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Immunomodulatory Properties of a Viral Homolog of Human Interleukin-10 Expressed by Human Cytomegalovirus during the Latent Phase of Infection▽

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Human cytomegalovirus (HCMV) establishes a latent infection in hematopoietic cells, from which it can reactivate to cause significant disease in immunocompromised individuals. HCMV expresses a functional homolog of the immunosuppressive cytokine interleukin-10 (termed cmvIL-10), and alternate splicing of the cmvIL-10 transcript results in expression of a latency-associated cmvIL-10 transcript (LAcmvIL-10). To determine whether LAcmvIL-10 encodes immunosuppressive functions, recombinant LAcmvIL-10 protein was generated, and its impact on major histocompatibility complex class II (MHC-II) expression was examined on granulocyte macrophage progenitor cells (GM-Ps) and monocytes. LAcmvIL-10 (and cmvIL-10) downregulated MHC-II on the surfaces of both cell types. This downregulation was associated with a decrease in total MHC-II protein and transcription of components of the MHC-II biosynthesis pathway. Unlike cmvIL-10, LAcmvIL-10 did not trigger phosphorylation of Stat3, and its ability to downregulate MHC-II was not blocked by neutralizing antibodies to the human IL-10 receptor, suggesting that LAcmvIL-10 either does not engage the cellular IL-10 receptor or utilizes it in a different manner from cmvIL-10. The impact of LAcmvIL-10 on dendritic cell (DC) maturation was also assessed. In contrast to cmvIL-10, LAcmvIL-10 did not inhibit the expression of costimulatory molecules CD40, CD80, and CD86 and the maturation marker CD83 on DCs, nor did it inhibit proinflammatory cytokines (IL-1α, IL-1β, IL-6 and tumor necrosis factor alpha). Thus, LAcmvIL-10 retains some, but not all, of the immunosuppressive functions of cmvIL-10. As GM-Ps and monocytes support latent infection, expression of LAcmvIL-10 may enable HCMV to avoid immune recognition and clearance during latency.

Human cytomegalovirus (HCMV) is a widely distributed human herpesvirus with a highly restricted host range. It is the prototypic betaherpesvirus with a life cycle that includes the ability to establish a life-long latent infection within the host. During latency, viral gene expression is highly restricted, and infectious virions are not produced (36). Periodically, HCMV is able to reactivate from latency, resulting in the production of new infectious virus, a process which often leads to life-threatening disease in immunosuppressed individuals. While HCMV infection causes mild or clinically nonapparent disease in the immunocompetent host, it is a major cause of morbidity and mortality in immunocompromised individuals such as patients with AIDS, allograft recipients (bone marrow and solid organ), and neonates (41). Despite the major clinical impact of this virus on select populations, many aspects of HCMV biology and pathogenesis remain poorly understood. In particular, little is known about the molecular mechanisms that underlie the ability of the virus to persist in a latent form.

The cell-mediated immune response plays an important role in the control of HCMV infection. In particular, clinical observations indicate that the severity of HCMV disease parallels the degree of impairment of T-cell responses (41). Both major histocompatibility complex class I (MHC-I)- and MHC-II-restricted HCMV-specific cytotoxic T lymphocyte responses are generated and are important for virus clearance (31, 49). MHC-II-restricted $CD4^+$ cells act as critical antiviral effectors as well as providing helper functions for maintaining HCMV-specific $CD8^+$ T-cell responses (26), and impairment of the HCMV-specific $CD4^+$ T-cell response has been associated with prolonged secretion of virus in young children (61). In a study of $CD4^+$ T-cell responses following primary HCMV infection in renal transplant recipients, van Leeuwen et al. (63) provided evidence for the emergence of HCMV-specific $CD4^+$ T cells that were represented during the latent phase of infection and that had the capacity to lyse HCMV antigen-expressing cells in an MHC-II-dependent manner (63). Thus, MHC-II-restricted $CD4^+$ T cells likely play an important role in the control of productive infection and latency/reactivation. These findings have led to the hypothesis that HCMV-encoded modulation of MHC-II would enhance the capacity of the virus to limit the host immune response during both productive and latent phases of infection. During latency, interference with MHC-II expression leading to impairment of $CD4^+$ T-cell surveillance of latently infected cells may enhance the chances for long-term maintenance of the virus. In this respect, experimental latent infection of cultured granulocyte macrophage progenitor (GM-P) cells has been reported to result in a decrease in the expression of cell surface MHC-II molecules (52).

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Human interleukin-10 (hIL-10) is a pleiotropic cytokine that exhibits potent immunomodulatory properties in hematopoietic cells (37). It has also been shown to be a critical determining factor in directing the induction of immunosuppression leading to persistent (i.e., long-term productive) lymphocytic choriomeningitis virus infection in vivo (7). During HCMV productive and latent infections, the virus expresses transcripts from the UL111A region which encode two homologs of hIL-10. In productively infected cells, this homolog has been denoted cmvIL-10 (29, 32). Although the 175-amino-acid cmvIL-10 protein exhibits only 27% identity with hIL-10, this viral cytokine is still able to bind to and signal through the IL-10 receptor (IL-10R) (22, 54). Indeed, cmvIL-10 has been shown to encode a broad range of immunosuppressive functions on myeloid cells comparable to those encoded by hIL-10. cmvIL-10 has been reported to inhibit peripheral blood mononuclear cell (PBMC) proliferation and proinflammatory cytokine synthesis (gamma interferon) and decrease surface expression of MHC-I and MHC-II molecules on monocytes (54). It has also been shown to induce functional paralysis of dendritic cells (DCs), impairing lipopolysaccharide (LPS)-induced upregulation of DC costimulatory molecules and altering cytokine production (9, 46). In addition, Yamamoto-Tabata et al. (66) demonstrated that cmvIL-10 decreased matrix metalloproteinase activity and altered cell-cell and cell-matrix interactions of HCMV-infected cytotrophoblasts and endothelial cells at the uterine-placental interface (66). The authors suggest that this may result in impaired cytotrophoblast remodeling of the uterine vasculature and consequently limit fetal growth.

During latent infection, altered splicing of the UL111A region transcript results in the expression of latency-associated cmvIL-10 (LAcmvIL-10) (19). A similar transcript, which appears to initiate from a slightly different site, but which is predicted to also encode LAcmvIL-10, has also been detected during productive infection of human foreskin fibroblasts (HFFs) (20). Thus, the LAcmvIL-10 open reading frame (ORF) appears to be latency associated rather than latency specific. Although colinear with cmvIL-10 at the amino terminus, LAcmvIL-10 is predicted to be a 139-amino-acid protein with a truncated C terminus due to retention of the second intron and the presence of an in-frame stop codon (19). The detection of LAcmvIL-10 transcription during latent infection raises the possibility that this variant of cmvIL-10 may encode similar immunosuppressive properties. Thus, we sought to answer a number of questions addressing the capacity of LAcmvIL-10 to exploit the immunosuppressive properties of hIL-10 as a potential viral mechanism for evading the host's immune system during the latent phase of infection. First, we applied a combination of flow cytometry, immunofluorescence and confocal microscopy, Western blotting, and real time reverse transcription-PCR (RT-PCR) to examine the effects of recombinant LAcmvIL-10 on the expression of MHC-II molecules by myeloid cell types shown to be important targets for HCMV latency. Second, we examined the impact of LAcmvIL-10 on Stat3 phosphorylation and its capacity to downregulate MHC-II in the presence of neutralizing antibodies to hIL-10R to gain insights into the mechanism of action of this viral cytokine. Finally, we sought to determine whether LAcmvIL-10 retained any of the inhibitory characteristics displayed by cmvIL-10 within the context of DC maturation by assessing the effect of this viral cytokine on the induction of DC maturation.

MATERIALS AND METHODS

Cells. HFFs and human embryonic kidney cells (HEK293) were grown in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (CSL, Parkville, Australia). 9.22.3 is a human B lymphoblastoid cell line with an HLA-DRα gene deletion (45) and was kindly provided by E. D. Mellins (Stanford University). Cells were grown in RPMI 1640 medium (GIBCO) supplemented with 10% heat-inactivated FCS. Human fetal liver-derived hematopoietic cells were isolated and cultured as GM-Ps, as previously described (27). On day 3 of culture, nonadherent cells were collected, exposed to the various purified protein preparations, and incubated at 37°C in a humidified atmosphere containing 5% CO_2.

PBMCs were isolated from healthy adult donors by density gradient sedimentation using Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) and $CD14^+$ monocytes isolated using anti-CD14 magnetic beads, according to manufacturer's instructions (Miltenyi Biotech, Auburn, CA). Isolated cells were typically >95% $CD14^+$ as determined by flow cytometry analysis following staining with anti-CD14 antibody (clone MϕP9; fluorescein isothiocyanate [FITC] conjugated; BD Biosciences, San Jose, CA).

Generation of monocyte-derived dendritic cells (MDDCs). $CD14^+$ monocytes were resuspended at 5×10^5 cells/ml in RPMI medium supplemented with 10% heat-inactivated FCS, 500 U/ml IL-4, and 400 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) (Schering Plough, Kenilworth, NJ) and allowed to adhere to 24-well tissue culture plates. Immature DCs were generated as previously described (1, 8). Typically >90% of cells were shown by flow cytometry analysis to be of an immature DC phenotype (i.e., $CD1a^+$ MHC-I^+ MHC-II^+ $CD80^{low}$ $CD83^-$ $CD86^{low}$). Mature DCs were generated by transferring the nonadherent immature DCs to new plates and culturing for a further 2 days in RPMI-GM medium containing 2 μg/ml LPS (Sigma, St. Louis, MO), 400 U/ml GM-CSF, and 500 U/ml IL-4 for 2 days at 37°C in a 5% CO_2 atmosphere (2). These cultures consisted of >90% mature DCs as determined by their characteristic stellate shape and their cell-surface phenotype (i.e., $CD1a^+$ MHC-I^{high} MHC-II^{high} $CD80^{high}$ $CD83^+$ $CD86^{high}$).

Antibodies. Flow cytometry was performed with allophycocyanin (APC)-conjugated mouse anti-human MHC-II (HLA-DR) monoclonal antibody (clone L243) and FITC-conjugated mouse anti-human CD14 monoclonal antibody (clone MϕP9) (BD Biosciences). Isotype antibodies used in parallel were APC-conjugated mouse anti-human immunoglobulin G2a (IgG2a) and FITC-conjugated mouse anti-human IgG clone (X39; BD Biosciences). Monoclonal antibodies specific for human MHC-I (clone Tu149; phycoerythrin [PE] conjugated) were from Caltag Laboratories (San Francisco, CA). PE-conjugated goat anti-mouse IgG, FITC-conjugated goat anti-human IgG, mouse IgG2a, PE-conjugated mouse IgG2a, and tricolor-conjugated mouse IgG2a antibodies were obtained from Caltag Laboratories (San Francisco, CA). PE-conjugated monoclonal antibodies specific to human CD83 (clone HB15a) and human CD80 (clone MAB104) and unconjugated CD83 (clone HB15a) were obtained from Immunotech (Marseilles, France). PE-conjugated anti-human CD86 (clone IT2.2) and APC-conjugated anti-human CD1a (clone HI149) were obtained from Pharmingen (Hamburg, Germany), and unconjugated anti-human HLA-DR (clone IQU9) was obtained from Novocastra (Wetzlar, Germany).

