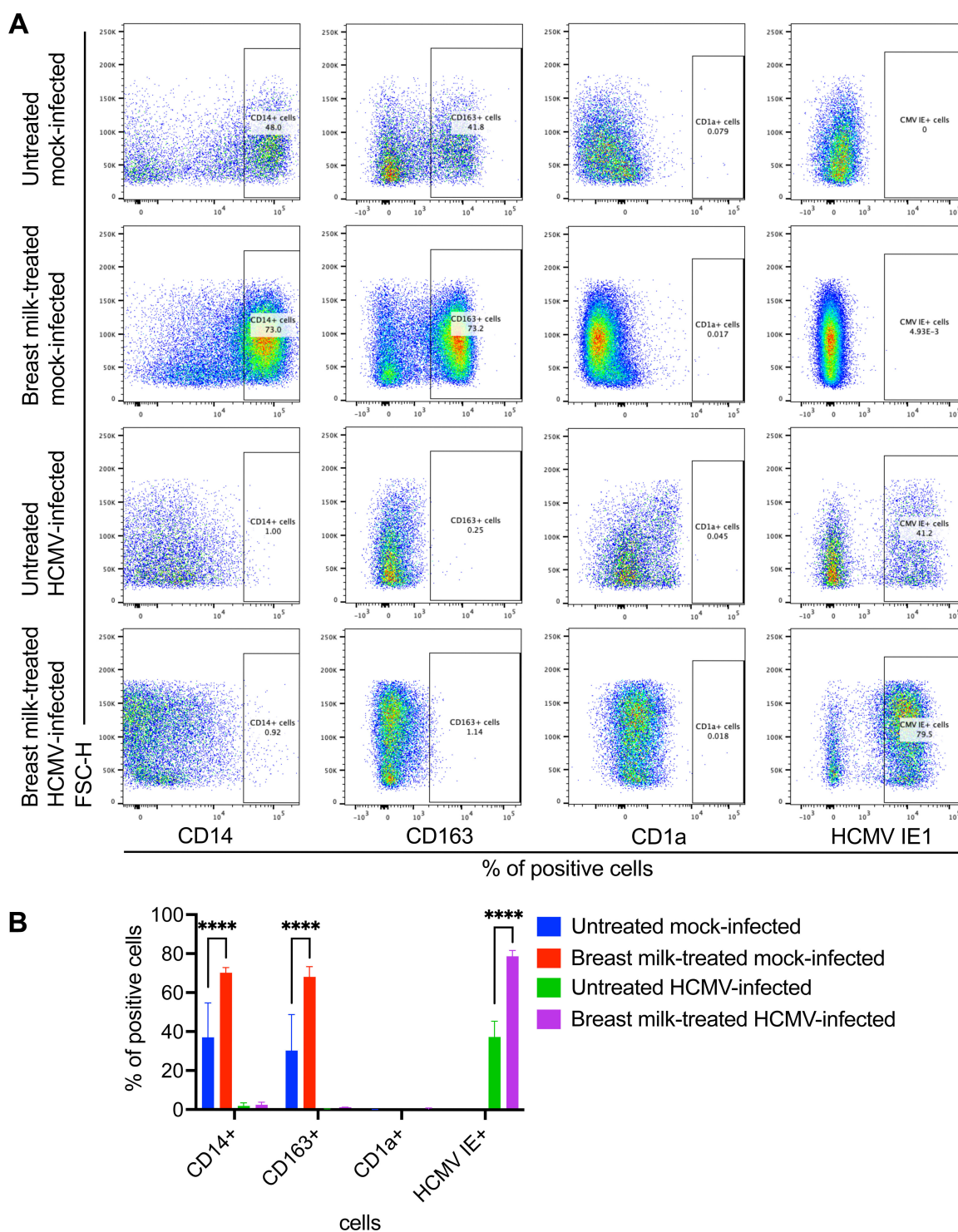


FIG 3 Breast milk-induced macrophages are more permissive for HCMV infection. Human CD14⁺ cells were left untreated or treated with breast milk (donor B) for 6 days. The treated and untreated cells were then infected with HCMV VR1814 at an MOI of 1. On day 3 post-infection, cells were harvested for flow cytometry analysis. Mock-infected treated and untreated cells served as negative controls for HCMV infection. (A) Percentages of cells expressing CD14, CD163, CD1a, and HCMV immediate early (IE) protein. (B) Phenotype of human CD14⁺ cells after treatment with breast milk and HCMV infection. Results are presented as mean $\pm$ SD and are representative of three independent experiments; **** P < 0.0001.

results, which were obtained using breast milk from donor B, were consistent with those obtained using breast milk from donor C (as shown in Fig. 1B and C). Taken together,

these results indicate that breast milk induces the differentiation of monocytes into macrophages. The absence of HCMV IE1 protein expression in the mock-infected cells confirmed that the monocytes used in the study did not express endogenous HCMV IE1.

HCMV infection of both untreated and breast milk-treated cells resulted in decreased expression of CD14 and CD163; expression of CD1a remained unchanged compared to results obtained from the corresponding mock-infected cells. However, our results revealed a nearly twofold increase in HCMV infection in cultures of breast milk-induced macrophages compared to cultures of untreated cells, with 78.8% vs 37.4% of the cells expressing HCMV IE1 protein, respectively ($P < 0.01$; Fig. 3A and B). This finding parallels the increases in the percentage of CD14⁺CD163⁺ differentiated macrophages observed in mock-infected breast milk-treated cells compared with mock-infected untreated cells. Additionally, 39.8% and 26.1% of the M-CSF-induced and GM-CSF-induced monocyte-derived macrophages, respectively, expressed HCMV IE1 (Fig. S6). Furthermore, 56.3% of the GM-CSF and IL-4-induced monocyte-derived dendritic cells also expressed HCMV IE1, representing a significant increase over the percentages observed in cultures of untreated cells ($P < 0.05$; Fig. S6; Fig. 3).

To exclude the effect of serum on HCMV infection in monocyte-derived cells cultured in RPMI 1640 medium containing 10% FBS, a similar experiment was performed by maintaining cells in X-VIVO 15 serum-free medium (Fig. 4). We observed a nearly sixfold increase in HCMV infection in cultures of breast milk-treated cells compared to cultures of untreated cells, with 51.3% vs 8.5% of the cells expressing HCMV IE protein, respectively ($P < 0.0001$; Fig. 4). Furthermore, our results revealed that 14.4% of the M-CSF-treated cells expressed HCMV IE. Although this was significantly higher than the percentage of untreated control cells that expressed this protein ($P < 0.05$; Fig. 4), the percentage of M-CSF-treated cells expressing HCMV IE was significantly lower than that of breast milk-treated cells ($P < 0.0001$; Fig. 4).

Overall, these data suggest two potentially overlapping possibilities: (i) breast milk accelerates differentiation of monocytes into macrophages. The increase in the number of HCMV-infected cells is a direct result of the observed increase in the rate of differentiation and thus the number of differentiated macrophages available for infection compared to untreated control; and/or (ii) in addition to differentiation, breast milk induces macrophage proliferation. This will also increase the number of differentiated macrophages that are susceptible to HCMV infection.

Breast milk-induced macrophages support lytic HCMV infection

Next, we determined the impact of breast milk on lytic HCMV infection of macrophages. As a first step, we evaluated the contributions of breast milk to HCMV attachment and entry into macrophages. In these experiments, human monocytes were left untreated or treated with breast milk for 6 days. HCMV attachment and entry assays were then performed, and HCMV DNA extracted from the cells was detected using qRT-PCR. Our results revealed that breast milk treatment significantly increased HCMV attachment to the cell surface compared to untreated cells (Fig. S9). While the results of the HCMV entry assay revealed an increased trend in breast milk-treated cells compared to untreated controls, the differences observed were not statistically significant ($P = 0.39$; Fig. S9).

To study the productive HCMV infection of macrophages, human monocytes were left untreated or treated with breast milk or M-CSF for 6 days followed by inoculation with HCMV VR1814 at an MOI of 1. The culture media were refreshed every 3 days. At day 9 post-infection, the cells were fixed, and expression of HCMV IE1 and glycoprotein B (gB) was evaluated by immunofluorescence assay.

We found that significantly more breast milk-induced macrophages became HCMV-infected under these conditions compared to untreated cells. Our results revealed that a significantly higher percentage of breast milk-treated cells expressed HCMV IE1 ($P < 0.001$) and gB ($P < 0.01$) compared to untreated cells (Fig. 5A and B). These findings are consistent with the results obtained by flow cytometry (Fig. 3A and B). M-CSF-induced macrophages, which have a phenotype that is similar to breast milk-induced

