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Viral Interleukin-10 Expressed by Human Cytomegalovirus during the Latent Phase of Infection Modulates Latently Infected Myeloid Cell Differentiation▽†

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The human cytomegalovirus UL111A gene is expressed during latent and productive infections, and it codes for homologs of interleukin-10 (IL-10). We examined whether viral IL-10 expressed during latency altered differentiation of latently infected myeloid progenitors. In comparison to infection with parental virus or mock infection, latent infection with a virus in which the gene encoding viral IL-10 has been deleted upregulated cytokines associated with dendritic cell (DC) formation and increased the proportion of myeloid DCs. These data demonstrate that viral IL-10 restricts the ability of latently infected myeloid progenitors to differentiate into DCs and identifies an immunomodulatory role for viral IL-10 which may limit the host's ability to clear latent virus.

Human cytomegalovirus (HCMV) is a species-specific betaherpesvirus that infects a majority of the world's population (29). HCMV establishes and maintains a lifelong latent infection in primitive myeloid lineage cells (14, 22, 27, 48, 53, 58). Following terminal cell differentiation of these cells into myeloid dendritic cells (DCs) and macrophages, latent virus has the ability to reactivate, resulting in the production of new, infectious virions and often severe disease in immunocompromised individuals (11, 14, 28, 37, 49, 50, 59, 63). Only a subset of viral genes are transcriptionally active during latency (2, 8, 12, 13, 17, 23, 34, 47), including HCMV UL111A, a gene that encodes homologs of the potent immunomodulatory cytokine human interleukin-10 (hIL-10). UL111A is transcriptionally active during both productive and latent phases of infection and encodes several viral IL-10 proteins (17, 24–26, 46) which exert a diverse range of immunomodulatory functions, including inhibition of cytokine synthesis and major histocompatibility complex (MHC) expression by myeloid cells, stimulation of B cells, and suppression of DC maturation and cytotrophoblast function (5–7, 9, 16, 18, 36, 51, 52, 61). The vast majority of characterization of functions has come from studies using recombinant viral IL-10 proteins, although some have also assessed function during productive infection with viruses with deficient viral IL-10 proteins. Much less is known about the function of viral IL-10 in the context of latent infection, which, to date, has been limited to a single report that viral IL-10 expressed during latent infection of primary human myeloid progenitor cells modulates cell surface MHC class II levels and renders these cells refractory to recognition by both allogeneic and autologous $CD4^+$ T cells (9). In this study, we examined (i) the production of proinflammatory cytokines linked to the control of cellular differentiation and (ii) the cellular differentiation pattern of primary human myeloid progenitor cells latently infected with parental virus or virus in which the gene encoding viral IL-10 has been deleted (viral IL-10 deletion viruses), providing evidence for a role of viral IL-10 in modulating the differentiation ability of latently infected cells.

Viral IL-10 inhibits mRNA and protein expression of proinflammatory cytokines by latently infected myeloid progenitor cells. To determine whether HCMV UL111A-encoded viral IL-10 affected expression of proinflammatory cytokines involved in modulating myeloid cell differentiation, we first evaluated mRNA expression of interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α) in cells latently infected with RVAdIL10C, a recombinant virus that cannot express any viral IL-10 (33), or its parent strain AD169. Primary human $CD34^+$ myeloid progenitors were latently infected as previously described (8, 22), and cells and culture supernatants were harvested at day 8 postinfection (p.i.). We have previously demonstrated that this viral IL-10 deletion virus infects and maintains a latent infection in $CD34^+$ myeloid progenitors as efficiently as the parental virus does (9). Our routine analyses of viral genome load when new virus stocks were generated or when new infection experiments were undertaken confirmed equal rates of latent infection with these two viruses (see Fig. 3C; also data not shown). We also confirmed expression of viral IL-10 transcripts throughout the 8-day time course in cells infected with the parental virus, whereas these transcripts were not detected at any time in cells infected with the viral IL-10 deletion virus (see Fig. S1 in the supplemental material).