Antibodies for immunofluorescence and confocal microscopy for the detection of MHC-II were mouse anti-human MHC-II (HLA-DR) monoclonal antibody (clone L243; Santa Cruz Biotechnology) and AlexaFluor 633-conjugated goat anti-mouse antibody (Molecular Probes, Eugene, OR). Western blotting for LAcmvIL-10 and cmvIL-10 was performed with goat anti-cmvIL-10 IgG polyclonal antibody (R&D Systems, Minneapolis, MN) and horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG polyclonal antibody (Dako, Glostrup, Denmark). For the detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), rabbit polyclonal IgG specific for GAPDH (clone FL335) and HRP-conjugated goat anti-rabbit IgG polyclonal antibody were utilized (Santa Cruz Biotechnology, CA). Western blot detection of Stat3 was carried out using antibodies against total Stat (polyclonal rabbit anti-mouse Stat3; catalog no. 9132) or phospho-Stat3Y705 (polyclonal rabbit anti-mouse; catalog no. 9131) (Cell Signaling Technology Inc., Danvers, MA). MHC-II detection was performed with monoclonal antibody specific for human MHC-II (HLA-DRα; clone DA6.147), kindly provided by E. D. Mellins (Stanford University), and HRP-conjugated rabbit anti-mouse IgG polyclonal antibody (Dako, Glostrup, Denmark). Neutralizing antibodies to hIL-10Rα and hIL-10Rβ were obtained from R&D Systems (Minneapolis, MN).

Construction of a LAcmvIL-10 bacterial cell expression vector and purification of recombinant LAcmvIL-10 protein. The LAcmvIL-10 amino acid sequence includes a signal peptide with a cleavage site between residues 25 and 26 (P and A, respectively). In order to generate a processed, i.e., cleaved, LAcmvIL-10 protein in a bacterial system, PCR primers were designed so that only the coding nucleotide sequence subsequent to the signal peptide was cloned into a bacterial cell expression vector (pET28a; Novagen). This vector tagged the amino terminus of the cloned protein with a His_6 tag. The cDNA sequence of LAcmvIL-10 expressed in GM-Ps latently infected with HCMV strain Toledo was PCR amplified using primers SigSecA (5′-TTCCGAGGAGGAGGGATCCGCGACGA CGACG-3′) and LAHinA (5′-GCTATGCCAAGCTTGTTACCTCTGTGCGG A-3′), which were designed to introduce BamHI and HindIII restriction endonuclease sites into the amplified product. The resultant PCR products were then cloned into the pET28a vector using BamHI and HindIII. This construction inserted the HCMV LAcmvIL-10 cDNA sequence, beginning 77 nucleotides downstream of the translation start site and extending to the translational stop codon of LAcmvIL-10.

Plasmid DNA encoding His-tagged LAcmvIL-10 was transformed into competent *E. coli* BL21(DE3) cells, and protein expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside. Final purification of recombinant LAcmvIL-10 was achieved by sequential ion exchange chromatography using a ProBond Resin column (Invitrogen). The concentration of recombinant LAcmvIL-10 collected from sequential fractions was determined by spectrophotometry.

Western blotting. Cells (5×10^5 cells/ml) to be analyzed for protein expression were washed once in 1× phosphate-buffered saline (PBS), lysed with cell lysis buffer (150 mM NaCl, 20 mM HEPES, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM EDTA, and protease inhibitor cocktail I; Calbiochem), and incubated on ice for 30 min with agitation every 5 min. Lysed cells were then collected and centrifuged at 20,800 × *g* for 10 min at 4°C. The cell supernatants were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrotransfer to a positively charged nylon membrane (polyvinylidene difluoride; Amersham Pharmacia Biotech, Uppsala, Sweden). Membranes were incubated in blocking solution (5% nonfat milk in PBS) for 1 h and then incubated with the relevant primary antibody diluted in blocking solution at room temperature for 16 h. Unbound antibody was removed by several washes in Tris-buffered saline–Triton X-100 (TBS-T; two washes for 30 s each and three for 5 min each); the blot was then rinsed in 1× TBS-T. The appropriate HRP-conjugated secondary antibody was added, and the membrane was incubated at room temperature for 30 min. Following antibody incubations, unbound antibody was removed by washing the membrane with TBS-T (two washes for 30 s each and three for 5 min each). Bound secondary antibody was detected with an ECL Plus Kit (Amersham Pharmacia Biotech), and exposed film was developed using a CP1000 automatic developer (AGFA, Australia). The molecular weights of proteins were determined using protein reference standards which were loaded onto each gel (Bio-Rad, CA).

Immunostaining and flow cytometry analyses. Cells to be analyzed for expression of cell surface molecules by flow cytometry were first harvested, washed in 1× PBS, pelleted by centrifugation, and washed in fluorescence-activated cell sorting (FACS) buffer (PBS with 1% FCS–0.2% sodium azide). Cells resuspended in 100 μl of FACS buffer were incubated with the relevant primary antibody at 4°C in the dark for 20 min. Following incubation, the cells were washed in FACS buffer and then centrifuged. If an unconjugated primary antibody was used, a second incubation with the appropriate fluorochrome-conjugated secondary antibody was performed in the same manner. Following a final wash in FACS buffer, the cell pellets were then resuspended in 150 μl of FACS buffer for immediate analysis or fixed in 150 μl of 1% (vol/vol) paraformaldehyde for analysis at a later time point using a FACSCalibur instrument and CellQuest software (BD Biosciences). Positive and negative staining with cell-surface-specific antibodies was determined by the level of fluorescence that exceeded, or did not exceed, levels obtained by 98% of the cells from the same starting population when incubated with isotype control primary antibodies.

Immunofluorescence staining and confocal microscopy. Cells (5×10^4) were cytospun onto glass slides, air dried, fixed in 4% paraformaldehyde in PBS for 10 min, and permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature. Slides were washed three times in PBS and incubated with blocking buffer (10% normal donkey serum in PBS) for 1 h at 37°C in a humidified atmosphere before staining. Slides were washed with PBS and incubated with primary goat anti-human CD14 (1:100 dilution), mouse anti-human HLA-DR (1:25 dilution), or the appropriate isotype control antibodies for 1 h. Cells were then either incubated with AlexaFluor 488 rabbit anti-goat IgG conjugate (1:200 dilution) for 30 min or AlexaFluor 633 goat anti-mouse IgG conjugate (1:50 dilution) for 1 h. All antibodies were diluted in blocking buffer and incubated at 37°C in a humidified atmosphere in the dark. Following each antibody incubation, slides were washed three times in PBS. Slides were mounted in SlowFade Gold antifade mounting medium (Molecular Probes), and cells were examined using a Leica laser scanning confocal microscope.

RNA extraction and real-time quantitative RT-PCR. Total RNA was isolated from monocytes using an RNAqueous kit (Ambion, Texas) according to the manufacturer's instructions. cDNA was synthesized from total RNA using random hexamers and Superscript III reverse transcriptase (Invitrogen, CA). The cDNA product was used for real-time quantitative PCR using a high-speed thermal cycler (Stratagene Mx3005P; Applied Biosystems, CA), and the quantitative PCR products were detected by using Sybr green PCR SuperMix (Invitrogen, CA) according to the manufacturer's instructions. The PCR cycling conditions were as follows: 1 cycle of 2 min at 50°C and 2 min at 95°C; 50 cycles of 15 s at 95°C and 45 s at 45°C; and then 1 cycle of 1 min at 95°C, 30 s at 55°C, and 30 s at 95°C. Three housekeeping genes, GAPDH, beta actin, and lactate dehydrogenase (LDHA), were included to assess the relative changes in gene expression between different treatments. The amplification efficiencies for each primer pair were determined by creating standard curves with 10-fold serial dilution of pooled cDNA. The log of the relative target quantity was plotted versus the cycle threshold values. Specific cDNA was quantified using this standard curve. All results are normalized with respect to the three housekeeping genes and expressed relative to the expression level of the mock treatment. Primer sequences were as follows: MHC-II alpha chain, 5′-CCACCAGTGGTC AATGTCAC-3′ (forward) and 5′-CCTGCAGTCGTAAACGTCCT-3′ (reverse); MHC-II beta chain, 5′-AGGCAGCATTGAAGTCAGGT-3′ (forward) and 5′-GGCAGGTGTAAACCTCTCCA-3′ (reverse); invariant chain, 5′-CTC CAGTGCTATGGGAGCAT-3′ (forward) and 5′-AGATCCTGCTTGGTCAC ACC-3′ (reverse); class II transactivator (CIITA), 5′-AGGTCTCCAACAAGC TTCCA-3′ (forward) and 5′-CTCTTGCTGCTGCTCCTCTC-3′ (reverse); GAPDH, 5′-GAGTCAACGGATTTGGTCGT-3′ (forward) and 5′-GACAAG CTTCCCGTTCTCAG-3′ (reverse); beta actin, 5′-AGAAAATCTGGCACCAC ACC-3′ (forward) and 5′-GGGGTGTTGAAGGTCTCAAA-3′ (reverse); and LDHA, 5′-TGGGAGTTCACCCATTAAGC-3′ (forward) and 5′-AGCACTCT CAACCACCTGCT-3′ (reverse).

Multiplex enzyme-linked immunosorbent assay (ELISA). The levels of multiple proinflammatory cytokines in clarified supernatants derived from DC cultures were measured using Fluorokine MAP (multianalyte profiling; R & D Systems) technology following the manufacturer's directions. Briefly, cytokine-specific antibodies (i.e., IL-1α, IL-1β, IL-6, tumor necrosis factor alpha [TNF-α], and GM-CSF) were precoated onto color-coated microparticles and were added to clarified DC culture supernatant samples to be assayed in a 96-well filter-bottomed microplate (R&D Systems). The mixture was incubated at room temperature for 3 h with gentle shaking to allow the cytokines of interest to bind to the immobilized antibodies. Bound analytes were washed three times, and biotinylated antibodies specific to the analytes of interest were added to each well and incubated for 1 h longer at room temperature with gentle shaking. Unbound biotinylated antibodies were removed by washing, and a streptavidin-PE conjugate was added to each well to bind to the biotinylated detection microparticles. Following washes to remove unbound streptavidin-PE-conjugated antibody, the samples were then read using a Bio-Plex analyzer, a dual laser, and a flow cytometry-based sorting and detection platform manufactured by Bio-Rad (Hercules, CA).