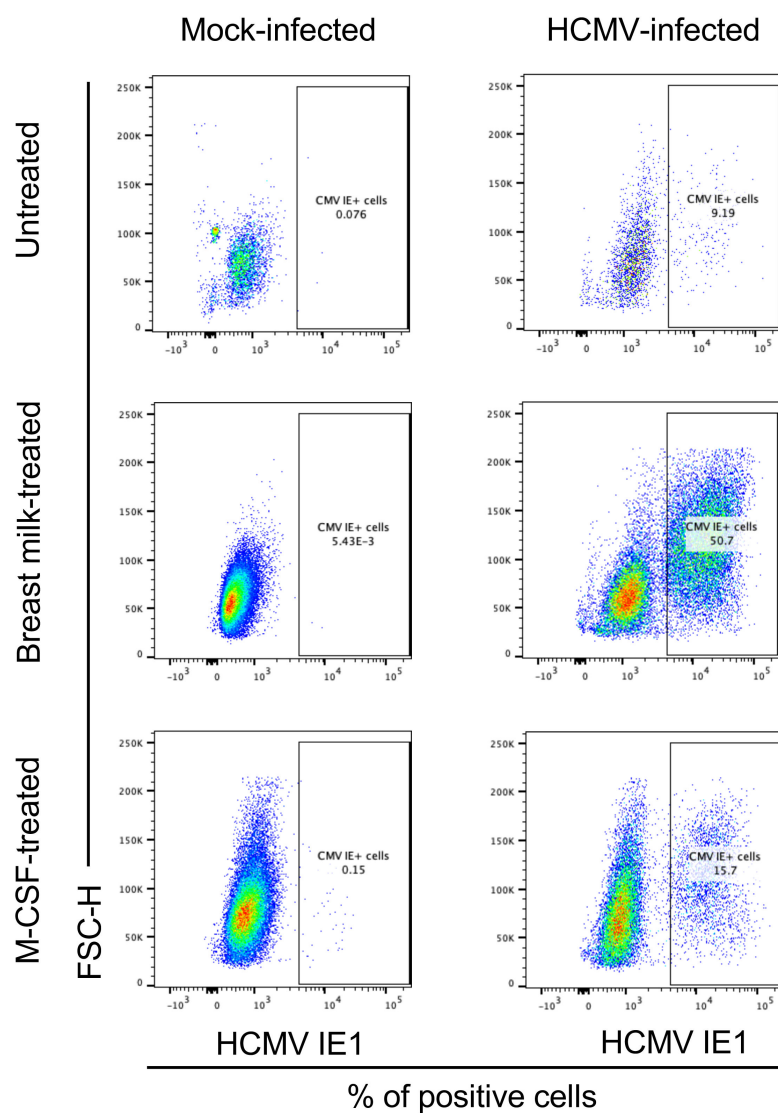
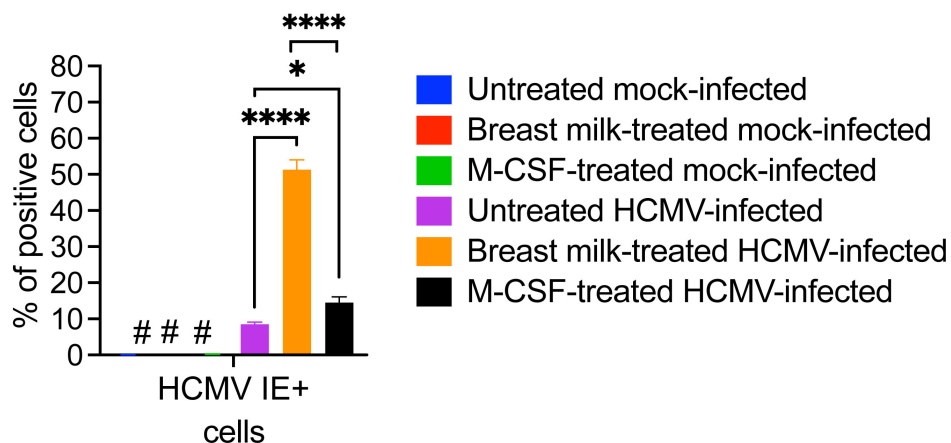
A**B**

FIG 4 Expression of HCMV IE in breast milk- or M-CSF-treated human CD14⁺ cells cultured in X-VIVO 15 serum-free medium. (A) Human CD14⁺ cells were left untreated or treated with breast milk or M-CSF in X-VIVO 15 serum-free medium for 6 days. On day 6, cells were infected with HCMV VR1814 at an MOI of 1. On day 3 post-infection, cells were harvested, and HCMV IE (Continued on next page)

FIG 4 (Continued)

expression was evaluated by flow cytometry with mock-infected cells serving as the negative control. (B) Percentages of cells expressing HCMV IE protein; #not detected. Results are presented as means $\pm$ SD and are representative of three independent experiments; * $P < 0.05$; **** $P < 0.0001$.

macrophages (both are CD68^{high}CD1a⁺; Fig. S4 to S6; Fig. 1B), were included in this experiment as positive controls. Interestingly, our results revealed that a significantly higher percentage of breast milk-induced macrophages expressed HCMV IE1 ($P < 0.0001$) and HCMV gB ($P < 0.001$) compared with the M-CSF-treated cells (Fig. 5A and B). These results show that breast milk-induced macrophages are significantly more susceptible to HCMV infection than the macrophages exposed to M-CSF alone. Collectively, these findings indicate that breast milk facilitates the differentiation of monocytes into macrophages that are highly permissive for HCMV. To verify the specific role of breast milk in the induction of monocyte-to-macrophage differentiation and thus HCMV infection, primary human fibroblasts were left untreated or treated with breast milk for 6 days, followed by inoculation with HCMV VR1814 at an MOI of 0.5. The results of these experiments revealed no increase in HCMV IE expression in breast milk-treated fibroblasts compared to untreated control cells (Fig. S10), thereby confirming the critical role of breast milk in monocyte-to-macrophage differentiation and thus HCMV infection.

HCMV virions are released from breast milk-induced macrophages

To determine whether breast milk-induced macrophages release infectious HCMV virions, we performed a series of experiments in which human monocytes were left untreated or treated with breast milk or M-CSF for 6 days. Cells were then inoculated with HCMV VR1814 at an MOI of 1. Culture supernatants were harvested on days 3, 6, 9, and 12 post-infection. After removing detached cells, the infectivity of the culture supernatants containing the released virus was tested on HCMV-susceptible human fibroblasts via an assay in which HCMV IE1 was detected by immunofluorescence methods (Fig. 6A).

We found that breast milk-induced macrophages released significantly higher levels of infectious HCMV virions on days 6, 9, and 12 after infection compared to their untreated counterparts ($P < 0.0001$, $P < 0.001$, and $P < 0.01$, respectively). Consistent with this finding, the percentages of HCMV IE1-positive indicator fibroblasts were approximately fivefold higher (Fig. 6B). Interestingly, while the M-CSF-induced macrophages released significantly more infectious HCMV virions than the untreated cells at day 9 post-infection ($P < 0.0001$), they released fewer virions than breast milk-induced macrophages. Breast milk-induced macrophages released significantly higher levels of infectious HCMV virions than the M-CSF-induced macrophages on both day 6 ($P < 0.0001$) and day 9 ($P < 0.01$) post-infection (Fig. 6B). Taken together, our data suggest that breast milk-induced macrophages are highly susceptible to HCMV infection and produce and release large quantities of infectious HCMV progeny.

HCMV infection in monocytes leads to viral latency; monocyte differentiation activates latent viruses and leads to the release of infectious virions. To determine the impact of breast milk on this process, monocytes with latent HCMV infection were left untreated or treated with breast milk or M-CSF in X-VIVO 15 serum-free medium (X-VIVO medium) or RPMI 1640 medium supplemented with 10% heat-inactivated FBS (RP10 medium) for 6 days. Cells then were co-cultured with primary human fibroblasts for 10 days, after which cell culture supernatants were harvested and added to an indicator monolayer of human fibroblasts to evaluate the infectivity of the virus contained in the supernatants, as previously reported (55–57). Our results revealed that cells in the RP10 medium that were treated with M-CSF (positive control) developed significantly more infectious centers (Fig. S11) and released significantly higher levels of infectious HCMV virions than the untreated control cells (Fig. S12). Collectively, these results demonstrate that M-CSF can induce HCMV reactivation in latently infected monocytes. Additionally, cells maintained in X-VIVO medium that were treated with M-CSF developed HCMV IE1-positive cells but no infectious centers (Fig. S11) but did release significantly higher

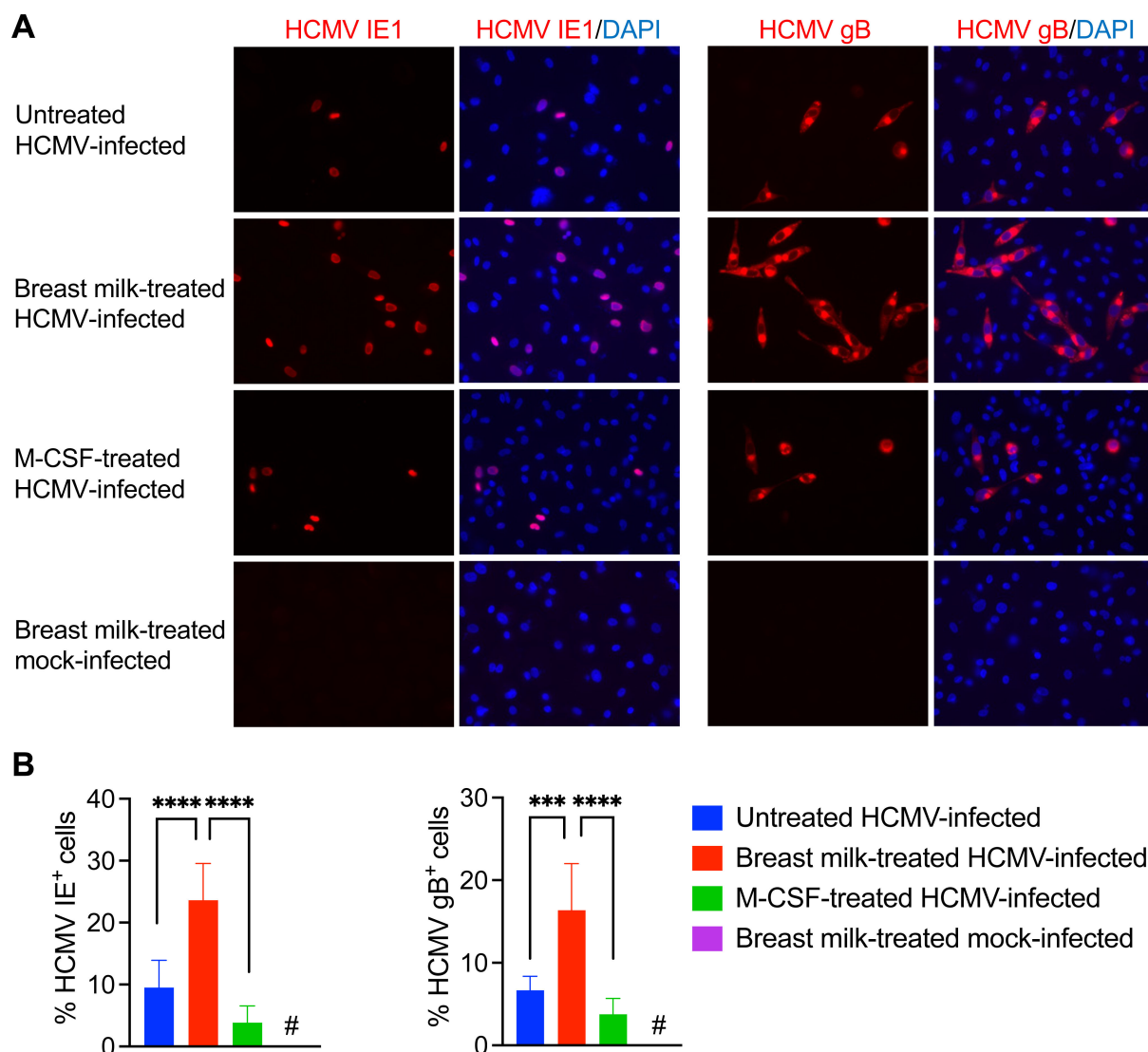


FIG 5 Breast milk-induced macrophages are highly susceptible to HCMV infection. Human CD14⁺ cells were left untreated or treated with breast milk (donor D) or M-CSF for 6 days. The cells were then inoculated with HCMV VR1814 at an MOI of 1. The media were refreshed every 3 days. On day 9 post-infection, cells were fixed and stained with anti-HCMV IE1 or anti-HCMV gB. The percentages of HCMV IE1- and gB-positive cells were determined quantitatively using images acquired from at least six randomly selected fields at $\times 400$ magnification. (A) HCMV-infected monocyte-derived cells expressing HCMV IE1 protein or gB. (B) Percentages of monocyte-derived cells expressing HCMV IE1 protein or gB; #not detected. Results are presented as means $\pm$ SD and are representative of three independent experiments; *** $P < 0.001$; **** $P < 0.0001$.