DNase-treated total RNA was reverse transcribed using SuperScript III (Invitrogen). mRNA expression was measured by quantitative reverse transcription-PCR (qRT-PCR) (Mx3000P qPCR system; Stratagene) at 50°C for 1 min, 95°C for 1 min, and then 50 cycles, with 1 cycle consisting of 95°C for 15 s and

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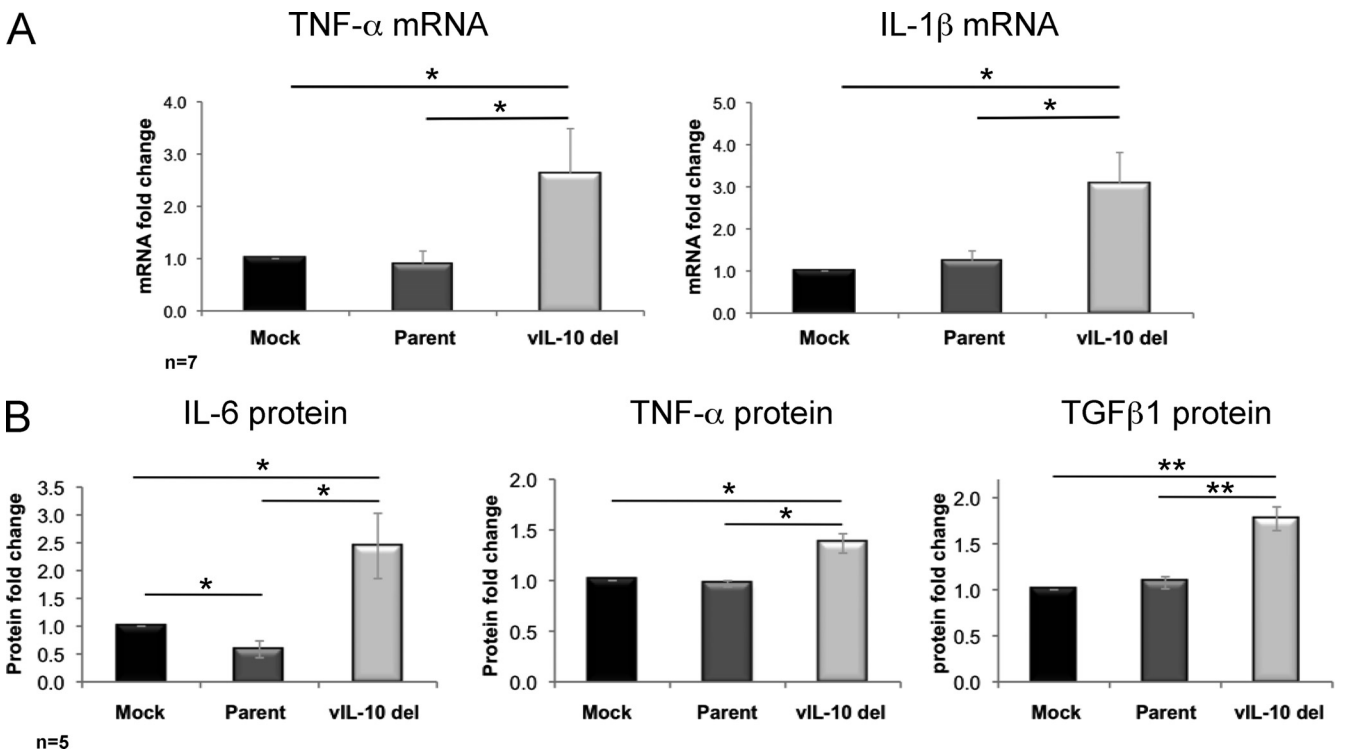