RESULTS

Impact of LAcmvIL-10 on cell surface MHC-II expression by myeloid cells. Latent HCMV infection has been shown to downregulate cell surface MHC-II expression on cultured $CD14^+$ primary human GM-Ps (52). As a consequence of the detection of LAcmvIL-10 transcripts in latently infected GM-Ps (19), we sought to determine whether LAcmvIL-10 protein could function to alter the constitutive expression of cell surface MHC-II on primary human GM-Ps using recombinant proteins generated using bacterial and mammalian cell expression systems.

LAcmvIL-10 protein was generated from *E. coli* cells transformed with a His-tagged LAcmvIL-10 expression vector. Recombinant LAcmvIL-10 or mock protein from parental vector-transformed cells was subjected to purification by sequential

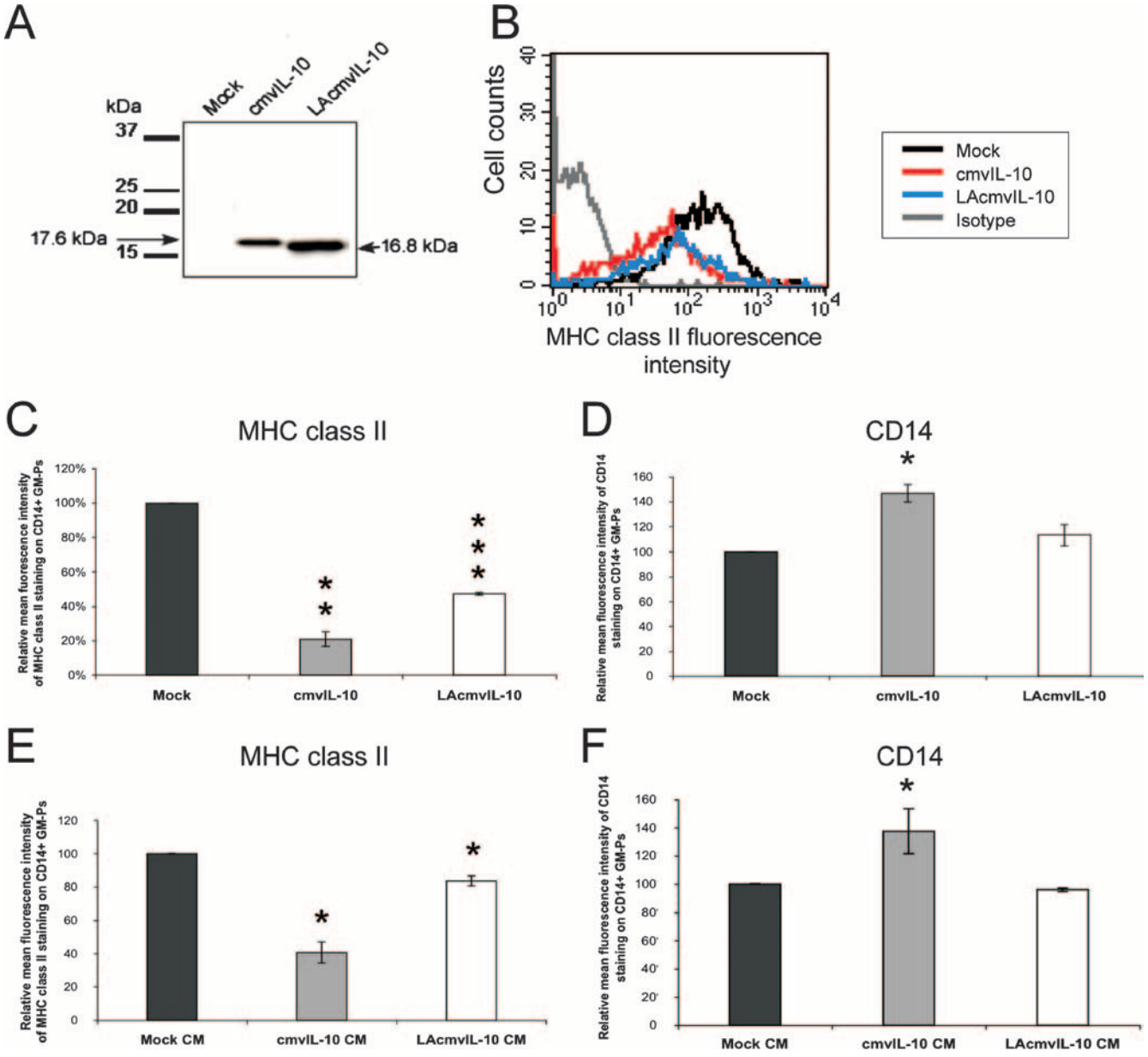

FIG. 1. MHC-II and CD14 expression on the surface of GM-Ps following treatment with purified LAcmvIL-10 and cmvIL-10. (A) Western blot of purified cmvIL-10 and purified LAcmvIL-10 separated by SDS-PAGE. Both proteins reacted with the polyclonal anti-cmvIL-10 antibody used to probe the blot with cmvIL-10 detected at 17.6 kDa and His-tagged LAcmvIL-10 detected at the predicted size of 16.8 kDa. Sizes of corresponding molecular mass markers are indicated in kilodaltons (kDa). (B) Histogram plot showing a decrease in surface MHC-II by LAcmvIL-10 and cmvIL-10 relative to mock protein preparation. Panels C to F show flow cytometric analyses of the expression of MHC-II (C and E) and CD14 (D and F) molecules on the surface of $CD14^+$ GM-Ps treated with either bacterial cell-expressed and purified LAcmvIL-10 and cmvIL-10 (C and D) or conditioned medium (CM) from mammalian cells transfected with LAcmvIL-10 and cmvIL-10 expression vectors (E and F). The relative MFIs of surface MHC-II and CD14 molecules are shown with the means and standard errors calculated from four replicate experiments. Each data point represented the relative MFI of a given surface molecule (relative MFI_M) on the various treatments of GM-Ps, calculated as follows: (MFI_M on $GM\text{-}Ps_{(treated)}$/MFI_M on $GM\text{-}Ps_{(mock)}$) $\times$ 100, where GM-Ps are treated with cmvIL-10, LAcmvIL-10, or mock preparation. Significant differences from the mock-treated control were determined using a one-tailed, paired Student's *t* test and are indicated as follows: *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

ion-exchange chromatography and analysis by SDS-PAGE and Western blotting using an anti-cmvIL-10 antibody (Fig. 1A). Recombinant epitope-tagged LAcmvIL-10 protein was readily detected at the predicted size of 16.8 kDa, as was the positive control, bacterial cell-expressed cmvIL-10 at 17.6 kDa (R&D Systems). Antibody did not react with the mock protein sample. It was concluded that recombinant LAcmvIL-10 was successfully expressed by and purified from *E. coli*. For production of mammalian cell-expressed proteins, LAcmvIL-10 and cmvIL-10 cDNAs were cloned, and recombinant proteins were expressed in HEK293 cells as previously described (20). A mock control consisting of HEK293 cells transfected

with the empty parental vector was also generated. This approach resulted in the successful expression and secretion of LAcmvIL-10 and cmvIL-10 proteins from transfected HEK293 cells (20).

MHC-II expression was first assessed on GM-Ps treated with bacterial cell-expressed, purified LAcmvIL-10 or cmvIL-10 proteins. GM-Ps isolated from fetal liver were grown in suspension culture in GM-P medium (27) for 7 days prior to treatment with either cmvIL-10 (25 ng/ml), LAcmvIL-10 (100 ng/ml), or a preparation derived from the purification of bacterial cells transformed with a parental vector, which constituted the mock treatment. Forty-eight hours posttreatment, GM-Ps were stained for CD14 and MHC-II and analyzed by flow cytometry (Fig. 1B to D). In four independent replicate experiments treatment of GM-Ps with either LAcmvIL-10 or cmvIL-10 resulted in a statistically significant decrease in the expression of cell surface MHC-II on $CD14^+$ GM-Ps (Fig. 1C). Treatment of GM-Ps with cmvIL-10 also resulted in modest enhancement of CD14 expression on the surface of cells (Fig. 1D).

In addition to assessment of GM-Ps treated with bacterial cell-expressed proteins, GM-Ps cultured for 7 days were also treated with 50% volumes of conditioned GM-P medium from either LAcmvIL-10-, cmvIL-10-, or parental vector-transfected HEK293 cells and cultured for a further 2 days. GM-Ps were then immunostained for the monocyte lineage cell marker CD14 and for MHC-II (HLA-DR) expression and were analyzed by flow cytometry. The mean fluorescence intensity (MFI) of cell surface MHC-II expression was determined from gated $CD14^+$ GM-Ps. Although the amount of cmvIL-10 and LAcmvIL-10 protein secreted into the culture medium by transfected cells could not be accurately quantitated or standardized, in comparison with GM-Ps treated with conditioned medium from parental vector-transfected cells, treatment with either LAcmvIL-10- or cmvIL-10-conditioned medium resulted in reduced levels of MHC-II staining (Fig. 1E). Treatment of GM-Ps with cmvIL-10 also resulted in modest enhancement of cell surface CD14 expression compared to parental vector-treated GM-Ps (Fig. 1F). In four independent replicate experiments using four separate GM-P cultures (i.e., different donors), treatment with either cmvIL-10- or LAcmvIL-10-conditioned medium resulted in a statistically significant ($P < 0.05$) reduction in the expression of MHC-II on the surfaces of $CD14^+$ GM-Ps. These results demonstrate that both the LAcmvIL-10 and cmvIL-10 derived from either mammalian cells or purified from a bacterial expression system exhibited the ability to downregulate MHC-II expression on $CD14^+$ GM-Ps, a cell type shown to support latent HCMV infection.

To determine whether LAcmvIL-10 downregulated MHC-II in a dose-dependent manner, GM-Ps were treated for 48 h with 10-fold decreasing concentrations of purified LAcmvIL-10 protein (100 ng/ml to 1 pg/ml) before being stained for CD14 and MHC-II and then analyzed by flow cytometry (Fig. 2A). GM-Ps were similarly treated with cmvIL-10 and mock protein preparations. Treatment with decreasing concentrations of either LAcmvIL-10 or cmvIL-10 resulted in a loss of the ability of these proteins to downregulate MHC-II on the surfaces of $CD14^+$ GM-Ps. There was little difference between these IL-10 homologs in their capacities to downregulate MHC-II, and this capacity was lost at the same dosage. This dose-dependent effect was observed in no fewer than three replicate experiments and was statistically significant. In addition to more primitive hematopoietic cells such as GM-Ps, PBMCs have also been shown to be an important reservoir of latent HCMV (53, 58–60). Thus, the impact of LAcmvIL-10 on these cells was also assessed. $CD14^+$ monocytes isolated from fresh peripheral blood were treated with decreasing concentrations of LAcmvIL-10, cmvIL-10, or mock protein preparations. As in GM-Ps, LAcmvIL-10 and cmvIL-10 downregulated surface MHC-II by $CD14^+$ monocytes in a dose-dependent manner (Fig. 2B). Thus, LAcmvIL-10 and cmvIL-10 possessed an ability to interfere with the expression of surface MHC-II molecules on both immature myeloid progenitor cells (i.e., GM-Ps) and primary human monocytes. Although previous studies demonstrating biological function of cmvIL-10 were performed with recombinant protein containing a histidine (His) epitope tag (54), our current work utilized a commercially available, tag-free cmvIL-10. As the LAcmvIL-10 protein we generated still contained the epitope tag, we generated our own His-tagged cmvIL-10 and compared it with commercially acquired cmvIL-10 for capacity to downregulate MHC-II on monocytes. This analysis confirmed that the His tag did not alter the immunosuppressive properties of virally encoded IL-10s (data not shown).