levels of HCMV virions than the untreated cells (Fig. S12). This may be because the X-VIVO medium does not provide optimal conditions for primary human fibroblast growth and thus supports only low cell densities in co-cultures with monocytes. The results of these experiments also revealed that breast milk did not reactivate HCMV in latently infected monocytes *in vitro* (Fig. S11 and S12). Thus, under these experimental conditions, breast milk does not induce HCMV reactivation in latently infected monocytes.

HCMV virions released from breast milk-induced macrophages can infect primary infant tonsil epithelial cells

MTCT of HCMV may occur via breastfeeding as virions in the breast milk are translocated through the infant's oral mucosal epithelia (7, 39). To characterize this potential transmission route, human monocytes were left untreated or treated with breast milk for

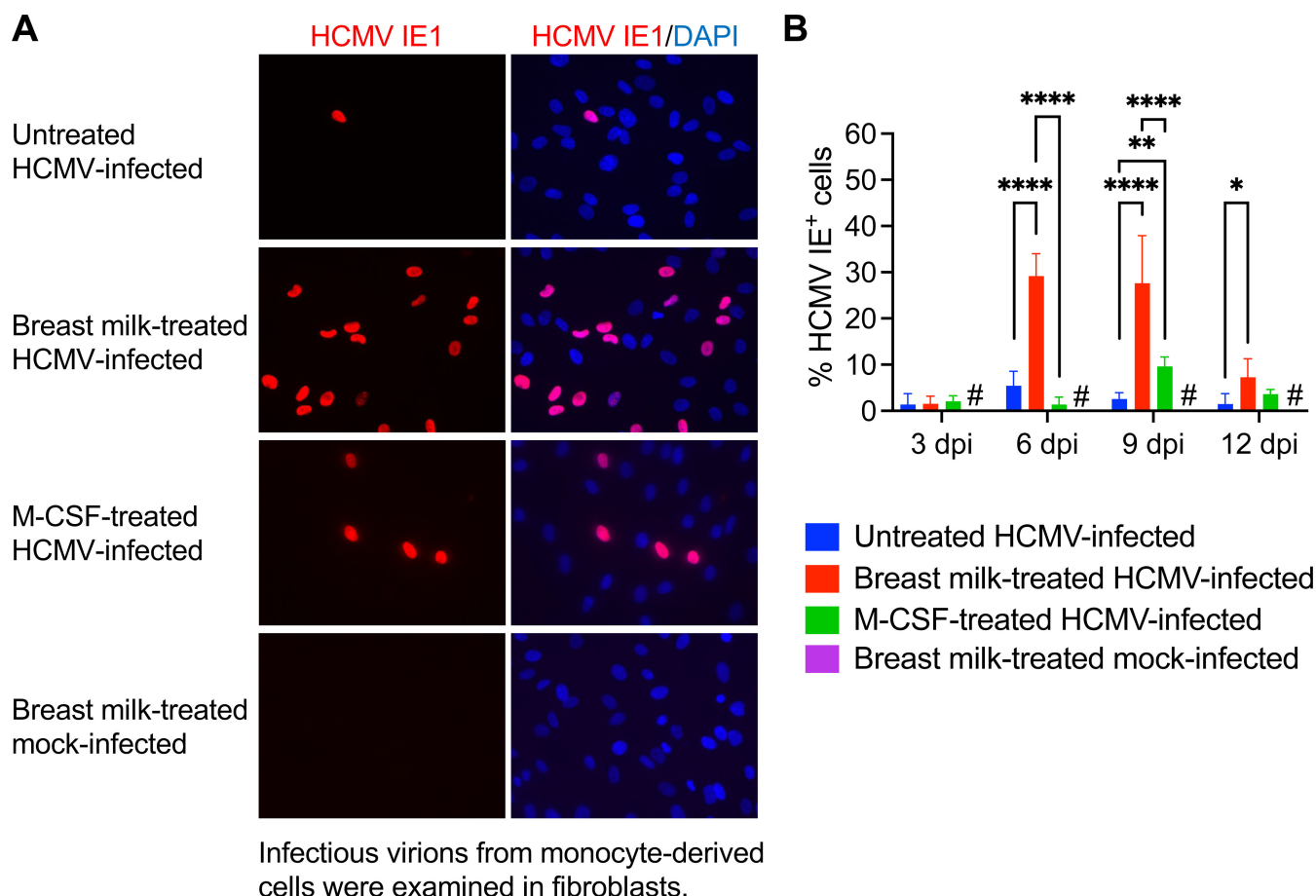


FIG 6 Breast milk-induced macrophages produce and release infectious HCMV progeny. Human CD14⁺ cells were left untreated or treated with breast milk (donor D) or M-CSF for 6 days. Cells were then infected with HCMV VR1814 at an MOI of 1. Cell culture supernatants were harvested on days 3, 6, 9, and 12 post-infection and used to infect human fibroblasts. Twenty-four hours later, the fibroblasts were fixed and immunostained for HCMV IE1 protein. The percentages of cells that were HCMV IE1-positive cells were determined quantitatively using images acquired from at least six randomly selected fields at $\times 400$ magnification. (A) Fibroblasts expressing HCMV IE1 protein after incubation with supernatants from untreated, breast milk-treated, or M-CSF-treated cells (day 9 post-infection). (B) Percentage of fibroblasts expressing HCMV IE1 protein; #not detected. Results are presented as means $\pm$ SD and are representative of three independent experiments; * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$.

6 days. These cells were then inoculated with HCMV VR1814 at an MOI of 1. Released virions were collected from the culture media on days 3, 6, and 9 post-infection. After removing any detached cells, the supernatants were used to infect primary infant tonsil epithelial cells isolated from two independent donors. After 24 hours, tonsil epithelial cells were fixed and immunostained for HCMV IE1 protein. Primary tonsil epithelial cells were also immunostained with an anti-cytokeratin antibody to verify culture purity (Fig. S13). A qRT-PCR assay was also performed to ensure that these cells contained no endogenous HCMV DNA (Fig. S2A).

HCMV IE1 protein was detected in the tonsil epithelial cells from two donors (#90 and #99) at 24 hours post-infection (Fig. 7A). Importantly, breast milk-induced macrophages supported productive HCMV infection, as revealed by the increase in the number of infectious virions collected between day 6 and day 9 post-infection (Fig. 7B). These data are consistent with our findings that documented an increase in the number of infectious HCMV virions released from breast milk-induced macrophages that were initially detected using human fibroblasts (Fig. 6). Taken together, these findings demonstrate that HCMV virions released from breast milk-induced macrophages can infect infant tonsil epithelial cells and thus may initiate systemic infections in infants.