FIG. 1. Upregulation of proinflammatory cytokines during latent infection with a viral IL-10 deletion virus. $CD34^+$ myeloid progenitor cells were mock infected or latently infected with either parental virus (Parent) or a viral IL-10 deletion virus (vIL-10 del). (A) Fold mRNA change (relative to mock-infected cells) measured by qRT-PCR of TNF-α (primers TNF-α-F [F stands for forward] [5′-CCGTCTCCTACCAGACCAA G-3′] and TNF-α-R [R stands for reverse] [5′-CTGAGTCGGTCACCCTTCTC-3′]) and IL-1β (primers IL-1β-F [5′-GCTGAGGAAGATGCT GGTTC-3′] and IL-1β-R [5′-GTGATCGTACAGGTGCATCG-3′]) following normalization to the housekeeping gene GAPDH (primers GAPDH-F [5′-TCACCAGGGCTGCTTTTAAC-3′] and GAPDH-R [5′-GACAAGCTTCCCGTTCTCAG-3′]). (B) Fold change (relative to mock-infected cells) of secreted IL-6, TNF-α, and TGFβ1 proteins measured by ELISAs. The number of independent biological replicate experiments (*n*) is shown. Error bars indicate the standard errors of the means. Significant differences between values for the samples were determined by a one-tailed, paired Student's *t* test and are denoted by horizontal lines and asterisks as follows: ∗, *P* value of <0.05; ∗∗, *P* value of <0.01.

60°C for 45 s. TNF-α and IL-1β mRNA were normalized to expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are presented as fold change of mRNA expression in infected samples relative to mock infection. Analysis of seven independent replicate experiments revealed a statistically significant increase in both TNF-α and IL-1β mRNA expression in cells latently infected with the viral IL-10 deletion virus compared to both mock-infected and parental virus-infected cells, which expressed comparable levels of these cytokine mRNAs (Fig. 1A).

We next determined whether secreted protein levels of these cytokines were altered in culture supernatants from cells latently infected with parental or viral IL-10 deletion viruses or with their mock-infected counterparts. An enzyme-linked immunosorbent assay (ELISA) (SABiosciences or R&D Systems) was performed on culture supernatants (day 8 p.i.) from five independent biological replicates. This analysis was expanded to include protein levels of IL-6 and transforming growth factor β1 (TGFβ1), which also play important roles in myeloid cell differentiation (4, 10, 19, 30, 40, 55, 57) (Fig. 1B).

Protein levels of IL-6, TNF-α, and TGFβ1 were significantly higher in culture supernatants from cells latently infected with the viral IL-10 deletion virus (mean values of 18.2 ng/ml, 2.1 pg/ml, and 37.0 ng/ml, respectively) compared to both mock-infected cells (means of 8.3 ng/ml, 1.6 pg/ml, and 21.6 ng/ml, respectively) and parental virus-infected cells (means of 6.7 ng/ml, 1.5 pg/ml, and 24.1 ng/ml, respectively). There was also a modest, but statistically significant reduction of IL-6 protein in cultures infected with parental virus compared to mock-infected cultures. IL-1β protein levels from all infection settings remained below the limits of detection. These results demonstrate that the loss of the ability to express viral IL-10 during latent infection of primary myeloid progenitor cells increases expression of proinflammatory cytokines which play important roles in modulating cellular differentiation.

As an adjunct to analyses of these cytokines, we also measured both granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 expression by qRT-PCR (50 amplification cycles) from mock-infected cells or cells latently infected with parental or viral IL-10 deletion viruses. Little, if any, expression of either of these two cytokines was detected. Specifically, in analyses of four independent replicate experiments, only one showed any evidence of GM-CSF mRNA, and this was detected at a point close to the limits of detection. IL-4 mRNA was detected in only 2 out of 4 replicate experiments, and like GM-CSF, in the samples which did yield detection, this was close to the limit of detection of the assay. Furthermore, there was no quantitative difference in either GM-CSF or IL-4

mRNA between mock-infected cells and cells infected with parental virus and viral IL-10 deletion virus (data not shown). It was therefore concluded that GM-CSF and IL-4 do not play a significant role in the viral IL-10-mediated control of myeloid cell differentiation during latency.

