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Shaping Phenotype, Function, and Survival of Dendritic Cells by Cytomegalovirus-Encoded IL-10

Martin J. Raftery,* Dörte Wieland,† Stefanie Gronewald,* Annette A. Kraus,* Thomas Giese,† and Günther Schönrich2*

Human dendritic cells (DCs) are essential for the antiviral immune response and represent a strategically important target for immune evasion of viruses, including human CMV (HCMV). Recently, HCMV has been discovered to encode a unique IL-10 homologue (cmvIL-10). In this study we investigated the capacity of cmvIL-10 to shape phenotype, function, and survival of DCs. For comparison we included human IL-10 and another IL-10 homologue encoded by EBV, which does not directly target DCs. Interestingly, cmvIL-10 strongly activated STAT3 in immature DCs despite its low sequence identity with human IL-10. For most molecules cmvIL-10 blocked LPS-induced surface up-regulation, confirming its role as an inhibitor of maturation. However, a small number of molecules on LPS-treated DCs including IDO, a proposed tolerogenic molecule, showed a different behavior and were up-regulated in response to cmvIL-10. Intriguingly, the expression of C-type lectin DC-specific ICAM-grabbing nonintegrin, a receptor for HCMV infection found exclusively on DCs, was also enhanced by cmvIL-10. This phenotypic change was mirrored by the efficiency of HCMV infection. Moreover, DCs stimulated with LPS and simultaneously treated with cmvIL-10 retained the function of immature DCs. Finally, cmvIL-10 increased apoptosis associated with DC maturation by blocking up-regulation of the antiapoptotic long form cellular FLIP. Taken together, these findings show potential mechanisms by which cmvIL-10 could assist HCMV to infect DCs and to impair DC function and survival.

H

uman CMV (HCMV)3 and EBV represent ubiquitous pathogens that are carried by a high percentage of healthy adults in a life-long infection (1, 2). In general, infections with these herpesviruses remain clinically apparent in immunocompetent hosts whereas severe disease and increased mortality is observed in immunosuppressed individuals, for example AIDS patients and transplant patients.

The successful coexistence of viruses with their host requires a variety of mechanisms of viral immune evasion (3–5). These fall into three different categories: viral escape from recognition by immune cells, resistance to apoptosis, and viral counterattack that can lead to generalized immunosuppression (6). The latter strategy could be particularly efficient if it targets dendritic cells (DCs), which play a pivotal role in both the innate and adaptive immune response (7–10). Immature DCs reside in the periphery at sites that represent the interface with the environment. In the skin and in mucosa of the lung and gastrointestinal tract, immature DCs take up Ags and process them into peptides, which are subsequently loaded onto MHC class I and class II molecules. After sensing infection through pattern-recognition receptors or inflammatory cytokines, DCs enter a maturation program and migrate into secondary lymphoid organs (11). Mature DCs represent the most potent professional APCs of the host immune system and are predisposed to initiate the antiviral T cell response (12). Numerous pathogens, including HIV-1, Mycobacterium tuberculosis, and HCMV can target DCs by binding to the DC-specific ICAM-grabbing nonintegrin (DC-SIGN) (13). HCMV and murine CMV have been shown to infect DCs and down-regulate immunologically important surface molecules (14–17). Besides phenotypic changes, HCMV-infected DCs are characterized by their ability to suppress the antiviral immune response and by enhanced apoptosis (16). However, the underlying mechanisms and the responsible viral proteins have not yet been defined.

The phenotype and function of human DCs can be profoundly altered by human IL-10 (hIL-10), a pleiotropic cytokine involved in regulation of inflammatory responses (18). Intriguingly, highly conserved IL-10 gene homologues have been found in the genome of several large DNA viruses, including EBV (19), equine herpesvirus 2 (20) and ovine Orf parapoxvirus (21). Recently, an IL-10 gene homologue encoded by HCMV (cmvIL-10) has been discovered that differs from other IL-10 genes in certain aspects (22). Firstly, the cmvIL-10 gene contains intervening sequences whereas other viral IL-10 genes lack introns. Secondly, the cmvIL-10 protein shows only 27% identity to the hIL-10 amino acid sequence whereas the EBV-encoded IL-10 protein (ebvIL-10) is 84% identical with hIL-10. Despite its low homology, cmvIL-10 binds to the IL-10R (22) and shows biological activity (23). Thus, cmvIL-10 could mediate HCMV-induced immunosuppression by targeting DCs.

In our comparative study we analyzed the action of cmvIL-10 on three different DC stages: immature DCs, LPS-induced DCs, and mature DCs. These stages model the situation in vivo, in which DCs may be exposed to cmvIL-10 in the absence, in the presence, or after an inflammatory signal.
Materials and Methods

Generation of DCs

Human DCs were generated from monocytes isolated from buffy coat preparations supplied by the German Red Cross, Berlin, Germany. CD14 monocytes were isolated from PBMC by Ab-coupled magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), and cultured in FCS-free CellGro DC medium (CellGenix, Freiburg, Germany) supplemented with 100 IU of penicillin, 100 μg/ml streptomycin, 4.5 mM glutamine, 500 U/ml GM-CSF, and 200 U/ml IL-4. Half of the medium was changed the third day after isolation, and the entire medium was changed at day 6, when the DCs were used for experiments. Whenever required, cells were matured with 1 μg/ml LPS (Sigma-Aldrich, Deisenhofen, Germany) for 2 days with or without recombinant hIL-10, cmvIL-10, or ebvIL-10 (R&D Systems, Wiesbaden, Germany).