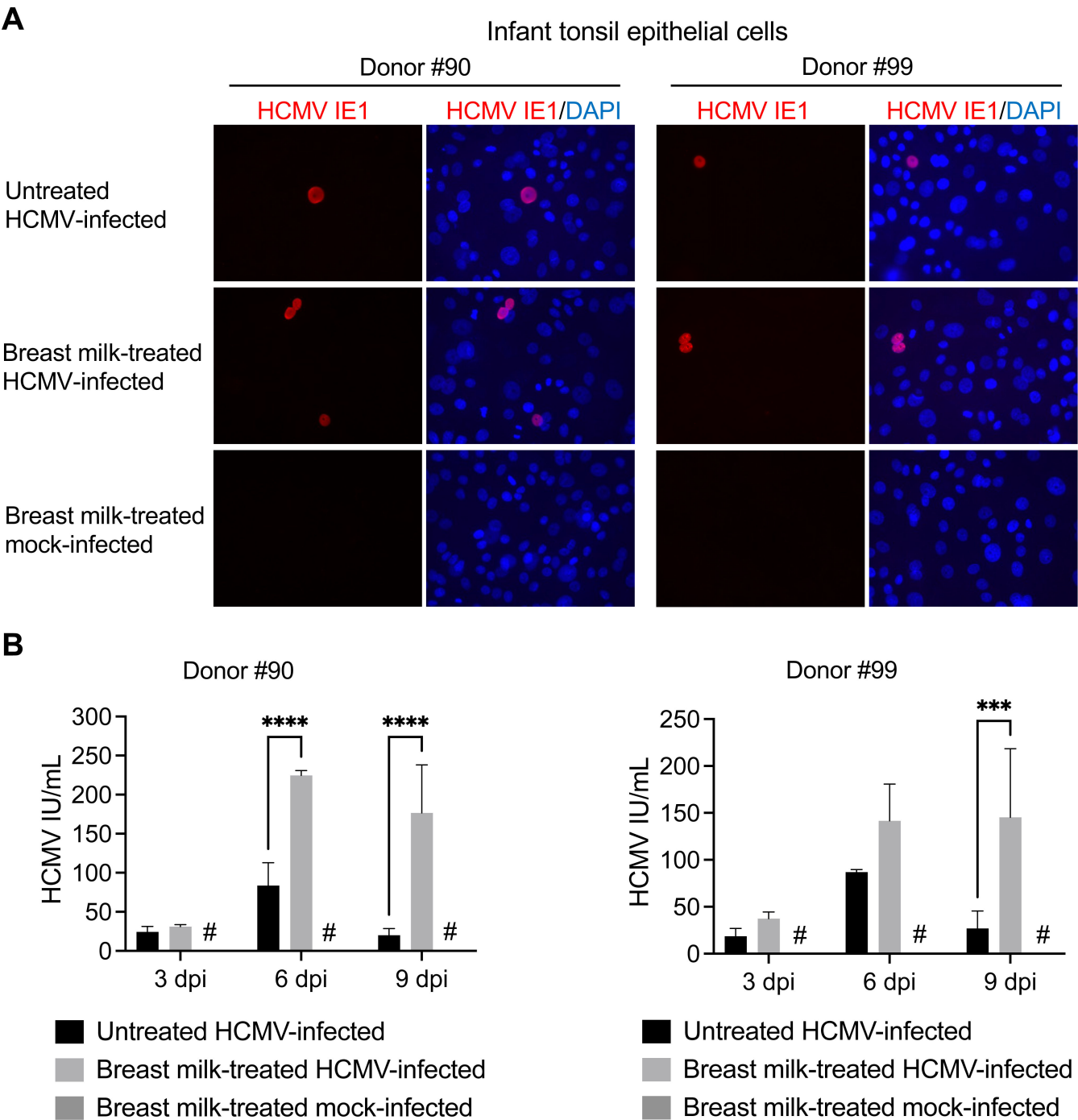


FIG 7 Infection of primary infant tonsil epithelial cells by HCMV virions released from breast milk-induced macrophages. Human CD14⁺ cells were left untreated or were treated with breast milk (donor B) for 6 days. Cells were infected with HCMV VR1814 at an MOI of 1. Released virions were collected from the culture media on days 3, 6, and 9 post-infection. Detached cells were removed by centrifugation; the remaining supernatants were used to infect primary infant tonsil epithelial cells (donor #90 or #99). After 24 hours, tonsil epithelial cells were fixed and stained with anti-HCMV IE1 to detect HCMV infection. The number of infectious virions in the supernatants from monocyte-derived cells was calculated using tonsil epithelial cells as an indicator monolayer and expressed as infectious units per milliliter (IU/mL). (A) Detection of HCMV IE1 expression in infant tonsil epithelial cells from donor #90 and donor #99 on day 6 post-infection. (B) Quantitative analysis of HCMV IE1-expressing tonsil epithelial cells from donors #90 and #99; #not detected. Results are presented as means $\pm$ SD; *** P < 0.001; **** P < 0.0001.

HCMV infection of infant tonsil epithelial cells is partially suppressed in the presence of breast milk

Breast milk contains a variety of antiviral factors, including immunoglobulins, defensins, glycoproteins (e.g., lactoferrin), glycosylated components (e.g., mucins), and human milk oligosaccharides (58). To determine whether these components might inhibit HCMV infection, we performed experiments to detect anti-HCMV IgG antibodies in breast milk samples. Only one of the five breast milk samples (donor E) was HCMV IgG positive (Table 1). We then evaluated the outcome of HCMV infection of infant tonsil epithelial cells in the presence of breast milk. Primary infant tonsil epithelial cells derived from two healthy donors were infected with HCMV VR1814 at an MOI of 1 in the presence of breast milk obtained from two healthy donors. Culture supernatants were harvested on days 3, 6, and 9 post-infection, and the release of infectious viral progeny was examined using human fibroblasts. To confirm that HCMV infection of infant tonsil epithelial cells is productive, tonsil epithelial cells infected in the presence or absence of breast milk were fixed on day 9 post-infection and immunostained for HCMV gB.

In the absence of breast milk, HCMV infection of tonsil epithelial cells remained productive, as indicated by expression of the HCMV late protein gB in 65.4%–96.2% of epithelial cells, with some donor variability (Fig. 8). The percentage of cells expressing HCMV gB was significantly lower (50.2%–62.4%; $P < 0.01$ and $P < 0.001$) when tonsil epithelial cells were infected with HCMV in the presence of breast milk. However, despite the diminished infection observed in the presence of breast milk, more than half of tonsil epithelial cells derived from two different donors remained susceptible and were productively infected with HCMV and expressed HCMV gB (Fig. 8). Furthermore, the virions released from tonsil epithelial cells infected with HCMV in the presence or absence of breast milk remained infectious, as indicated by the detection of HCMV IE1 protein in cells of an indicator monolayer of human fibroblasts (Fig. 9). Consistent with diminished expression of HCMV gB, breast milk also had a significant impact on the production of infectious virions in tonsil epithelial cells.

Thus, our results showed that HCMV can infect infant tonsil epithelial cells in the presence of breast milk, and these cells can sustain a productive infection for up to 9 days. The reduction in the percentage of HCMV-infected cells observed under these conditions suggests that while breast milk may contain some antiviral factors, these mediators cannot fully abolish HCMV infection of target infant tonsil epithelial cells.

DISCUSSION

While postnatal HCMV infection is generally assumed to take place during breastfeeding (3, 4, 6, 10), the anatomical site of virion transmission has not been identified. The results presented in this study highlight a potential oropharyngeal route that facilitates breast milk-mediated HCMV transmission. Specifically, we have shown that HCMV can be transmitted from breast milk-induced macrophages to infant tonsil epithelial cells. Our results revealed that breast milk triggers the differentiation of monocytes into macrophages, stimulates their proliferation, and promotes their susceptibility to productive HCMV infection. HCMV virions released from breast milk-induced macrophages generate a productive infection in primary infant tonsil epithelial cells. Collectively, our results suggest a mechanism that explains how breast milk may mediate virion transmission

TABLE 1 HCMV IgG in breast milk samples^a

Sample tested	HCMV IgG (standard units)
Breast milk #A	2.09 ± 0.19
Breast milk #B	2.02 ± 1.15
Breast milk #C	1.29 ± 0.10
Breast milk #D	2.43 ± 0.08
Breast milk #E	18.77 ± 0.71

^aNegative: <9 standard units; positive: >11 standard units.

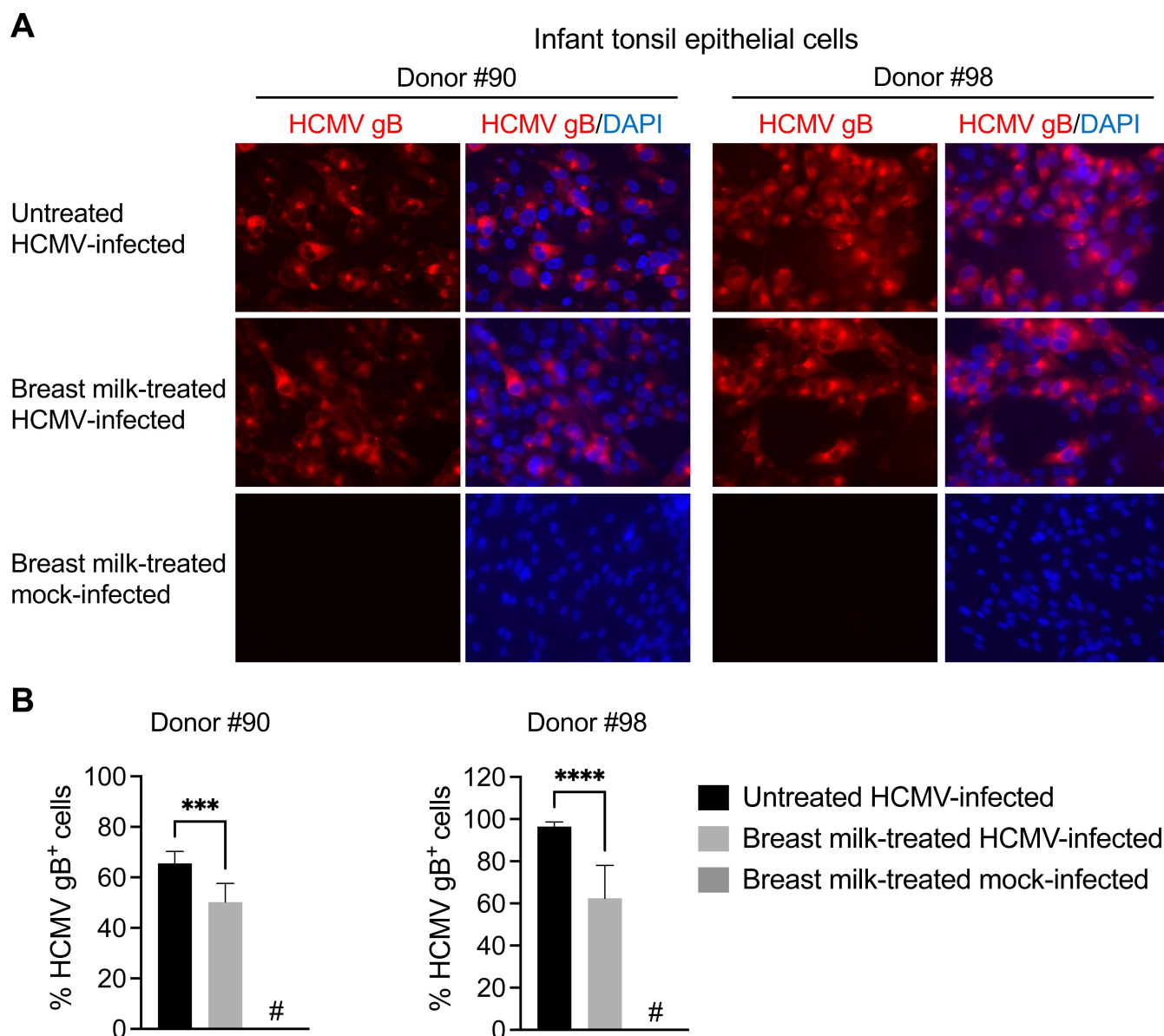


FIG 8 Breast milk partially inhibits HCMV infection of primary infant tonsil epithelial cells. Primary infant tonsil epithelial cells from donors #90 and #98 were infected with HCMV VR1814 at an MOI of 1 in the presence of breast milk obtained from donor B or C. At day 9 post-infection, tonsil epithelial cells were fixed and stained with anti-HCMV gB. (A) Infant tonsil epithelial cells expressing HCMV gB after infection in the presence of breast milk. (B) The percentage of HCMV gB positive cells was determined quantitatively using images from at least six randomly selected fields acquired at $\times 400$ magnification; #not detected. Results are presented as means $\pm$ SD; *** $P < 0.001$; **** $P < 0.0001$.

from an HCMV-seropositive mother to a breast-feeding infant via the oropharyngeal mucosa.