Viral IL-10 expressed during latency inhibits differentiation of latently infected myeloid progenitor cells toward a DC phenotype. Myeloid DCs, together with monocytes, originate from $CD34^+$ myeloid progenitor cells (20, 31, 38, 39, 45, 60). TNF-α, IL-6, IL-1β, and TGFβ1 have all been linked to initiation of DC differentiation. A cocktail of TNF-α, IL-1β, and IL-6 drives DC differentiation from $CD14^+$ monocytes (10, 19), and studies have also demonstrated that TGFβ1 promotes DC differentiation from $CD34^+$ myeloid progenitor cells (4, 30, 40, 55, 57). Our finding that a lack of ability to express viral IL-10 expressed during latency resulted in the upregulation of these proinflammatory cytokines (Fig. 1) raised the possibility that the latently infected myeloid cell differentiation state may be regulated by viral IL-10. In this respect, we observed notable differences in the morphology of cells of myeloid progenitor cultures mock infected or latently infected with parental or viral IL-10 deletion viruses (Fig. 2). Specifically, in comparison to mock-infected or parental virus-infected cells, loosely adherent cells in cultures latently infected with the viral IL-10 deletion virus were often more irregular in shape with more-pronounced, numerous spiky projections and were frequently grouped together in loose clumps (Fig. 2). These features were consistent with a DC morphology (1, 3, 32, 41, 43, 44, 62).

We therefore performed flow cytometry to quantify myeloid DC formation from myeloid progenitors after mock infection or infection with parental virus or viral IL-10 deletion virus. On day 8 p.i., cells were stained with anti-CD11c antibody conjugated to allophycocyanin (anti-CD11c–APC) (BD Biosciences), anti-HLA-DR–peridinin chlorophyll protein complex (PerCP; BD Biosciences), anti-lineage 1 cocktail–fluorescein isothiocyanate (FITC) (containing antibodies against CD3, CD14, CD16, CD19, CD20, and CD56; BD Biosciences), or their respective isotype control antibodies. Data were acquired using FACSCalibur or FACSCanto flow cytometers and analyzed by CellQuest or FlowJo software, respectively (BD Biosciences).

The gated lineage-negative (Lin^-) cell population was analyzed for expression of CD11c and HLA-DR, with Lin^- $CD11c^+$ $HLA\text{-}DR^+$ cells defined as myeloid DCs. Six independent replicate experiments were analyzed. In contrast to mock- and parental virus-infected cultures, which yielded a comparable proportion of myeloid DCs, latent infection with the viral IL-10 deletion virus resulted in the formation of a significantly higher proportion of myeloid DCs (Fig. 3A and B). Addition of 100 ng/ml of recombinant viral IL-10 proteins (18) to viral IL-10 deletion virus infected-cultures on day 4 and day 6 p.i. completely abrogated this increased myeloid DC formation (Fig. 3A and B), indicating that the increased proportion of myeloid DC formed in cultures latently infected with the viral IL-10 deletion virus was due to a lack of ability to express viral IL-10.

We also measured viral DNA by qPCR to determine whether the increase in the proportion of DCs resulted in any change to viral genome load. In 4 independent replicate infection experiments, viral DNA levels remained highly comparable in cells infected with the viral IL-10 deletion virus and parental virus-infected counterparts (Fig. 3C). In addition, viral transcription from the major immediate-early region was detected only at very low levels, and there was no difference between parental and viral IL-10 deletion virus. These data indicated that there is no increased viral activity, for example due to initiation of spontaneous reactivation, in cells infected with the viral IL-10 deletion virus.

In an extension of the analyses of cell phenotype, we included staining with anti-CD207–phycoerythrin (PE; Immuno-Tech) to detect langerin, a marker expressed by Langerhans cells (a subset of myeloid DCs). Analysis of three independent