Total cellular MHC-II protein synthesis and localization in monocytes treated with LAcmvIL-10 and cmvIL-10. Successful expression of MHC-II at the cell surface is the result of a multistep biosynthesis and assembly pathway (12, 44). To examine levels of total cellular MHC-II protein, we performed Western blotting of $CD14^+$ monocytes treated either with mock or with LAcmvIL-10- or cmvIL-10-purified protein preparations. Forty-eight hours after treatment, lysates of 2×10^5 $CD14^+$ monocytes were prepared, and proteins were separated by SDS-PAGE. Lysates of the B cell line 9.22.3, which has a deletion in the HLA-DRα gene and consequently does not express MHC-II (HLA-DR), was included as a negative control (44). Immunoblot analysis was carried out with the anti-MHC-II (HLA-DRα) antibody DA6.147 in conjunction with the ECL detection system (Amersham Pharmacia Biotech). A 33-kDa fragment corresponding to the MHC-II HLA-DRα chain was detected in mock-, LAcmvIL-10-, and cmvIL-10-treated samples, but the amount of MHC-II expressed by cells treated with LAcmvIL-10 and cmvIL-10 was less than that of the mock-treated sample (Fig. 3A). Comparable protein loading of samples was confirmed by reprobing the membrane for the housekeeping gene GAPDH. Densitometer analysis of MHC-II bands was performed, and the data were normalized to GAPDH expression from three replicate experiments. This analysis demonstrated that, compared to mock-treated cells, LAcmvIL-10 and cmvIL-10 significantly decreased total cellular MHC-II expression (Fig. 3B).

To determine whether LAcmvIL-10 or cmvIL-10 altered MHC-II protein localization, immunofluorescence staining was performed. Human $CD14^+$ monocytes were incubated with either 100 ng/ml purified LAcmvIL-10 or cmvIL-10 protein or with mock protein preparations and analyzed for MHC-II (HLA-DR) expression at 48 h using monoclonal antibody (clone L243) and an AlexaFluor 633-conjugated goat anti-mouse IgG as a secondary antibody (Fig. 4). The pattern

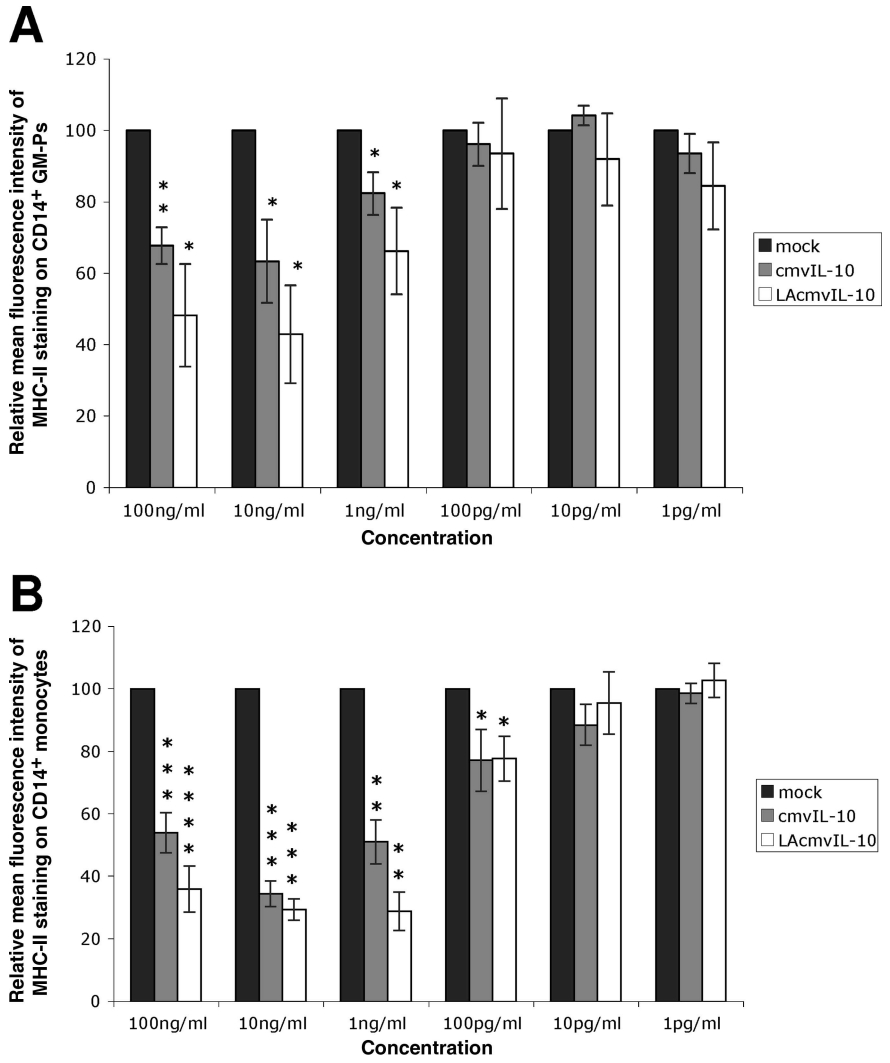

FIG. 2. Ability of LAcmvIL-10 and cmvIL-10 to downregulate cell surface MHC-II on GM-Ps and monocytes in a dose-dependent manner. GM-Ps (A) and monocytes (B) were treated for 48 h with 10-fold decreasing concentrations of purified LAcmvIL-10, cmvIL-10, and mock protein preparations before being stained for CD14 and MHC-II and analyzed by flow cytometry. The relative MFIs of MHC-II surface molecules are shown with the means and standard errors calculated from at least three separate experiments. Each data point represents the relative MFI of a given surface molecule (relative MFI_M) on the various treatments of GM-Ps, calculated as follows: (MFI_M on $GM\text{-}Ps_{(treated)}$/MFI_M on $GM\text{-}Ps_{(mock)}$) $\times 100$, where GM-Ps are treated with cmvIL-10, LAcmvIL-10, or mock preparation. Significant differences from the mock-treated control were determined using a one-tailed, paired Student's *t* test and are indicated as follows: *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$, ****, $P < 0.00005$.

of MHC-II staining was then determined by counting a minimum of 300 cells from each treatment under a magnification of ×400. In nine independent replicate experiments, 73.1% ± 6.2% (± standard error of the mean) of monocytes treated with the mock protein preparation displayed a clear and strong surface staining pattern of MHC-II. In contrast, only 15.7% ± 11.8% of monocytes treated with LAcmvIL-10 and 33.1% ± 9.4% of cmvIL-10-treated cells stained positive for cell-surface MHC-II. In addition to reduced cell surface MHC-II staining, a punctate vesicular pattern was observed in a very small proportion of cells treated with either LAcmvIL-10 (3.5% ± 1.5%) or cmvIL-10 (5.4% ± 2.1%). This pattern of punctate staining was not observed in any cells treated with the mock protein preparation. No staining was detected in monocytes incubated with isotype control antibody. These results indicate that both LAcmvIL-10 and cmvIL-10 reduce total MHC-II protein expression in monocytes and, in a very small proportion of cells, lead to the accumulation of MHC-II molecules within cytoplasmic vesicles.

Impact of LAcmvIL-10 and cmvIL-10 on transcription of components of the MHC-II biosynthesis pathway. MHC-II molecules are heterodimers consisting of an α and β chain which present peptides to $CD4^+$ T lymphocytes. The α and β chains form a heterodimer in the endoplasmic reticulum and associate with the invariant chain. Processing of the invariant chain in cytosolic endosomes ultimately results in its removal, enabling peptides to be loaded onto the α/β chain heterodimer, and this peptide-loaded MHC-II molecule is presented on the cell surface (12, 44, 65). To determine whether LAcmvIL-10 and cmvIL-10 inhibited the transcription of com-

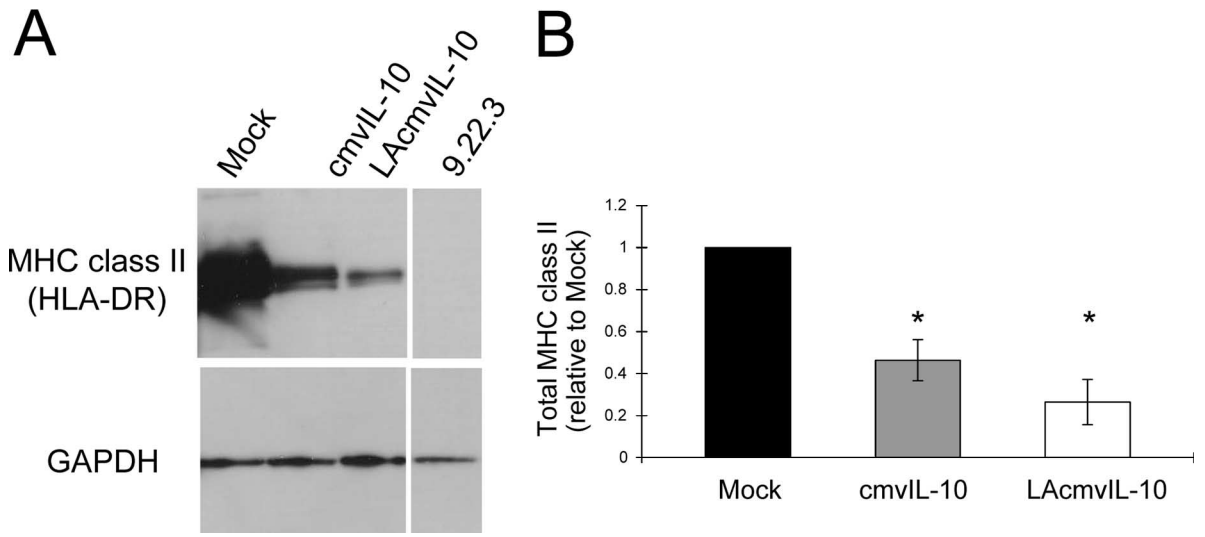

FIG. 3. Expression of MHC-II protein by $CD14^+$ monocytes treated with LAcmvIL-10 and cmvIL-10. (A) MHC-II (HLA-DRα) Western blotting performed on whole-cell lysates from monocytes treated with mock, cmvIL-10, or LA-cmvIL-10 protein preparations. Lysates from $HLA\text{-}DR^-$ 9.22.3 cells were included as a negative control. The immunoblot was reprobed for the housekeeping gene GAPDH (bottom panel). (B) Graph depicting densitometer analysis of MHC-II bands from three replicate Western blotting experiments following normalization to GAPDH expression. Significant differences from the mock-treated control were determined using a one-tailed, paired Student's *t* test.∗, $P < 0.05$.

ponents of the MHC-II pathway, RNA extracted from monocytes 48 h after incubation with mock, LAcmvIL-10, or cmvIL-10 proteins was assessed by real-time RT-PCR using primers specific for MHC-II (HLA-DR) α, β, and invariant chains. The level of transcription of each MHC-II component was determined following normalization to three housekeeping genes, GAPDH, β-actin, and LDHA. In a total of three independent replicate experiments, by comparison with mock protein-treated cells, both LAcmvIL-10 and cmvIL-10 significantly inhibited transcription of α, β, and invariant chains (Fig. 5A to C). A master regulatory factor, CIITA, regulates the transcription of genes involved in the MHC-II biosynthesis pathway, including α, β, and invariant chains (48). Real-time RT-PCR was therefore also extended to assess CIITA RNA levels. This analysis demonstrated that both LAcmvIL-10 and cmvIL-10 significantly inhibited CIITA transcription (Fig. 5D). These data are consistent with the idea that LAcmvIL-10 and cmvIL-10 downregulate MHC-II by interfering with CIITA.