While nearly all seropositive women shed HCMV into breast milk, this is typically self-limited and usually ends 2–3 months after delivery (59). Interestingly, not all infants exposed to HCMV-positive breast milk become infected. Previous studies reported that $\sim 7\%$ of preterm infants acquired the infection via breast milk from HCMV-seropositive mothers (3, 60). HCMV transmission can be prevented by anti-HCMV specific antibodies found in breast milk that can directly neutralize virus infectivity (61, 62). However, the results of one study that focused on functional anti-HCMV antibodies in a group of 18 seropositive women revealed that greater HCMV neutralization activity (as determined by NT-50 assays) could be found in plasma compared with breast milk

A Infectious virions released from tonsil epithelial cells were examined in fibroblasts

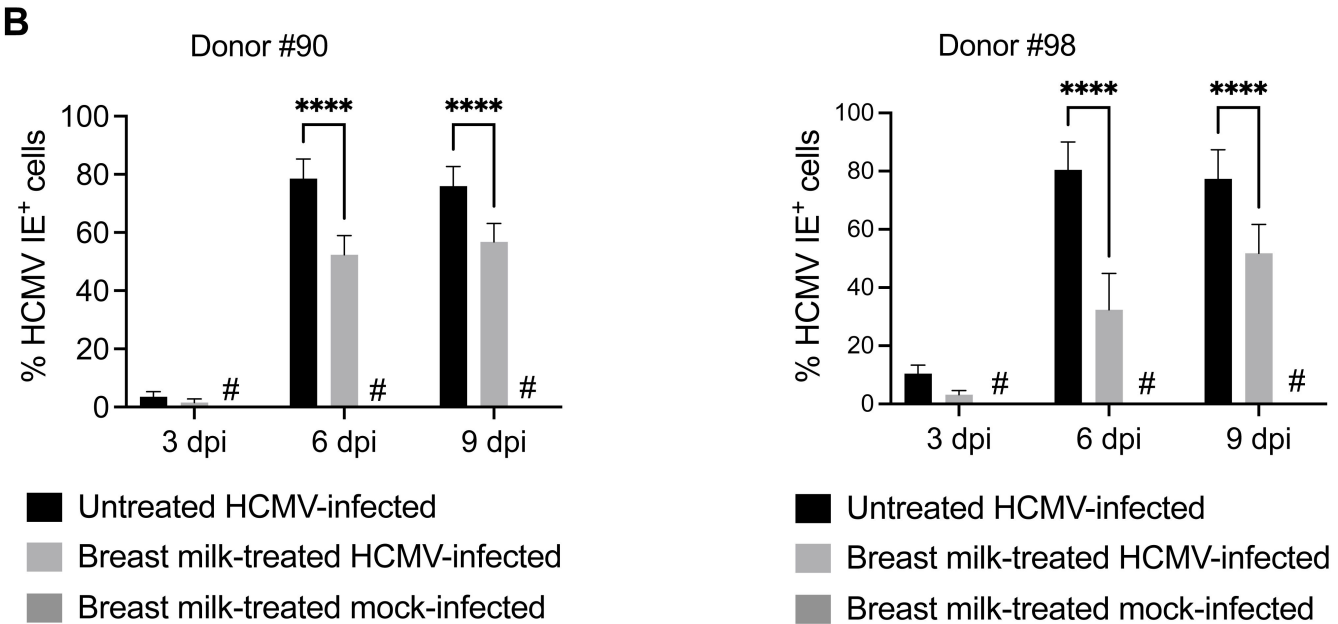
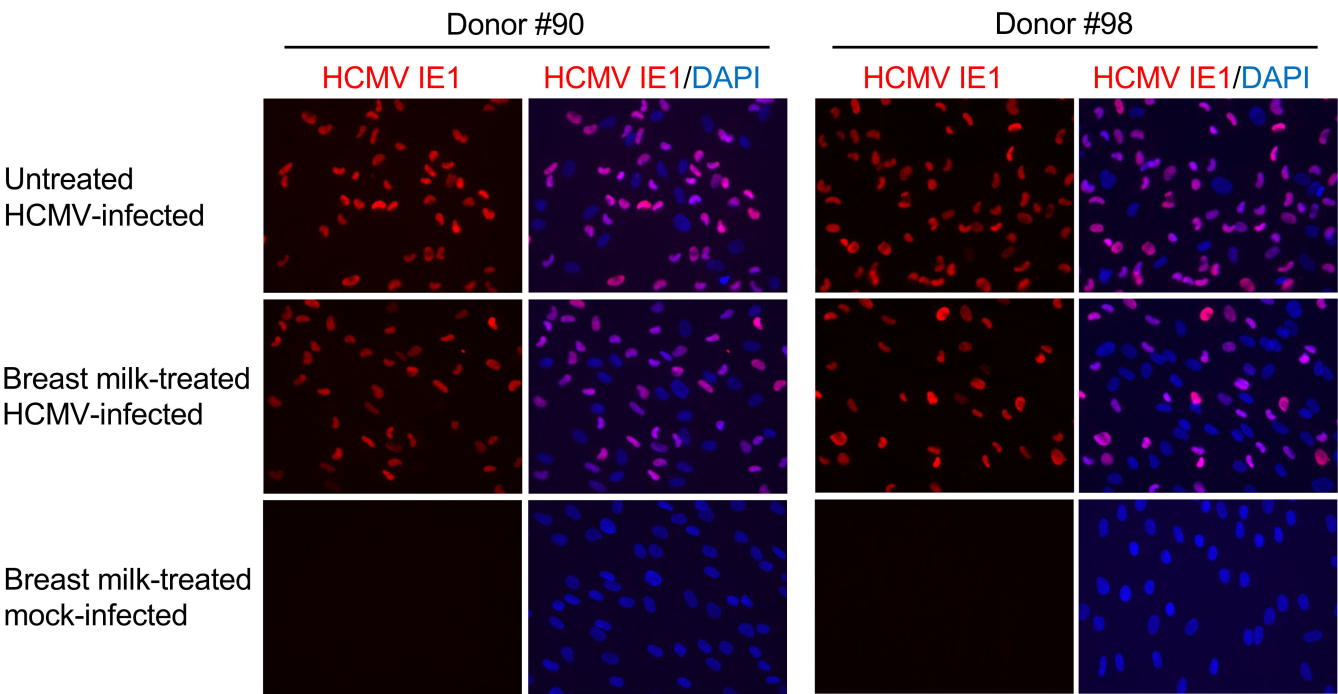


FIG 9 Breast milk inhibits but does not abolish the production of infectious HCMV virions in infant tonsil epithelial cells. Infant tonsil epithelial cells from donors #90 and #98 were infected with HCMV VR1814 at an MOI of 1 in the presence of breast milk obtained from donor B or C. Cell culture supernatants were harvested on days 3, 6, and 9 post-infection. Infectivity was examined in human fibroblasts by immunostaining for HCMV IE1 at 24 hours after infection. The percentage of HCMV-IE1-positive cells was determined quantitatively using images from at least six randomly selected fields acquired at $\times 400$ magnification. (A) Fibroblasts expressing HCMV IE1 protein were detected after incubation with supernatants from tonsil epithelial cells (9 dpi). (B) Percentage of fibroblasts expressing HCMV IE1 protein; #not detected. Results are presented as means $\pm$ SD; **** $P < 0.0001$.

whey as early as 2 months postpartum (59). These data suggest maternal neutralizing antibodies were more prominent in systemic circulation as opposed to the mucosa.

HCMV reactivation during lactation occurs in nearly all HCMV-seropositive women (10). Reactivation can take place locally in the mammary glands and often occurs without the detection of viral DNA in blood or urine (63). Peripheral blood monocytes migrate to the mammary gland tissue through systemic circulation and the lymphatic vessels (14), and CD14⁺ macrophages are among the most abundant leukocytes found in breast milk (14, 16–18). HCMV-infected CD14⁺ monocytes/macrophages were detected in breast milk, suggesting that they may ultimately be responsible for viral transmission from mother to child (20). The impact of breast milk on viral reactivation was not addressed in previous studies. Experimental results using our *in vitro* model (i.e., CD14⁺ monocytes with latent HCMV infection) revealed that breast milk did not activate latent HCMV. However, breast milk does induce monocyte-to-macrophage differentiation, which facilitates HCMV infection. Macrophage differentiation is a complex and multistep process, and the specific step or phenotype of differentiation that supports HCMV reactivation is not known. It is possible that breast milk-induced macrophage differentiation may not express the critical factor(s) for HCMV reactivation.

CD14 is expressed on myeloid cells, including monocytes and macrophages, and is a critical mediator of innate immune cell activation (41). Peripheral blood monocytes migrate into the tissues where they differentiate into macrophages with specific phenotypes that develop in response to tissue-specific stimuli and microenvironments (64, 65). Macrophages are divided phenotypically and functionally into two subtypes, including classically activated M1 (CD80⁺/CD86⁺) and alternatively activated M2 (CD163⁺/CD206⁺) macrophages (42). One recent report described a unique lactation-associated population of macrophages found in murine mammary tissue and human breast milk (15). “Lactation-induced macrophages” (LiMacs) in mice are predominantly monocyte-derived and were found to proliferate rapidly *in situ* during lactation.