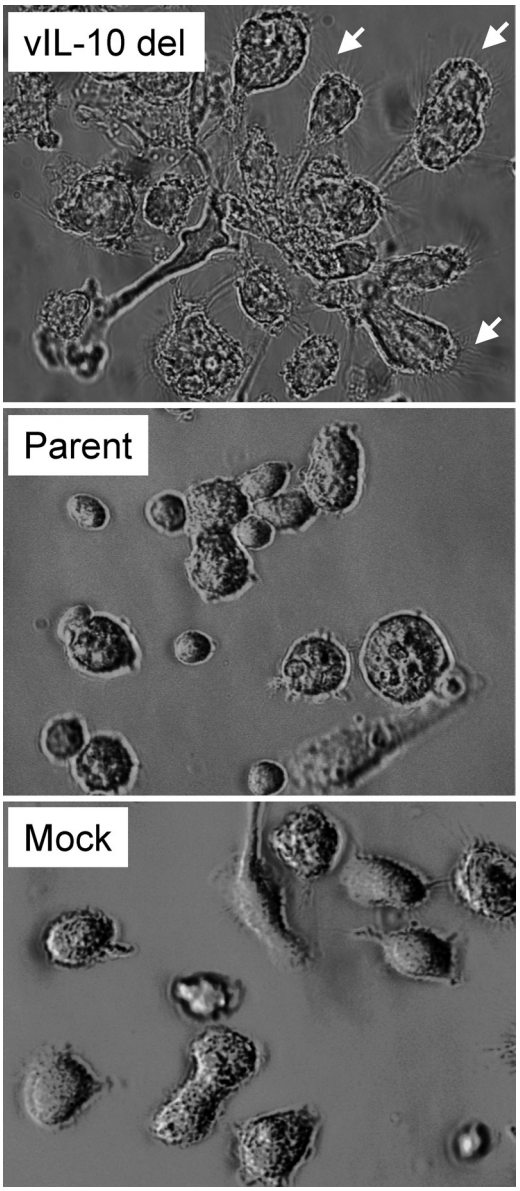

FIG. 2. Cell morphology changes during latent infection with a viral IL-10 deletion virus. Light microscopy views of myeloid progenitor cells on day 8 after latent infection with a viral IL-10 deletion virus (vIL-10 del) or parental virus (Parent) or mock infection (Mock). The small white arrows highlight fine spiky projections on clumping cells in cultures infected with vIL-10 del virus.