Ability of LAcmvIL-10 to signal through hIL-10R. cmvIL-10 has been shown previously to bind and signal through hIL-10R (22, 29, 54). To examine the signaling events induced by LAcmvIL-10, monocytes were treated with recombinant hIL-10, cmvIL-10, or LAcmvIL-10 for 10 min. Cell lysates were then analyzed for the activation of the transcription factor Stat3, a well-documented signaling outcome following activation of IL-10R (14). Western blotting of cell lysates revealed that Stat3 became phosphorylated on tyrosine 705 in response to treatment with both cmvIL-10 and hIL-10 (Fig. 6A). However, no phosphorylation of Stat3 was detected in monocytes treated with LAcmvIL-10. Polyclonal antiserum directed to the Stat3 protein was used to visualize the total amount of Stat3 in each lysate and to confirm that each lane contained the same amount of protein.

To investigate whether LAcmvIL-10 triggered Stat3 phosphorylation with delayed kinetics relative to hIL-10 or cmvIL-10, monocytes were treated with 10 ng/ml cytokine and incubated at 37°C for 10, 30, or 60 min. Cell lysates were then prepared and analyzed for Stat3 phosphorylation (Fig. 6B). For cells treated with cmvIL-10, the most intense signal occurred in cells treated for 10 min, with the amount of phospho-Stat3 decreasing slightly at the 30- and 60-min time points. In contrast, LAcmvIL-10 treatment did not trigger phosphorylation of Stat3, even at the extended time points, suggesting either that this protein does not engage the cellular IL-10R or that it utilizes this receptor in a manner that differs from that of hIL-10 and cmvIL-10. An alternative explanation for this finding is that there may have been some inhibitor in the LAcmvIL-10 preparation which prevented Stat3 activation. To examine this possibility, cells were treated with both cmvIL-10 and LAcmvIL-10 in combination, and then Stat3 activation was evaluated by Western blotting. As shown in Fig. 6C, Stat3 phosphorylation was observed in response to treatment with cmvIL-10, whether alone or in combination with LAcmvIL-10. These findings confirm that LAcmvIL-10 neither induces Stat3 phosphorylation nor blocks Stat3 activation by cmvIL-10.

To assess binding of LAcmvIL-10 to the hIL-10R, GM-Ps were either left untreated or treated for 2 h with a combination of neutralizing antibodies to hIL-10Rα (25 μg/ml) and hIL-10Rβ (25 μg/ml) before being incubated with mock-, cmvIL-10-, or LAcmvIL-10-treated proteins and incubated for a further 48 h. Cells were immunostained with anti-CD14-FITC and anti-HLA-DR-APC or with their isotype controls, mouse IgG2a-FITC and mouse IgG2a-APC, respectively. HLA-DR expression on $CD14^+$ cells was assessed by flow cytometry for each treatment (Fig. 7A), with the MFI of antibody staining for HLA-DR and CD14 calculated relative to mock-treated cells. The impact of hIL-10R neutralizing antibodies on the down-modulation of MHC-II (HLA-DR) following treatment with LAcmvIL-10 and cmvIL-10 was assessed in a total of four independent replicate experiments (Fig. 7B). Incubation with

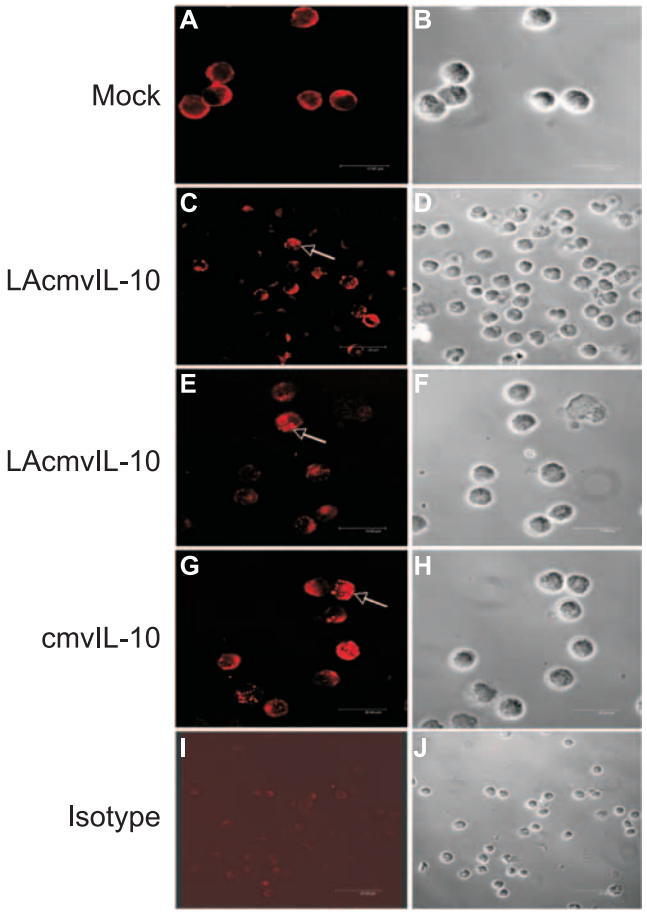

FIG. 4. Analysis of MHC-II (HLA-DR) protein localization in LAcmvIL-10- and cmvIL-10-treated CD14$^+$ monocytes. Immunofluorescence staining and confocal microscopy visualization of MHC-II (HLA-DR; red staining) in monocytes treated with mock protein preparation (A), LAcmvIL-10 (C and E), and cmvIL-10 (G). Cells stained with isotype control antibody are shown in panel I. Corresponding phase-contrast images from each treatment are shown (B, D, F, H, and J). Arrows indicate the appearance of punctate cytoplasmic MHC-II staining in a small proportion of cells treated with LAcmvIL-10 and cmvIL-10. White bars indicate size.

either cmvIL-10 or LAcmvIL-10 significantly reduced HLA-DR expression on CD14$^+$ GM-Ps that had not been pretreated with hIL-10R neutralizing antibodies. When neutralizing antibodies were added, the downregulation of HLA-DR by cmvIL-10 was almost completely inhibited. In contrast, LAcmvIL-10 continued to downregulate HLA-DR in the presence of IL-10R neutralizing antibodies. Thus, neutralizing antibodies to the hIL-10R inhibited the MHC-II modulating function of cmvIL-10 but did not affect the function of LAcmvIL-10. It was concluded that LAcmvIL-10 functioned to downregulate HLA-DR cell surface expression on CD14$^+$ GM-Ps using a mechanism independent of cmvIL-10 and possibly independent of hIL-10R.

Impact of LAcmvIL-10 on the maturation of DCs. In response to pathogens, DCs undergo maturation, which is characterized by profound phenotypic changes that enable subsequent T-lymphocyte activation. This includes the upregulation of the costimulatory and adhesion molecules CD40, CD80, CD83, and CD86 as well as an increase in MHC expression and the secretion of proinflammatory cytokines such as TNF-α, IL-6, and IL-1-β (3, 24, 43, 62). These phenotypic changes are significantly impaired by immunosuppressive cytokines such as hIL-10, which inhibits DC maturation (34). In MDDCs, Chang et al. (9) and Raftery et al. (46) showed that cmvIL-10 mimics the effects of hIL-10 by impairing upregulation of DC costimulatory and adhesion molecules and proinflammatory cytokine production in response to the DC maturation stimulus LPS (9, 46).

To determine whether LAcmvIL-10 could function to inhibit DC maturation, DCs generated from human CD14$^+$ monocytes by culture with GM-CSF and IL-4 were induced to mature with LPS (1 μg/ml) in the presence of increasing concentrations of purified LAcmvIL-10 (60 ng/ml, 120 ng/ml, or 750 ng/ml) or mock protein preparation. Purified cmvIL-10 (25 ng/ml) was included as a positive control for impairment of DC maturation. Immature DCs incubated in the absence of LPS or viral IL-10s were also included to establish baseline levels of immune molecule expression prior to maturation.

Treatment of DC with LPS in the presence of cmvIL-10 resulted in impaired upregulation of the costimulatory molecules CD40, CD80, and CD86, the maturation marker CD83, and also MHC-I (Fig. 8). In stark contrast, treatment of DCs with LAcmvIL-10 did not result in any significant change in molecule expression in comparison to the LPS-treated DCs incubated with the mock protein preparation (i.e., mature DC control). Analysis of data from four replicate experiments demonstrated significant inhibition of upregulation of CD40, CD80, CD83, CD86, and MHC-I in the presence of cmvIL-10 but not in the presence of LAcmvIL-10, despite concentrations of LAcmvIL-10 as high as 750 ng/ml. Similarly, conditioned medium from HEK293 cells transfected with the cmvIL-10 expression vector inhibited the upregulation of immune molecules on immature DCs induced to mature with LPS, whereas conditioned medium from LAcmvIL-10-transfected cells did not (data not shown). It was concluded that cmvIL-10, but not LAcmvIL-10, functions to inhibit the upregulation of costimulatory and adhesion molecules by DCs in response to LPS treatment.