In the present study, we modeled the generation of lactation-induced macrophages *in vitro* using human breast milk and peripheral blood CD14⁺ monocytes obtained from healthy donors. We found that treatment of peripheral blood monocytes with breast milk induces their differentiation into large CD14^{high}CD163^{high}CD68^{high}CD206^{high}CD1a[−] macrophages that displayed numerous protrusions (Fig. 1A; Fig. S3). These findings are consistent with previous descriptions of breast milk macrophages as large cells with numerous inclusions in the cytoplasm that express the CD14 marker (16, 18). LiMac-like macrophages with a similar phenotype (CD11b⁺CD11c⁺CD64⁺HLA-DR⁺CD163⁺CD14⁺CD16⁺) were also identified in human breast milk (15). The high level of CD163 and CD206 expression indicates macrophage polarization toward the alternatively activated M2 phenotype (Fig. 1B; Fig. S4 and S5). Although HCMV can establish and sustain productive infection in both types of macrophages, M2 macrophages are more susceptible to infection (35). Interestingly, the high level of CD14 and CD163 expression detected on breast milk-induced macrophages is reduced substantially at 3 days after HCMV infection (Fig. 3A and B). While the mechanism underlying this dramatic change remains unclear, productive HCMV infection in macrophages also resulted in reductions in the surface expression of CD11b (66) as well as CD11c and CD64 with a distinct impact on macrophage identity and function (67). Future studies will be needed to define the molecular mechanisms leading to these changes.

Freshly isolated breast milk macrophages spontaneously produce GM-CSF (16). Furthermore, the development and maintenance of LiMacs required colony-stimulating factor 1 (CSF-1, also called M-CSF) (15). Given these findings, we compared the responses of breast milk-induced macrophages to those induced by M-CSF and GM-CSF. We found that the phenotype of breast milk-induced macrophages is similar to that of M-CSF-induced macrophages (both are CD68^{high}CD1a[−]). However, despite these similarities, breast milk-induced macrophages supported significantly higher levels of HCMV infection than those that were M-CSF-induced (Fig. 5A and B). These results suggest that breast milk-induced macrophages may have some additional features that support HCMV replication. For example, breast milk may induce high-level expression of HCMV receptors in macrophages; it may also facilitate the development of a

unique macrophage phenotype and the expression of multiple HCMV receptors. Our finding that breast milk promotes increased HCMV binding to macrophages supports this notion. Several specific HCMV receptors have been described and characterized. Platelet-derived growth factor- α (PDGFR α) has been identified as the cellular receptor for HCMV in fibroblasts (68), while neuropilin-2 (Nrp2) serves as an HCMV receptor in epithelial and endothelial cells (69). Olfactory receptor family 14 subfamily I member 1 (OR14I1) has also been identified as a receptor for HCMV in epithelial cells (70). Other factors that facilitate virus infection have also been characterized. For example, epidermal growth factor receptor activation is required for HCMV infection in fibroblasts (71), CD34⁺ progenitor cells (72), and monocytes (73), while HCMV binding to DC-SIGN is required for dendritic cell infection (74). Future studies will be needed to define the molecular mechanism underlying the increased susceptibility to HCMV observed in breast milk-induced macrophages.

Our finding that breast milk induces macrophage proliferation is quite intriguing and consistent with results presented in another recent report that detailed the rapid *in situ* expansion of monocyte-derived LiMacs during lactation (15). Many studies have focused on macrophage proliferation and its critical role in promoting tissue homeostasis and the immune response. For example, the human macrophage population expands in areas of necrosis secondary to the recruitment of circulating monocytes as well as the proliferation of resident macrophages (75). Similarly, alveolar macrophage proliferation contributes to local macrophage expansion in patients with chronic lung inflammation (76) and the proliferation of donor macrophages contributes to the reconstitution of the alveolar macrophage population after allogeneic bone marrow transplantation (77). Results from mouse model studies revealed that (i) local proliferation of resident macrophages leads to their accumulation in adipose tissue during obesity development (78); (ii) the myelocytomatosis viral oncogene (Myc) is required for M-CSF-driven proliferation in macrophages (51), and (iii) macrophage proliferation is facilitated by M-CSF-induced Akt signaling (49, 50). To the best of our knowledge, this study is the first to demonstrate that breast milk induces human macrophage proliferation *in vitro*. In future studies, we plan to identify the specific molecules or factors in breast milk that induce macrophage proliferation.

Breast milk-induced macrophages are highly susceptible to lytic HCMV infection compared to both controls and M-CSF-induced macrophages, despite the latter having a similar phenotype (Fig. S4 to S6). In contrast, treatment of fibroblasts with breast milk elicited no change in their susceptibility to virus infection (Fig. S10), i.e., breast-milk-induced macrophage differentiation and proliferation have a specific role in HCMV infection. Previous studies indicated that M-CSF-induced macrophages produce significantly higher levels of viral progeny than GM-CSF-induced macrophages at 4–6 days after HCMV infection (35). Here, we found that breast milk-induced macrophages present significantly higher levels of infectious virions at 6 and 9 days post-infection (dpi) compared to macrophages induced with M-CSF alone. Furthermore, breast milk-induced macrophages can sustain a productive HCMV infection for up to 12 dpi (Fig. 6B). It has been shown that the continuous production of infectious virions by differentiated macrophages that can survive for months to years (26) represents a long-term viral persistence strategy (15, 30). Enhanced HCMV replication in breast milk-induced macrophages may contribute to the observed chronic shedding of HCMV in the breast milk of postpartum mothers (79).

While HCMV is transmitted from seropositive mothers to their infants via breastfeeding in the postpartum period, the mechanisms leading to this transfer have not been elucidated (3, 4, 6, 10). Previous studies have revealed that HCMV infects polarized human colon epithelial cells via the apical surface. This result suggests that infectious HCMV in breast milk may infect gut epithelium, thereby establishing MTCT (8). We recently demonstrated that HCMV infected both polarized infant tonsil epithelial cells and *ex vivo* tonsil explants from their apical/mucosal surface; this might be the first target for HCMV MTCT through breast milk (7). Notably, we did not include polarized tonsil

epithelial cells or tonsillar organoids in these experiments, which is a limitation of this study. We found that HCMV virions released from infected breast milk-induced macrophages can infect infant tonsil epithelial cells (Fig. 7), thereby highlighting this important potential route for HCMV MTCT. HCMV-infected breast milk macrophages might interact with both the tonsil and intestinal surface, thereby spreading the virus into both these epithelia and initiating HCMV MTCT. Moreover, diminished HCMV infection of infant tonsil epithelial cells observed in the direct presence of breast milk (Fig. 8) suggests the presence of one or more antiviral factors including immunoglobulin, defensins, glycoproteins, glycated components, human milk oligosaccharides, polyunsaturated fatty acids, and extracellular vesicles (58, 80). However, consistent with previous findings (81), we show here that HCMV infection of infant tonsil epithelial cells still occurs in the presence of breast milk, and it can be sustained productively for up to 9 days (Fig. 9).

Collectively, these findings suggest that HCMV could be transmitted from seropositive mothers to their infants by virus transfer from HCMV-infected breast milk macrophages to infant tonsil epithelial cells. Thus, our studies have uncovered a potential mechanism in which breast milk induces the differentiation of monocytes into macrophages as well as macrophage proliferation and both contribute to HCMV MTCT via the oropharyngeal mucosal route. These findings suggest that specific intervention strategies might be considered to prevent the long-term neurodevelopmental sequelae of postnatal HCMV transmission.

MATERIALS AND METHODS

Viruses and cells

HCMV clinical strain VR1814 was kindly provided by Dr. Lenore Pereira, University of California–San Francisco. HCMV VR1814 was propagated in primary human umbilical vein endothelial cells (HUVECs; Lonza, catalog: C2519A). HUVECs were infected with VR1814 at an MOI of 0.01. Cell culture media were harvested when cells reached 100% cytopathic effect. Cell-free virions released into the culture media were concentrated by centrifugation at 20,000 rpm at 4°C for 90 min. The virions were resuspended in Dulbecco's Modified Eagle Medium (DMEM; Corning, catalog: 10-017-CM) containing 20% heat-inactivated FBS (Hyclone, catalog: SH30396.03). VR1814 viral stocks were titrated in normal human neonatal dermal fibroblasts (Lonza, catalog: CC-2509). Fibroblasts were incubated with 200 μ L of serial dilutions of the viral stock solution at 37°C for 2 hours. The cells were then washed and cultured for 24 hours and then fixed and immunostained for HCMV immediate early (IE) protein. The titer of the viral stock (in IU/mL) was calculated with the following formula: number of HCMV IE-positive nuclei $\times$ 5 $\times$ (1/dilution of the inoculum).

HUVECs were maintained in an endothelial cell growth medium (Lonza, EGM-2 BulletKit Medium, catalog: CC-3162). Normal human neonatal dermal fibroblasts (Lonza, catalog: CC-2509) were maintained in a fibroblast growth medium (Lonza, FGM-2 BulletKit, catalog: CC-3132).

PBMCs were isolated from heparinized blood from HIV-negative donors using a Ficoll-Paque Plus density gradient (Sigma). CD14⁺ cells were isolated from PBMCs using human CD14 MicroBeads (Miltenyi Biotec, catalog:130-050-201) following the manufacturer's instructions. The purity of CD14⁺ cells used in the experiments was ~95% as determined by flow cytometry.

Primary tonsil epithelial cells were established from palatine tonsil tissue collected from HIV-negative children who were less than 5 years of age and had undergone routine tonsillectomy at the clinic of the Department of Otolaryngology, University of California–San Francisco. Epithelial cells were isolated from small palatine tonsil tissue fragments by treatment with 0.2% type I collagenase at 37°C for 1 hour under stirring conditions. After digestion, cells were suspended in RPMI 1640 medium (Corning, catalog: 10-040-CM) supplemented with 10% heat-inactivated FBS and passed through a 70 μ m cell strainer. After washing, the cells were resuspended in keratinocyte

growth medium (KGM Gold; Lonza, catalog: 00192060) supplemented with 1% antibiotic antimycotic solution and seeded into collagen-coated flasks (Corning, catalog: 353136). Epithelial cells were frozen in liquid nitrogen. Experiments were performed with early passages (2–5) only. Epithelial cells obtained from each donor were tested to verify the absence of HIV-1 and HCMV infection.