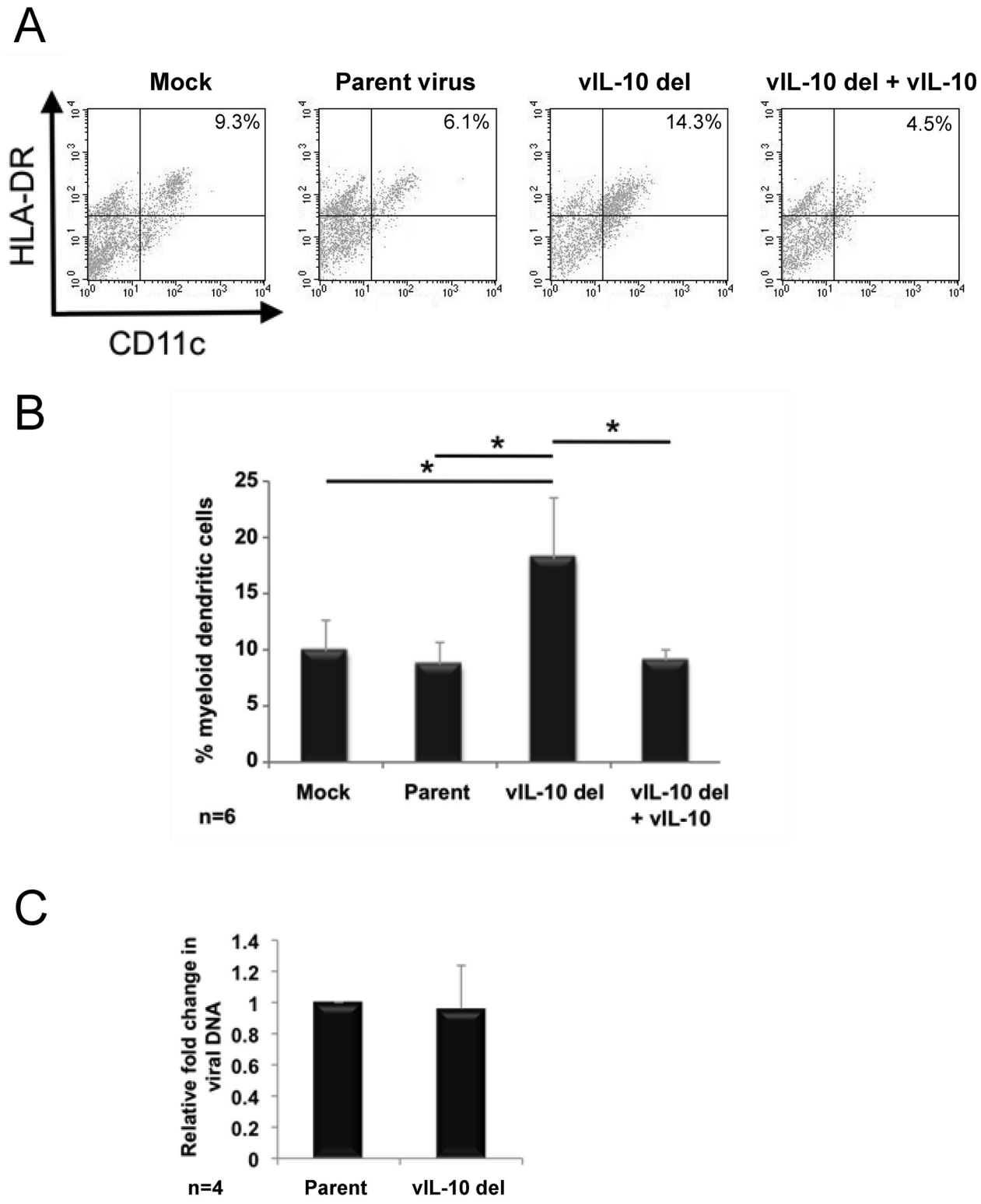

FIG. 3. Increased generation of myeloid dendritic cells from myeloid progenitor cells latently infected with a viral IL-10 deletion virus. $CD34^+$ myeloid progenitor cells were mock infected or latently infected with either parental virus (Parent) or a viral IL-10 deletion virus (vIL-10 del). Cultures of cells infected with vIL-10 del virus supplemented with recombinant viral IL-10 proteins were also generated (vIL-10 del + vIL-10). (A) Flow cytometry scatter plots of Lin^- cells expressing HLA-DR and CD11c to identify myeloid DCs. (B) Graph depicting the percentage of myeloid DCs. (C) Graph depicting the fold change in viral genome load in cells infected with the viral IL-10 deletion virus relative to parental virus-infected counterparts. The number of independent biological replicate experiments (n) is shown. Error bars indicate the standard errors of the means. Significant differences (P value of <0.05) between the values for samples were determined by a one-tailed, paired Student's t test and are denoted by an asterisk.