Both hIL-10 and cmvIL-10 have previously been shown to inhibit LPS-induced secretion of proinflammatory cytokines such as TNF-α, IL-6, and IL-1-β by DCs (9, 34, 46). Consequently, we also sought to determine whether LAcmvIL-10 altered cytokine secretion from DCs induced to mature with LPS. Immature DCs were stimulated with LPS (1 μg/ml) in the presence of 50% volumes of conditioned medium from either LAcmvIL-10-, cmvIL-10-, or parental vector-transfected (mock) HEK293 cells and cultured for 3 days. Purified cmvIL-10 (25 ng/ml) was used as a positive control for inhibition of DC maturation, and immature DCs not stimulated with LPS were used as a negative control. Supernatants from four replicate DC cultures were collected and clarified by low-speed centrifugation, and the levels of IL-1α, IL-1β, IL-6, TNF-α, and GM-CSF were analyzed using a multiplex sandwich ELISA (Fig. 9). Mock-treated DC induced with LPS showed a marked induction of secretion of the proinflammatory mediators IL-1α, IL-1β, IL-6, and TNF-α. In comparison, the addition of purified cmvIL-10 protein or cmvIL-10-conditioned medium in the presence of LPS stimulation significantly inhib-

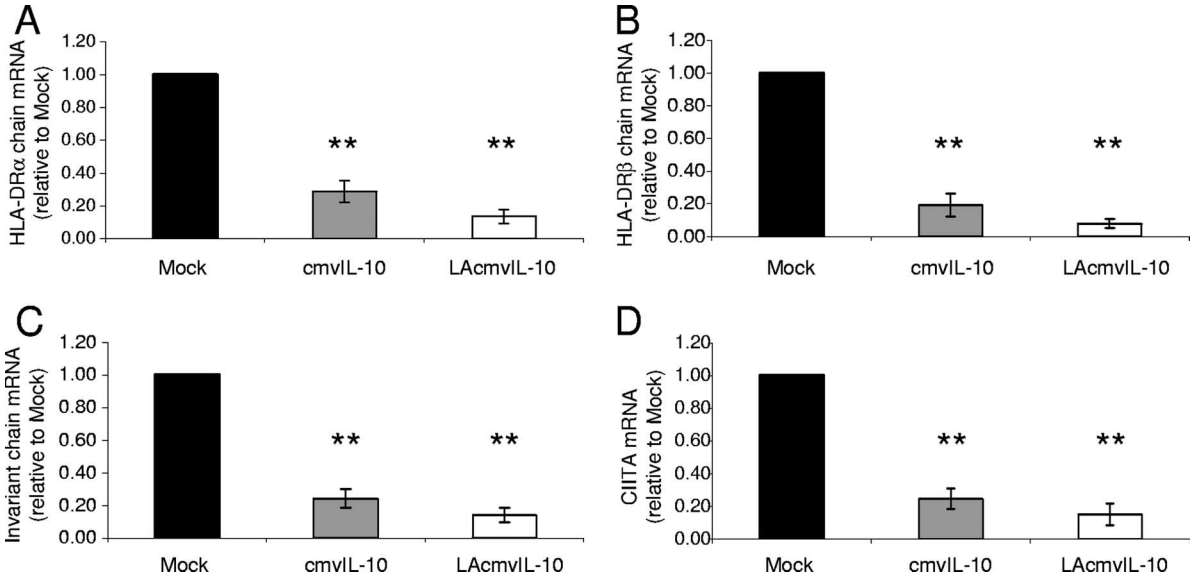

FIG. 5. Analysis of mRNA expression of components of the MHC-II biosynthesis pathway. Quantitative real-time RT-PCR analysis of HLA-DRα chain (A), HLA-DRβ chain (B), invariant chain (C), and CIITA (D) mRNA expression in monocytes treated for 48 h with mock, cmvIL-10, or LAcmvIL-10 protein preparations. Significant differences from the mock-treated control were determined using a one-tailed, paired Student's *t* test. **, $P < 0.005$.

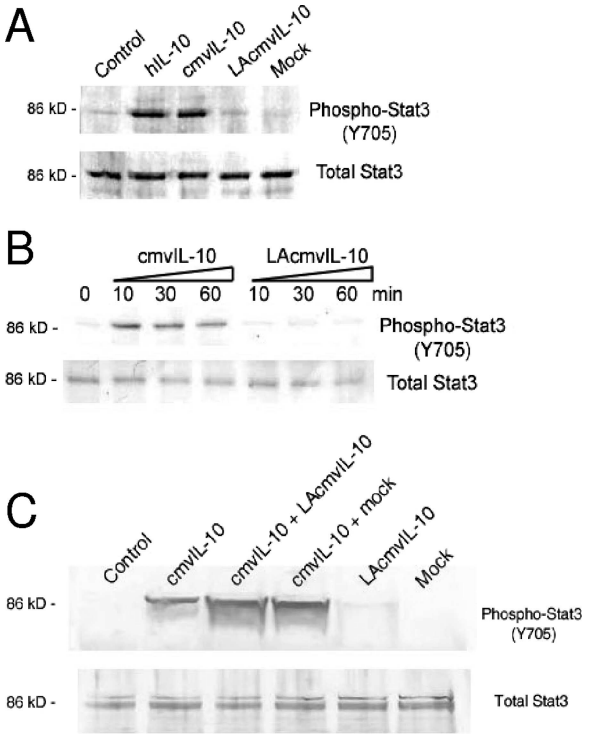

FIG. 6. Activation of Stat3 by cmvIL-10 but not LAcmvIL-10. (A) Primary human $CD14^+$ monocytes were left untreated (Control) or treated with 10 ng/ml purified hIL-10, cmvIL-10, or LAcmvIL-10 protein preparations for 10 min. Cell lysates were then immunoblotted with polyclonal antiserum to detect phosphorylated or total unmodified Stat3 protein as indicated. (B) Monocytes were treated with 10 ng/ml purified cmvIL-10 or LAcmvIL-10 for increasing times and then immunoblotted as described above. (C) Monocytes were incubated with 10 ng/ml for each indicated cytokine or combination of cytokines for 10 min before lysis and blotting.

ited IL-1α, IL-1β, IL-6, and TNF-α production. In contrast, treatment of DCs with LPS in the presence of LAcmvIL-10-conditioned medium showed no inhibition of any of these cytokines in comparison with mock-treated cells. Production of the cytokine GM-CSF was not altered significantly in the presence of either purified cmvIL-10 or conditioned medium from cmvIL-10- or LAcmvIL-10-transfected cells compared to levels detected in mock-treated DCs. Taken together with the cell surface molecule analyses, these data demonstrate that cmvIL-10, but not LAcmvIL-10, inhibits LPS-induced maturation of MDDCs.

DISCUSSION

This study represents the first functional assessment of HCMV LAcmvIL-10 protein, addressing the capacity of LAcmvIL-10 to exploit the immunosuppressive properties of hIL-10 as a viral mechanism for potentially limiting the effectiveness of the host's immune system during the latent phase of infection. Significantly, recombinant LAcmvIL-10 exhibited a dose-dependent ability to downregulate constitutive levels of MHC-II on the surfaces of both GM-Ps and monocytes, cell types known to be important sites of HCMV latency. The ability of LAcmvIL-10 to interfere with MHC-II expression was comparable to that of the related protein cmvIL-10, a functional homolog of hIL-10 which is expressed from the UL111A region of the HCMV genome during productive infection (29, 32, 54).

Consistent with the flow cytometry analysis, immunofluorescence and confocal microscopy demonstrated a reduction in the number of LAcmvIL-10- and cmvIL-10-treated monocytes expressing surface MHC-II. In the vast majority of these cells, the loss of cell surface staining corresponded with a general loss of cellular MHC-II staining. However, in a very small proportion of cells, the loss of cell surface staining was asso-

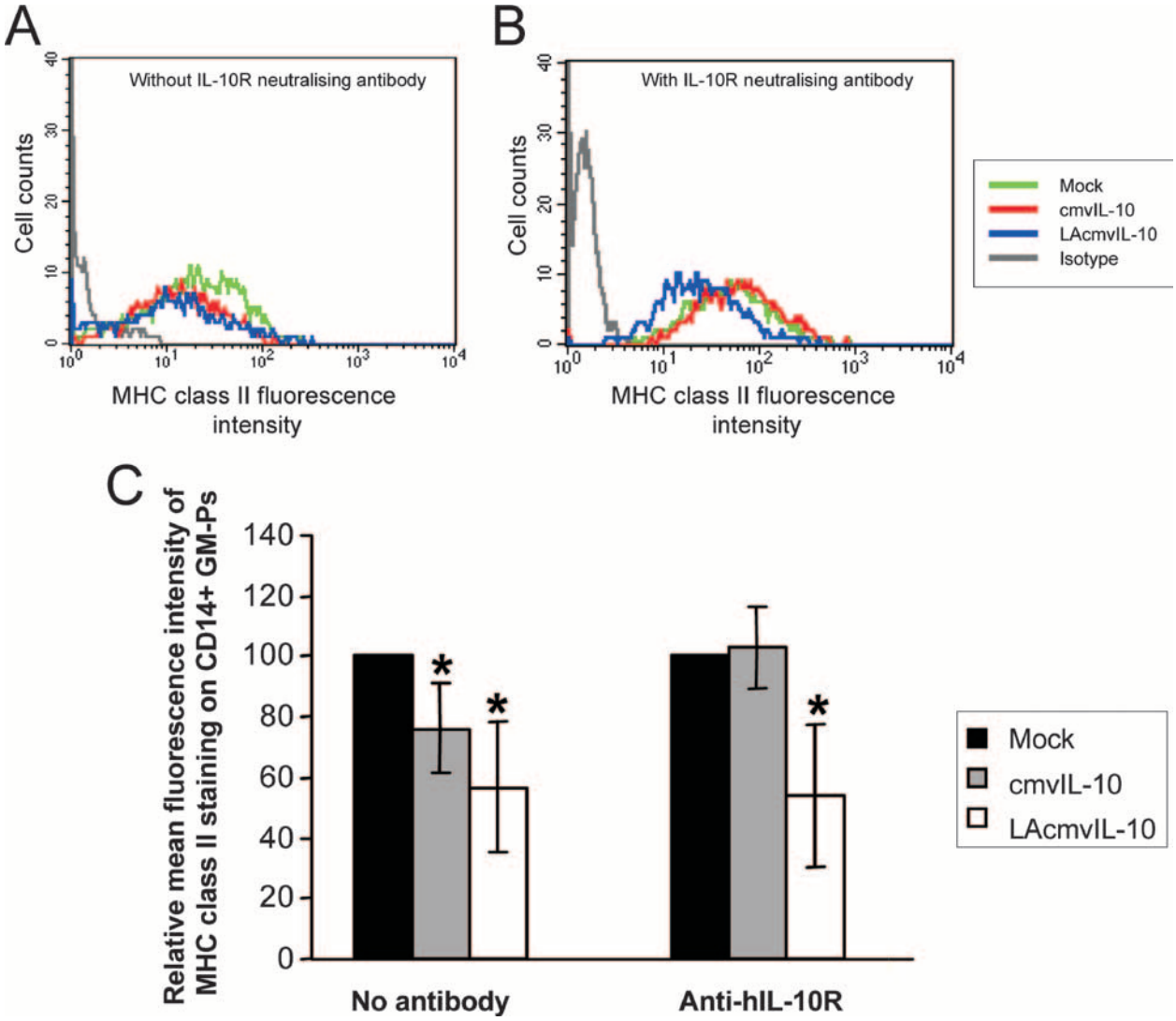

FIG. 7. Capacity of LAcmvIL-10 and cmvIL-10 to downregulate MHC-II in the presence of neutralizing antibody to hIL-10R. Flow cytometric analysis of MHC-II (HLA-DR) expression on $CD14^+$ GM-Ps following incubation without (A) or with (B) anti-hIL-10Rα/β neutralizing antibodies, prior to treatment with either LAcmvIL-10, cmvIL-10, or mock protein preparations. (C) The relative MFI values of surface MHC-II are shown with the means and standard errors calculated from four independent replicate experiments. Each data point represents the relative MFI of a given surface molecule (relative MFI_M) on the various treatments of GM-Ps, calculated as follows: (MFI_M on $GM\text{-}Ps_{(treated)}$/MFI_M on $GM\text{-}Ps_{(mock)}$) ×100, where GM-Ps are treated with the cmvIL-10, LAcmvIL-10, or mock preparation. Significant differences from mock treatments were determined using a one-tailed, paired Student's *t* test. *, $P < 0.05$.