Preparation of human breast milk samples

Fresh samples of human breast milk were obtained from HIV-negative healthy volunteers within 2–5 months postpartum and were subjected to centrifugation at $600 \times g$ for 15 min to separate the lipid phase, milk supernatant, and cell pellets. The lipid phase was aspirated. The milk supernatant was filtered through a 0.45 μm filter, aliquoted, and stored at -80°C . Cell pellets were resuspended in 90% FBS containing 10% dimethyl sulfoxide and stored in liquid nitrogen. For the cell treatment experiments, the human breast milk supernatants were diluted in RPMI 1640 medium containing 10% heat-inactivated FBS.

Cell viability assays

CD14⁺ cells were seeded in clear flat-bottom 96-well plates (Corning, catalog: 3595) at a density of 2×10^5 cells per well and incubated for 6 days both with and without breast milk supernatants at dilutions of 1:2, 1:4, or 1:8. The culture media were refreshed on day 3. On day 6, CellTiter-Glo 2.0 assays were performed following the manufacturer's instructions (Promega). Control wells containing medium alone without cells were used to determine the background luminescence. After equilibrating the plate and its contents to room temperature for approximately 30 min, 100 μL of CellTiter-Glo 2.0 Reagent was added to 100 μL of medium with or without cells. The plate was placed on an orbital shaker for 2 min to induce cell lysis and additionally at room temperature for 10 min to stabilize the luminescence signal. Triplicate 50 μL samples were then transferred to white flat-bottom 96-well plates (Costar, catalog: 3917). Luminescence was measured using a Promega Glomax machine.

Immunophenotyping the cells by flow cytometry

Flow cytometry analysis was performed as described previously (82). Briefly, human CD14⁺ cells were seeded in non-treated six-well plates (Corning) at a density of 1×10^6 cells/well. On day 0 and day 3, 1 mL of RPMI 1640 medium containing 10% FBS and (i) human breast milk (1:4 dilution), (ii) 25 ng/mL recombinant human M-CSF (rhM-CSF; Peprotech, catalog: 300–25), (iii) 25 ng/mL rhGM-CSF (Peprotech, catalog: 300–03), or (iv) 25 ng/mL rhGM-CSF and 25 ng/mL rhIL-4 (R&D Systems) was added to specific well. On day 5, cells were harvested for immunophenotyping by flow cytometry. To determine the phenotype of HCMV-infected cells, 6 days after treatment, the cells were washed with Dulbecco's phosphate-buffered saline (DPBS, Corning, catalog: 21–031-CM) and then inoculated with HCMV VR1814 at an MOI of 1 at 37°C for 2 hours. Cells were washed with DPBS and cultured in RPMI 1640 medium containing 10% FBS. At 3 dpi, cells were washed with DPBS and harvested after incubation with 1 mL of Cellstripper solution (Corning, catalog: 25–056-CI) at room temperature for 20 min. One milliliter of RPMI 1640 medium containing 10% FBS was then added to the cells. Any cells that remained adherent were detached using a cell scraper (Thermo Scientific, catalog: 179693). Harvested cells were centrifuged at $400 \times g$ at 4°C for 4 min, resuspended in 200 μL of DPBS, and transferred to U-bottom 96-well plates. Cells were stained with Live/Dead Fix Aqua solution (Invitrogen, catalog: L34966) at 4°C for 20 min while protected from light. After incubation, cells were washed twice with DPBS. To prevent non-specific antibody binding, cellular Fc receptors were blocked by incubating the cells with an FcR blocking solution containing 2.5 μL of Human TruStain FcX (BioLegend Human TruStain FcX, catalog: 422302) per 50 μL BD brilliant stain buffer per sample. Cells were blocked at room temperature for 5–10 min while

remaining protected from light. Without removing the FcR blocking solution, cells were stained at 4°C for 1 hour with the following antibodies: PE-Dazzle 594-conjugated mouse anti-human CD14 IgG2a (BioLegend, catalog: 301852, 1:200 dilution), Brilliant Violet 711-conjugated mouse anti-human CD163 IgG1 (BioLegend, catalog: 333630, 1:200 dilution), APC-conjugated mouse anti-human CD206 (BioLegend, catalog: 321110, 1:200 dilution), and Brilliant Violet 421-conjugated mouse anti-human CD1a IgG1 (BioLegend, catalog: 300128, 1:200 dilution) in the BD brilliant stain buffer. Cells were then washed with DPBS containing 2% FBS and fixed with 4% paraformaldehyde at 4°C for 20 min while remaining protected from light. Then, the cells were washed and permeabilized with 0.1% Triton X-100 at 4°C for 10 min. Intracellular HCMV IE1 protein was detected by staining the fixed and permeabilized cells with the Alexa Fluor 488-conjugated mouse anti-HCMV IE1 IgG2a (MilliporeSigma, catalog: MAB810X, 1:1,000 dilution) at 4°C for 1 hour. To detect intracellular CD68 and Ki67, cells were first incubated with antibodies against surface markers. Cells were then fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (Invitrogen, catalog: 00-5523-00) following the manufacturer's instructions. Fixed and permeabilized cells were incubated with APC/Cyanine7-conjugated mouse anti-human CD68 antibody (BioLegend, catalog: 333822, 1:20 dilution) and PE-conjugated mouse anti-human Ki-67 antibody (BioLegend, catalog: 350504, 1:20 dilution). Cells were washed with DPBS containing 2% FBS and analyzed on a BD LSRFortessa flow cytometer (BD Biosciences) using FACSDiva software.

Cell proliferation assays

Click-iT EdU proliferation assay for microplates

The Click-iT EdU Proliferation assay for microplates (Invitrogen, catalog: C10499) was used following the manufacturer's instructions. Briefly, human CD14⁺ cells were seeded at a density of 1×10^5 cells/well in clear flat-bottom 96-well plates (Corning, catalog: 3595). Cells were left untreated or treated with breast milk (1:4 dilution) or 25 ng/mL rhM-CSF (Peprotech, catalog: 300-25); the culture media were refreshed on day 3. On day 5, 10 μ M EdU (final concentration) diluted in RPMI 1640 medium supplemented with 10% FBS with or without breast milk or rhM-CSF was added to the cell cultures. On the following day, cells were fixed for 5 min using Click-iT EdU fixative at room temperature. Cells were then washed with the Click-iT EdU wash buffer. The 1 $\times$ Click-iT EdU reaction cocktail was then added followed by a 30 min incubation at room temperature. Cells were then blocked for 5 min with a 1.5% bovine serum albumin (BSA) blocking solution at room temperature. After washing cells with the Click-iT EdU wash buffer, the Amplex UltraRed reaction mixture was added followed by incubating at room temperature for 15 min protected from light. The Amplex UltraRed stop solution was used to stop the reaction. The absorption signal that indicated the incorporation of EdU into newly synthesized DNA was measured by a Molecular Devices EMax precision microplate reader using a filter (562 nm) appropriate for Amplex UltraRed reagent.

Click-iT plus EdU cell proliferation imaging assay and immunofluorescence staining for Ki67

Cell proliferation was measured using Click-iT Plus EdU Cell Proliferation imaging kits (Invitrogen, catalog: C10637) and rabbit monoclonal anti-Ki67 (Abcam, catalog: ab16667, 1:250 dilution) following the manufacturer's instructions. Briefly, human CD14⁺ cells were seeded at a density of 2×10^5 cells/well on Nunc Lab-Tek chamber slides (Thermo Scientific, catalog: 177402PK). Cells were left untreated or treated with breast milk (1:4 dilution) or 25 ng/mL rhM-CSF (Peprotech, catalog: 300-25); the culture media were refreshed on day 3. On day 3 or day 5, 10 μ M EdU (final concentration) diluted in RPMI 1640 medium supplemented with 10% FBS with or without breast milk or M-CSF was added to the cells. On the following day, cells were fixed for 15 min in 4% paraformaldehyde in PBS and then washed with 3% BSA in PBS and permeabilized for 20 min in 0.5% Triton X-100. Cells were washed with 3% BSA in PBS; 5% BSA in PBS was then added,

and cells were incubated for 1 hour. The 1× Click-iT Plus reaction cocktail was added, and cells were incubated for 30 min at room temperature while protected from light. Cells were washed with 3% BSA in PBS; rabbit monoclonal anti-Ki67 diluted in PBS with 3% BSA was added. Cells were then incubated at 4°C overnight. After washing, Alexa Fluor 594 donkey anti-rabbit secondary antibody (Invitrogen, catalog: A-21207, 1:1,000 dilution in PBS with 3% BSA) was added, and cells were incubated for 1 hour at room temperature while protected from light. Cells were then washed three times with PBS and were mounted on microscope slides using ProLong Diamond Antifade Mountant with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, catalog: P36962) and analyzed on a fluorescence microscope.

Quantitative reverse transcription-PCR

DNA was extracted from 2×10^5 monocyte-derived cells, 2×10^5 tonsil epithelial cells, or 200 μ L whole breast milk using QIAamp DNA Blood Mini Kit (QIAGEN, catalog: 51104) following the manufacturer's instructions. The HCMV genome was quantified based on five standards prepared from HCMV VR1814 stock. HCMV VR1814 stock (titer: 5.6×10^6 IU/mL) was diluted in PBS to create four 10-fold diluted standards. This yielded five standards at 5.6×10^2 to 5.6×10^6 IU/mL, or 2.75, 3.75, 4.75, 5.75, and 6.75 log₁₀ IU/mL, respectively. Buffer AE (10 mM Tris-Cl; 0.5 mM EDTA; pH 9.0) from QIAamp DNA Blood Mini Kit served as a negative control. The β -actin housekeeping gene was used as an internal control to normalize gene expression data.

The qRT-PCR assay used a PowerUp SYBR Green Master Mix (Applied Biosystems, catalog: A25742) following the manufacturer's instructions. The total reaction volume was 20 μ L, and reactions were carried out using the Real-Time PCR Detection System (ATILA BioSystems). Primers (IE1 FW: 5'-CACGACGTTCTGCAGACTA-3') and (IE1 RV: 5'-TTTTCAGCATGTGCTCCTTG-3') were used to amplify HCMV IE1 DNA (83); primers (β -actin FW: 5'-CCCTGGACTTCGAGCAAGAG-3') and (β -actin RV: 5'-ACTCCATGCCAGGAAGGAA-3') were used to amplify β -actin DNA (84). Each reaction mixture contained 10 μ L of the 2× master mix, 0.4 μ L of 10 μ M forward (FW) primer, 0.4 μ L of 10 μ M (RV) reverse primer, 5.2 μ L of nuclease-free water, and 4 μ L of DNA. The qPCR cycling conditions were as follows: 50°C, 2 min; 95°C, 2 min; 40 cycles at 95°C, 15 s and 60°C, 1 min; 95°C, 15 s; 60°C, 1 min; as well as 95°C, 15 s; with a final cooling step at 4°C.