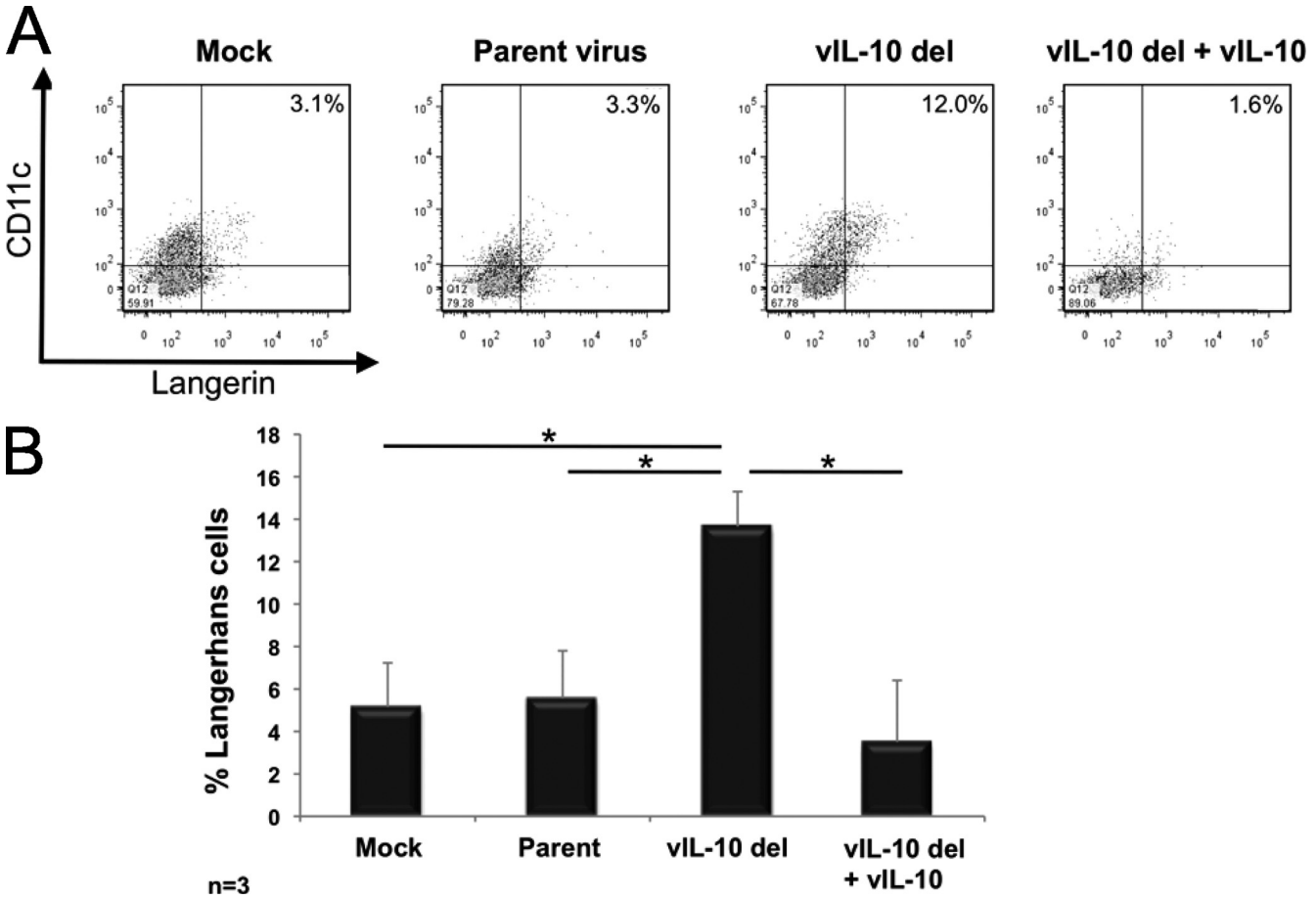

FIG. 4. Increased generation of Langerhans cells from myeloid progenitor cells latently infected with a viral IL-10 deletion virus. $CD34^+$ myeloid progenitor cells were mock infected or latently infected with either parental virus (Parent) or a viral IL-10 deletion virus (vIL-10 del). Cultures of vIL-10 del virus-infected cells supplemented with recombinant viral IL-10 proteins were also generated (vIL-10 del + vIL-10). (A) Flow cytometry scatter plots of cells expressing CD11c and langerin to identify Langerhans cells. (B) Graph depicting the percentage of Langerhans cells. The number of independent biological replicate experiments (*n*) is shown. Error bars indicate the standard errors of the means. Significant differences (*P* value of <0.05) between the values for samples were determined by a one-tailed, paired Student's *t* test and are denoted by an asterisk.

replicates revealed that in comparison to mock infection or latent infection with parental virus, latent infection of myeloid progenitors with the viral IL-10 deletion virus resulted in the formation of more $CD11c^+$ $langerin^+$ Langerhans cells (Fig. 4). As before, this increase was blocked by addition of recombinant viral IL-10 proteins to viral IL-10 deletion virus-infected cultures (Fig. 4).