ciated with the appearance of a punctate vesicular staining pattern in the cytoplasm. We have previously reported a decrease in MHC-II expression on the surfaces of GM-Ps latently infected with HCMV, concomitant with a retention of MHC-II protein within $HLA\text{-}DM^+$ cytoplasmic vesicles (52). Furthermore, Koppelman et al. (28) demonstrated that hIL-10 downregulates MHC-II expression at the plasma membrane of monocytes by affecting the exocytosis and recycling of MHC-II peptide complexes and by suppressing the reexpression of internalized MHC-II on the plasma membrane affecting the recycling pool of these molecules (28). MHC-II was shown to localize to vesicles that costained with the endosomal markers HLA-DM and CD63. It was suggested that hIL-10 affects the biosynthetic pool of MHC-II, an idea which is based on the observation that HLA-DM identifies lysosomal-like structures through which the majority of newly synthesized MHC-II must negotiate on the way to the plasma membrane (5, 64). It is therefore tempting to speculate that LAcmvIL-10 may function to suppress the host's ability to successfully mount an immune response against the virus in its latent form by affecting MHC-II exocytosis and recycling in latently infected cells. Although the observations reported previously in latently HCMV-infected GM-Ps and in hIL-10-treated monocytes share similarities with the observations of altered MHC-II localization by LAcmvIL-10 and cmvIL-10 in the current study, a decrease in total MHC-II in these cells was not reported in infected GM-Ps or hIL-10-treated monocytes. A more detailed examination of the MHC-II biosynthesis pathway, together

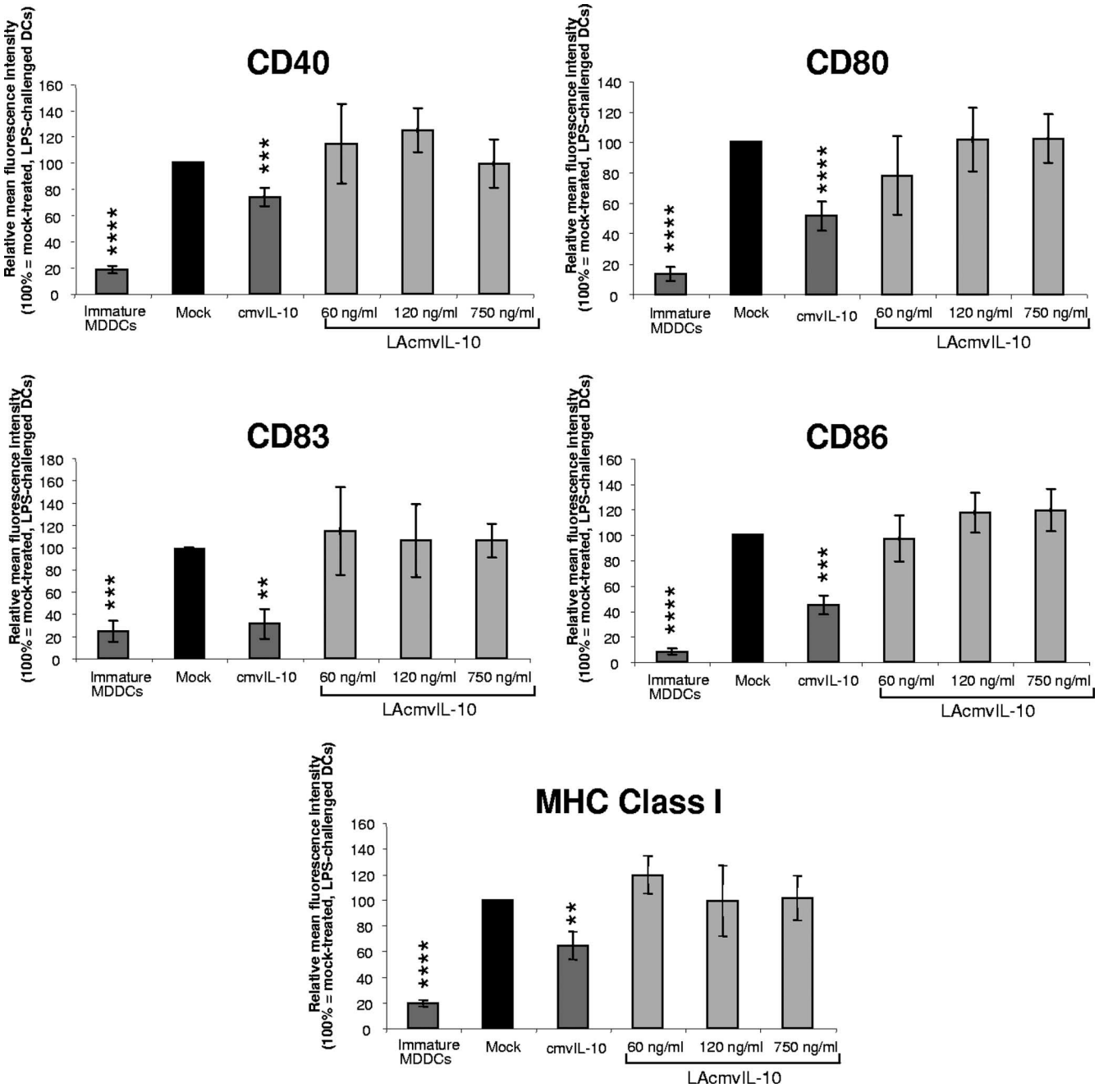

FIG. 8. Effect of LAcmvIL-10 and cmvIL-10 on MDDC maturation following LPS stimulation. Flow cytometric analysis of the expression of CD40, CD80, CD83, CD86, and MHC-I molecules on the surface of LPS-induced DCs. The relative MFIs of surface molecules are shown with the means and standard errors calculated from four separate experiments. Each data point represents the relative MFI of a given surface molecule (relative MFI_M) on the various treatments of LPS-induced DCs, calculated as follows: (MFI_M on $DCs_{(treated\ or\ immature)}$/$MFI_M$ on $DCs_{(mock)}$) $\times 100$, where DCs are immature or treated with the cmvIL-10, LAcmvIL-10, or mock preparation. Significant differences from the mock-treated control were determined using a one-tailed, paired Student's *t* test and are indicated as follows: **, $P < 0.005$; ***, $P < 0.0005$; ****, $P < 0.00005$.

with determination of the site of accumulation of MHC-II molecules in the small proportion of cells displaying a punctate staining pattern, will therefore be important components of future studies to compare and contrast the mechanisms of action of these viral IL-10 proteins. Analysis of transcription of components of the MHC-II biosynthesis pathway revealed that MHC-II α chain, β chain, and invariant chain and the master regulator of MHC-II, CIITA, were inhibited by both LAcmvIL-10 and cmvIL-10. Thus, in addition to any potential posttranslational interference, these data provide evidence that both viral IL-10s act as inhibitors of CIITA, with the consequence of decreased transcription of MHC-II components and reduced MHC-II protein expression.