HCMV infection of monocyte-derived cells

Human CD14⁺ cells were seeded at a density of 2×10^5 cells/well on Nunc Lab-Tek chamber slides (Thermo Scientific, catalog: 177402PK) in RPMI 1640 medium containing 10% FBS with or without human breast milk (1:4 dilution) or 25 ng/mL rhM-CSF (Peprotech, catalog: 300-25). The media were refreshed on day 3. On day 6, cells were washed with DPBS and inoculated with HCMV VR1814 at an MOI of 1 at 37°C for 2 hours. After incubation with the virus, cells were washed three times with DPBS. RPMI 1640 medium containing 10% FBS was added to all wells. Cell culture media were harvested on 3, 6, 9, and 12 dpi. The supernatants were centrifuged to remove the detached cells and then added to an indicator monolayer of human fibroblasts to evaluate the infectivity of the virus contained in the supernatants. The indicator fibroblasts were fixed 24 hours after infection and immunostained for the HCMV IE1 protein. Monocyte-derived cells were fixed and immunostained for HCMV IE1 or HCMV gB on day 9 post-infection.

The infectivity of HCMV virions released from monocyte-derived cells was also tested on a monolayer of infant tonsil epithelial cells. At 3, 6, and 9 days after HCMV infection of monocyte-derived cells, the culture media were harvested and centrifuged to remove detached cells. The supernatants were then incubated with the indicator infant tonsil epithelial cells. After 24 hours, tonsil epithelial cells were fixed and immunostained for the HCMV IE1 protein as described above.

HCMV infection of infant tonsil epithelial cells in the presence of breast milk

Primary infant tonsil epithelial cells obtained from two donors (#90 and #98) were seeded at a density of 2×10^5 cells/well on Nunc Lab-Tek chamber slides (Thermo Scientific, catalog: 177402PK). Confluent cells were infected with HCMV VR1814 at an MOI of 1 in the presence of breast milk obtained from two donors (B and C, 1:4 dilution) at 37°C for 2 hours. After incubation with the virus, cells were washed three times with DPBS. At 3, 6, and 9 dpi, the culture media were harvested and centrifuged to remove the detached cells. The infectivity of the virus in the supernatants was evaluated on an indicator monolayer of human fibroblasts. At 24 hours after infection, fibroblasts were fixed and immunostained to detect the HCMV IE1 protein. At 9 dpi, tonsil epithelial cells were fixed and immunostained for HCMV gB protein.

Immunofluorescence assay

Cells were fixed for 20 min with 4% paraformaldehyde in PBS at room temperature. Fixed cells were washed twice with PBS and permeabilized with 0.1% Triton-X 100 in PBS for 10 min. After washing with PBS, cells were blocked with 5% BSA in PBS or 5% normal serum from the sources of the secondary antibodies (i.e., donkey or goat serum) at room temperature for 1 hour. Cells were then incubated with a primary antibody in PBS with 1% BSA or normal serum at room temperature for 1 hour or at 4°C overnight. The primary antibodies included mouse monoclonal anti-HCMV IE1 (Virusys, catalog: P1203, 1:2,000 dilution), mouse monoclonal anti-HCMV gB (clone CH177, 1:500 dilution, a gift from Dr. Lenore Pereira), rabbit polyclonal anti-wide spectrum cytokeratin (Abcam, catalog: ab9377, 1:50 dilution), or rabbit IgG isotype control (Abcam, catalog: ab37415, used at the same concentration as the rabbit primary antibody). After washing three times with PBS, the cells were incubated for 1 hour with the secondary antibodies diluted in PBS supplemented with 1% BSA at room temperature. Secondary anti-mouse or anti-rabbit antibodies labeled with DyLight 488 or DyLight 594 were purchased from Vector laboratories and used at a 1:500 dilution. After washing three times with PBS, cells were mounted on microscope slides using ProLong Diamond Antifade Mountant with DAPI (Invitrogen, catalog: P36962) and analyzed using a Nikon Eclipse E400 fluorescence microscope (Nikon).

Giemsa staining

Giemsa stain (Sigma, catalog: G5500-500ML) was used following the manufacturer's instructions. Briefly, cells were fixed in methanol for 7 min, air dried, and then incubated with Giemsa stain (1:20 dilution in deionized water) for 40 min. The slides were then rinsed with deionized water, air-dried, and mounted using VectaMount Permanent Mounting Medium (Vector Laboratories, catalog: H-5000-60). Cells were examined by a Nikon Eclipse E400 fluorescence microscope (Nikon) using the bright field.

HCMV attachment assay

Isolated human CD14⁺ cells were seeded at a density of 2×10^5 cells/well in Nunc Lab-Tek chamber slides (Thermo Scientific, catalog: 177402PK) and were incubated with or without breast milk (1:4 dilution) for 6 days with the culture media refreshed on day 3. On day 6, cells in the chamber slides were cooled for 20 min in a 4°C refrigerator, washed with chilled DPBS, and then incubated with a chilled suspension of HCMV VR1814 at an MOI of 1 at 4°C for 2 hours. Cells were then washed four to five times with chilled DPBS to remove unbound cell-free virions. Cell-associated DNA was extracted using QIAamp DNA Blood Mini Kit (QIAGEN, catalog: 51104) following the manufacturer's instructions.

HCMV entry assay

Isolated human CD14⁺ cells were seeded at a density of 2×10^5 cells/well in Nunc Lab-Tek chamber slides (Thermo Scientific, catalog: 177402PK) and were incubated with

or without breast milk (1:4 dilution) for 6 days with the culture media refreshed on day 3. On day 6, cells were washed with DPBS and then incubated with HCMV VR1814 at an MOI of 1 at 37°C for 2 hours. Cells were then washed three times with DPBS. Surface-bound virions were removed by treatment with 2 mg/mL proteinase K (Invitrogen, catalog: AM2546) in DPBS at 4°C for 1 hour. Cell culture medium containing 10% FBS was then added to stop the enzymatic reaction, and cells were washed four times with DPBS to remove any cell-free virus. Cell-associated DNA was extracted using QIAamp DNA Blood Mini Kit (QIAGEN, catalog: 51104) as described above.

Detection of CMV IgG antibodies

HCMV-specific IgG antibodies in breast milk samples were measured using a human anti-CMV IgG ELISA kit (Abcam, catalog: ab108724) following the manufacturer's instructions. Results were determined based on the following parameters (in Standard Units): cut-off, 10; gray zone, 9–11; negative, <9; positive, >11.

HCMV infection of primary human fibroblasts

Normal human neonatal dermal fibroblasts (Lonza, catalog: CC-2509) were seeded at a density of 2×10^4 cells/well on Nunc Lab-Tek chamber slides (Thermo Scientific, catalog: 177402PK) in fibroblast growth medium (Lonza, FGM-2 BulletKit, catalog: CC-3132). When the culture reached ~80% confluency, fibroblasts were left untreated or treated with human breast milk (1:4 dilution) for 6 days with the media refreshed on day 3. On day 6, cells were washed with DPBS, inoculated with HCMV VR1814 at an MOI of 0.5, and cultured at 37°C for 2 hours. Cells were then washed three times with DPBS and cultured in fibroblast growth medium. At 24 hours post-infection, fibroblasts were fixed and immunostained for HCMV IE1 protein.

HCMV latency and reactivation assays

HCMV latency and reactivation assays were performed as previously reported (55–57). Briefly, human CD14⁺ cells were seeded at a density of 2×10^5 cells/well on Nunc Lab-Tek chamber slides (Thermo Scientific, catalog: 177402PK) in X-VIVO 15 serum-free medium (X-VIVO medium) and cultured overnight. Cells were washed with DPBS and inoculated with HCMV VR1814 at an MOI of 4 and cultured at 37°C for 2 hours. Cells were then washed three times with DPBS and cultured in X-VIVO medium for 6 days with the media refreshed on day 3 to establish HCMV latency.

Cells with latent HCMV infection were left untreated or treated with human breast milk (1:4 dilution) or 50 ng/mL rhM-CSF (Peprotech, catalog: 300–25) in X-VIVO medium or RPMI 1640 medium supplemented with 10% heat-inactivated FBS (RP10 medium) for 6 days with the media refreshed on day 3. Infected cells were co-cultured with primary human fibroblasts (Lonza, catalog: CC-2509) in X-VIVO medium or RP10 medium for 10 days, with the media refreshed every other day. Co-cultured cells were fixed and stained with anti-HCMV IE1 antibody, and infectious centers containing five or more HCMV IE1-positive cells were counted. On day 10 after co-culture, cell culture supernatants were also harvested and added to an indicator monolayer of human fibroblasts to evaluate the infectivity of the HCMV virions released into the supernatants. The indicator fibroblasts were fixed 24 hours after infection and immunostained for the HCMV IE1 protein. The number of infectious virions in the supernatants was calculated and expressed as infectious units per milliliter.

Statistical analysis

Statistical comparisons were performed using two-tailed Student's *t* tests for two groups or analysis of variance for three or more groups (GraphPad PRISM 10). Values of *P* < 0.05 were considered significant. Results are presented as means ± SD.

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AUTHOR CONTRIBUTIONS

Xiaodan Cai, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review and editing | Nicole T. Padilla, Investigation | Kristina Rosbe, Resources | Sharof M. Tugizov, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – review and editing

DATA AVAILABILITY

All data are fully available without restriction.

ETHICS APPROVAL

This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the Committee on Human Research of the University of California–San Francisco (IRB approval #H8597-30664-03). All subjects provided written informed consent for the collection of samples and their subsequent analysis.

ADDITIONAL FILES

The following material is available [online](#).

Supplemental Material

Supplemental material (JV101177-24-s0001.pdf). Fig. S1 to S13.

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