It is not certain why latent HCMV would encode a function via viral IL-10 to inhibit formation of lymphoid cells (LCs). However, the known roles of LCs may provide some insights. LC differentiation from $CD34^+$ myeloid progenitors latently infected with viral IL-10 deletion virus could be explained by increased TGFβ1 expression by these cells. TGFβ1 stimulation is required for LC differentiation from $CD34^+$ myeloid progenitors both *in vivo* and *in vitro* (4, 15, 56). Immature LCs are particularly effective in processing antigens into MHC class II complexes for presentation to antigen-specific T cells (42). Upon a maturation stimulus, surface MHC class II expression increases (35), resulting in efficient antigen cross-presentation and $CD8^+$ cytotoxic T cell activation (21, 54). Therefore, blocking of LC differentiation by viral IL-10 may result in the reduced ability of latently infected cells to present viral antigens.

We did not identify a significant difference in the timing or frequency of reactivation of viral IL-10 deletion virus in comparison to parental virus using our previously described reactivation assay, whereby latently infected cells were cocultured with monolayers of primary human foreskin fibroblasts to stimulate reactivation, and monolayers were monitored daily for the appearance of cytopathic effect (CPE) as an indicator of virus reactivation (8, 9, 22). Specifically, in four independent experiments, both parental virus and viral IL-10 deletion virus reactivated at the same time (mean time of 12 days after reactivation stimulus), and the frequencies of reactivation were very similar for the parental virus (1.2×10^{-4}) and viral IL-10 deletion virus (1.3×10^{-4}). These results were comparable to the rates of reactivation that we have previously reported for these viruses (9).

Reeves et al. demonstrated that HCMV could be reactivated from naturally infected $CD34^+$ progenitors after their *ex vivo*

differentiation to become mature DCs (37). On this basis, if the presence of viral IL-10 suppressed DC differentiation and reactivation by maintaining the cells in the DC progenitor stage, it could be predicted that cells infected with the viral IL-10 deletion virus might reactivate at a higher rate than the parental strain. However, it is possible that the presence of viral IL-10 promotes differentiation to a different mature myeloid cell type, such as a macrophage. In this respect, *ex vivo* differentiation of naturally infected myeloid cells to a macrophage has been shown to result in reactivation from latency (49, 50). Thus, both macrophages and mature DCs support virus reactivation, and so any skewing of differentiation by viral IL-10 between these two terminally differentiated cell types may not alter the frequency of reactivation. If viral IL-10 did indeed suppress reactivation by maintaining DCs in the progenitor stage and did not skew differentiation to a different cell type such as a macrophage, the magnitude of the increase in the proportion of DCs may not have been sufficient to result in a detectable difference in reactivation frequency as measured by this assay. Defining whether the presence of viral IL-10 expression during latency inhibits differentiation/maturation of DC-committed progenitors or whether it acts at an earlier stage of differentiation by reprogramming more-primitive myeloid progenitors to differentiate toward a different cell type such as a macrophage, will be important components of future studies to fully delineate the role of viral IL-10 during latency and reactivation. Examination of the role of viral IL-10 in modulating myeloid cell differentiation during latent infection with viruses based upon a low-passage clinical strain (in addition to the laboratory strain AD169 used in the current study) will also be an important consideration for subsequent analyses of viral IL-10 function.

In summary, this study identifies a role for viral IL-10 in modulating the differentiation of latently infected myeloid progenitor cells. Latent infection of these progenitors with a virus unable to express viral IL-10 resulted in upregulation of cellular cytokines which were likely to create a favorable environment for DC formation, together with skewing of differentiation toward a myeloid DC type, at least some of which were Langerhans cells. Inhibition of the DC differentiation pathway by viral IL-10 may provide an advantage to latent HCMV in the context of evasion of the host's immune system. As DCs are the most potent antigen-presenting cell type, suppression of differentiation of latently infected myeloid progenitors toward a DC is likely to enhance the ability of latent virus to limit presentation of latency-associated viral peptides to HCMV-specific T cells. In this respect, we reported that latently infected cells were unable to evade detection by $CD4^+$ T cells in the absence of the ability to express viral IL-10 and that this was concomitant with increased MHC class II by these latently infected cells (9). Thus, modulation of DC differentiation by viral IL-10 may act to render latently infected cells less immunogenic and so limit/evade immune detection to enhance the ability of HCMV to persist in a latent state in the human host.

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