While LAcmvIL-10 and cmvIL-10 both functioned to down-

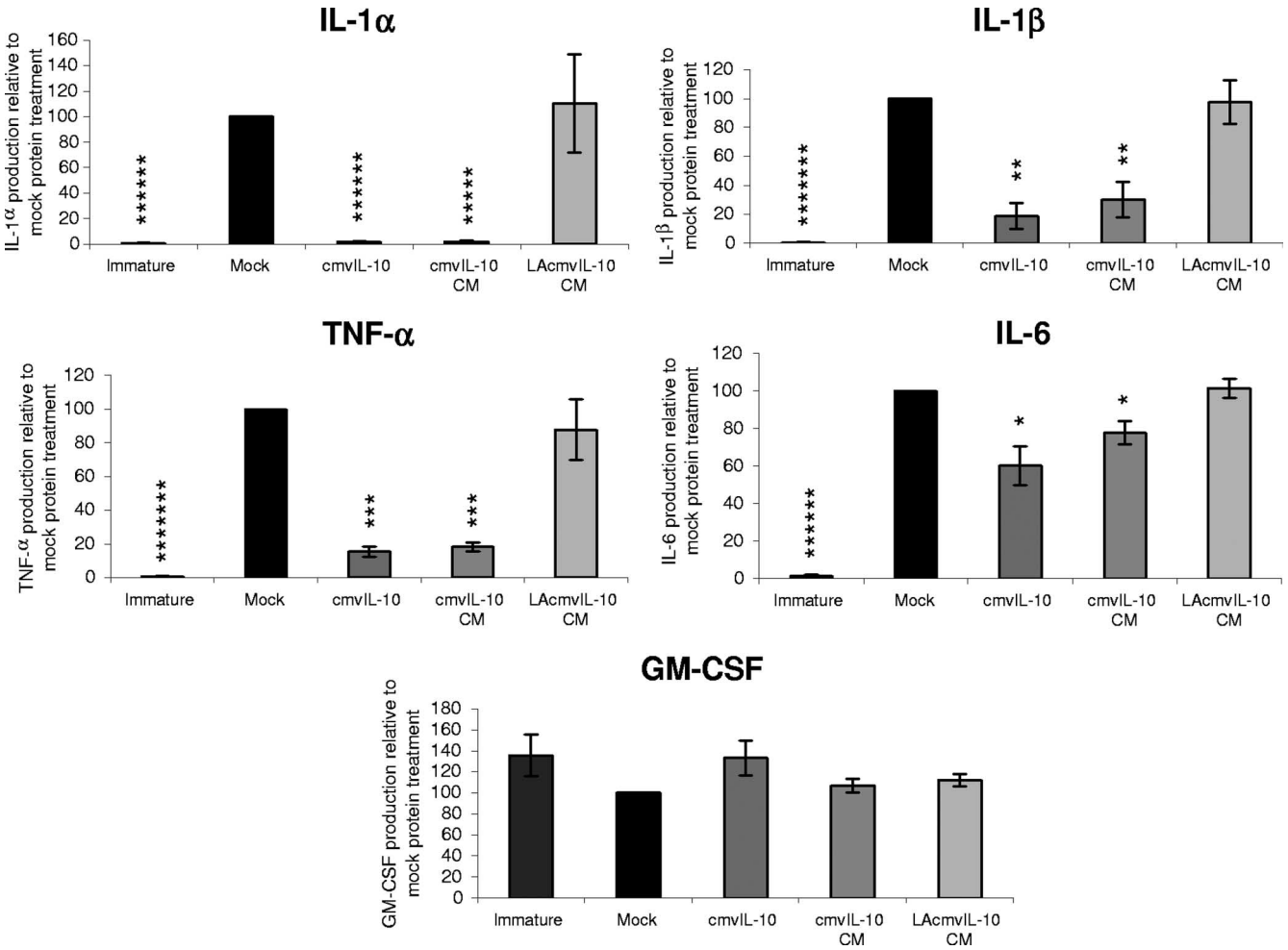

FIG. 9. Impact of LAcmvIL-10 and cmvIL-10 on proinflammatory cytokine production by MDDCs following LPS stimulation. Multiplex ELISA-based analysis of the levels of cytokines production by DCs treated for 3 days with LPS in the presence of either purified cmvIL-10 protein or conditioned medium (CM) from HEK293 cells transfected with either cmvIL-10 or LAcmvIL-10 expression vectors. Uninduced DCs (i.e., immature DCs) and LPS-induced DCs treated with mock-conditioned medium are also included. The levels of IL-1α, IL-1β, IL-6, TNF-α, and GM-CSF in the supernatants of DCs relative to LPS-induced DCs treated with mock-conditioned medium are shown, with significant differences determined using a one-tailed, paired Student's *t* test and indicated as follows: *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; *****, $P < 0.000005$; ******, $P < 0.0000005$; *******, $P < 0.00000005$.

regulate MHC-II on GM-Ps and monocytes, there were profound differences in the ability of these two viral IL-10 homologs to interfere with the maturation of DCs. Maturation of DCs leads to phenotypic changes including upregulation of the costimulatory and adhesion molecules CD40, CD80, CD83, and CD86, which are involved in T-lymphocyte activation, and increased MHC-I and -II expression on the surface of maturing DCs (3, 43, 62). A number of studies have demonstrated that productive HCMV infection interferes with the maturation of DCs and their antigen-presenting function by inhibiting the expression of these immune molecules (4, 18, 30, 38, 51). Furthermore, Chang et al. (9) and Raftery et al. (46) showed that stimulation of immature DCs to mature in the presence of cmvIL-10 results in the functional paralysis of DCs (9, 46). In our study, DCs treated with cmvIL-10 exhibited impaired LPS-induced upregulation of the DC costimulatory molecules CD40, CD80, and CD86 and the maturation marker CD83. cmvIL-10 was also shown to significantly inhibit the upregulation of MHC-I expression on the surface of DCs in response to LPS. However, in stark contrast to cmvIL-10, LAcmvIL-10 treatment (even at high concentrations) did not inhibit upregulation of CD40, CD80, CD83, CD86, or MHC-Ion the surfaces of DCs in response to LPS-induced maturation. Assessment of cytokine production by DCs treated with cmvIL-10-conditioned medium showed that cmvIL-10 had the capacity to inhibit the secretion of IL-1α, IL-1β, IL-6, and TNF-α. These data are consistent with the cytokine profiles reported following HCMV infection of monocytes/macrophages and cytotrophoblasts (16, 17, 40, 54, 66). Importantly, treatment of LPS-induced DCs with LAcmvIL-10-conditioned medium did not result in the alteration of cytokine production compared to mock controls. Thus, LAcmvIL-10 and cmvIL-10 differ in their ability to alter DC maturation, but both are able to downregulate constitutive expression of MHC-II in GM-Ps and monocytes. Despite the impact of cmvIL-10 and LAcmvIL-10 on MHC-II expression on myeloid lineage cells, neither affected

the expression of MHC-II on Daudi human B cell lymphoblastoma cells (55), further indicating cell-type-specific immunosuppressive functions of these viral cytokines.

The inability of LAcmvIL-10 to impair LPS-induced DC maturation in a manner similar to cmvIL-10 may be a consequence of differences in the structure of these two proteins. Mapping of the HCMV latency-associated UL111A region transcript encoding LAcmvIL-10 has demonstrated retention of a second intron which is ordinarily spliced in the transcript encoding cmvIL-10 (19). The presence of an in-frame stop codon within intron 2 results in the translation of the LAcmvIL-10 protein that is truncated at the C terminus in comparison to cmvIL-10. This truncation is predicted to have structural implications on LAcmvIL-10 that may affect binding to and signaling through the IL-10R complex. Investigations into the functions of cmvIL-10 have also assessed the binding and signaling of the hIL-10 homolog through both IL-10R1 and IL-10R2 (29, 54). Jones et al. (22) resolved the crystal structure of the cmvIL-10 bound to soluble IL-10R1 (sIL-10R1), demonstrating that cmvIL-10 bound to two sIL-10R1 molecules as a disulfide-linked homodimer of two interpenetrating polypeptide chains (22). cmvIL-10 and hIL-10 share similar topologies and sIL-10R1 binding sites even though the interdomain angle of cmvIL-10 is ~40° larger than that of hIL-10. Computer predictions supported by epitope and peptide mapping data (47, 64, 67, 68) suggest that the regions of hIL-10 likely to be involved in contact with IL-10R1 include the N terminus, helix A, the AB loop, part of helix B, and the C-terminal helices E/F. Jones et al. (22) demonstrated that cmvIL-10 uses essentially the same structural epitope as described for hIL-10 with helix A, the AB loop, and helix F contacting sIL-10R1 (22). Studies by Gesser et al. (15) assessed the function(s) of the C-terminal portion of hIL-10 using a nonapeptide with homology to residues 152 to 160 (15). This peptide was found to mimic some of the activities of intact hIL-10 such as inhibition of IL-1β-induced and spontaneous IL-8 production, downregulation of TNF-α production, and inhibition of MHC-II expression on gamma interferon-stimulated monocytes.

In the case of LAcmvIL-10, the absence of amino acid residues coded by exon 3 of the UL111A transcript results in the loss of the C-terminal helices E/F present in cmvIL-10 (19). This truncation may therefore result in a diminished capacity of LAcmvIL-10 to dimerize or signal through the IL-10R complex. Indeed, we found that in contrast to treatment of monocytes with cmvIL-10 or hIL-10, LAcmvIL-10 did not induce the phosphorylation of Stat3, a signaling molecule which is rapidly phosphorylated upon activation of hIL-10R (14). In addition, neutralizing antibodies to hIL-10R blocked the ability of cmvIL-10, but not LAcmvIL-10, to downregulate MHC-II, providing evidence of a mechanistic basis for the differences between these proteins. LAcmvIL-10 does retain an immunomodulatory capacity as it downregulates constitutive MHC-II expression on GM-Ps and monocytes. Thus, LAcmvIL-10 either functions through a different, as yet unidentified receptor, or it is still able to exert a function through the IL-10R complex in a manner which differs from that utilized by hIL-10 and cmvIL-10. The hIL-10 dimer is thought to have evolved from a monomeric protein by a mechanism known as protein oligomerization or three-dimensional domain swapping. This process may have lead to the exchange of α-helices E and F with another monomer to create the dimer with intertwining α-helical domains (6). A monomeric form of hIL-10 engineered by Josephson et al. (23) was able to bind to the IL-10R1 with a 1:1 stoichiometry and retained biological activity, although with reduced affinity (60-fold) and specific activity (10-fold) compared to wild-type hIL-10 (23). In addition, Gesser et al. (15) also assessed the function(s) of the N-terminal portion of hIL-10 by generating a nonapeptide correlating to residues 8 to 16 of hIL-10 (15). While this peptide did not exhibit any cytokine synthesis-inhibitory properties, it did downregulate MHC-II expression. It therefore remains possible that LAcmvIL-10 has the ability to bind to and signal in some way through the IL-10R complex. Consequently, an extension of the work presented here will be to further examine LAcmvIL-10–hIL-10R interactions to better define the mechanism of action of this protein in the context of the hIL-10 receptor-mediated signaling pathway and also to examine the ability of LAcmvIL-10 to form homodimers or heterodimers with hIL-10 or cmvIL-10.

Many of the latency-associated gene products expressed by herpesviruses have also been reported to be expressed during the productive phase of infection. These include latency-associated transcripts (LAT) expressed by herpes simplex virus type 1; EBNA-1, the LAT of Epstein-Barr virus; the latently expressed varicella-zoster virus genes ORF21, ORF29, ORF62, ORF63 and ORF4; the pseudorabies virus LAT gene; and HCMV major immediate-early region sense LATs (CLTs) (10, 11, 13, 21, 25, 33, 35, 39, 50, 56, 57). We have detected RNA with features of LAcmvIL-10 transcription in RNA extracted from productively infected HFFs (20), and Chang et al. (9) detected a protein product by Western blotting consistent with LAcmvIL-10 when extracts from productively infected MRC-5 cells were reacted with anti-cmvIL-10 antibody (9). The relative contributions of cmvIL-10 and LAcmvIL-10 expression during productive infection await a more detailed analysis of the expression from the UL111A region during this phase of infection. In the context of latent infection, a UL111A deletion mutant virus unable to express LAcmvIL-10 or cmvIL-10 (42) established and reactivated from latency in experimentally infected, cultured GM-Ps in a manner comparable to its parent virus (data not shown). Together with our identification of an immunomodulatory function, this suggests that LAcmvIL-10 is not required directly for latent infection of myeloid progenitors but, rather functions as an immunosuppressor to limit the immune response to latently infected cells.

To conclude, the data presented here demonstrate that a homolog of hIL-10 expressed by HCMV during the latent phase of infection shares some, but not all, of the immunomodulatory functions of the productive infection-associated protein cmvIL-10. While cmvIL-10 was very effective at inhibiting the LPS activation of DCs by preventing the upregulation of costimulatory and adhesion molecules critical to antigen presentation, LAcmvIL-10 treatment neither hindered the upregulation of DC maturation markers nor inhibited the secretion of proinflammatory cytokines. Most significant, however, is the finding that LAcmvIL-10 was able to downregulate constitutive MHC-II expression on the surfaces of primary human $CD14^+$ GM-Ps and monocytes. As GM-Ps and monocytes represent a latent reservoir of HCMV, modulation of MHC-II expression by LAcmvIL-10 during the latent phase of infection represents a strategy that could afford the virus escape from

immunosurveillance and increase the chances for lifelong survival.

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