Antibodies

The following Abs were used for phenotypic analysis of cell markers by flow cytometry: anti-CD11c (B-Ly6), anti-CD18 (clone 6.7), anti-CD25 (clone M-A251), anti-CD40 (clone LOB7/6), anti-CD54 (clone HA58), anti-CD83 (clone HB15e), anti-CD86 (clone IT2.2), anti-CD95 (clone DX2), anti-CD150 (clone A12), anti-DC-SIGN (clone DCN46), anti-HLA-DR, -HLA-DP, and -HLA-DQ (clone TU39), anti-HLA-DM (clone MaP.DM1), and isotype-matched control Abs were purchased from BD Pharmingen (Heidelberg, Germany); anti-MHC class I (clone W6/32) and anti-CD200 (clone MRC OX104) were obtained from Serotec (Düsseldorf, Germany); anti-CD55 (clone M-A251), anti-CD40 (clone LOB7/6), anti-CD54 (clone HA58), anti-CD80 (clone MAB104) was obtained from Southern Biotechnology Associates (Birmingham, U.K.); anti-CD44 (clone M-A251), anti-CD200 (clone MRC OX104) were obtained from Serotec (Düsseldorf, Germany); anti-CD55 (clone M-A251), anti-CD40 (clone LOB7/6), anti-CD54 (clone HA58), anti-CD80 (clone MAB104) was obtained from Southern Biotechnology Associates.

Western blot analysis

To detect STAT3 activation, immature DCs were stimulated with 25 ng/ml hIL-10, cmvIL-10, or ebvIL-10 in culture medium for 15 min, harvested and washed once with ice-cold PBS before resuspension in lysis buffer consisting of ice-cold PBS with 1% Nonidet P-40 and proteinase inhibitors (Sigma-Aldrich). After 1 h on ice, cells were harvested and washed once with ice-cold PBS before resuspension in 1% Nonidet P-40 and proteinase inhibitors (Sigma-Aldrich) at 25°C. The cells were then incubated in cell lysis buffer consisting of ice-cold PBS with 1% Nonidet P-40 and proteinase inhibitors (Sigma-Aldrich) at 25°C for 5 min, centrifuged for 10 min at 13,000 × g, and the supernatant was stored at −80°C. The proteins were separated by SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in TBS, and probed with antibodies directed against phospho-STAT3 (Cell Signaling Technology) or the following anti-human mAb: c-FLIPL (clone NF6) (24), Bcl-2 (clone 7; BD Biosciences, Heidelberg, Germany), or Bcl-xL (clone 44; BD Biosciences). Thereafter, blots were stripped and restained with anti-human β-actin (clone AC-15) (Abcam). In all cases the staining with primary Ab was followed by staining with HRP-coupled secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1/10,000 dilution and detection by ECL (Pierce, Rockford, IL).

Flow cytometry

For surface immunofluorescence by flow cytometry cells in suspension were washed once with ice-cold FACScalibur solution (PBS with 1% FCS and 0.02% sodium azide) before being resuspended with the first Ab in ice-cold blocking solution (PBS with 10% heat inactivated FCS and 0.2% sodium azide) for 1 h. The cells were then washed in ice-cold FACScalibur solution and the staining repeated with PE-coupled goat anti-mouse secondary Ab. After the final staining step, cells were washed in ice-cold FACScalibur and then resuspended in 100 μl of PBS with 0.3% formaldehyde. Flow cytometry was performed on a FACScalibur (BD Biosciences).

HCMV infection of DCs

Immature DCs and DCs exposed to LPS with or without IL-10 for 2 days were infected with an endothelial-cell adapted strain of virus at a multiplicity of infection of 1. The cells were then incubated for 3 days in DC

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** Strong cmvIL-10-induced signaling in immature DCs. Western blot analysis of phosphorylated STAT3 expression in immature DCs treated for 15 min with 25 ng/ml hIL-10, cmvIL-10, or ebvIL-10. Expression of β-actin was determined to control the proper loading of SDS-PAGE gels. In addition, Western blots were analyzed by densitometry to determine the mean band intensity. The results are given as mean ± SD and are derived from three individual experiments with DCs from different donors.

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** Enhanced DC-SIGN expression on immature DCs treated with cmvIL-10. A, Histograms showing DC-SIGN expression on immature DCs left untreated or treated for 2 days with 25 ng/ml cytokine as indicated. On the x-axis, the fluorescence intensity (log scale, 4 decades) is given, whereas the y-axis depicts the relative cell number. The mean fluorescence intensity (upper right and left corner) is shown. B, Immature (iDC) or mature DCs (mDC) were left untreated or treated with 25 ng/ml cytokine as indicated. After 2 days, DC-SIGN expression was determined by cytofluorimetric analysis. The respective mean fluorescence intensities (MFI) on untreated immature or mature DCs were set as 100%. The relative percentage of mean fluorescence intensity is given on the y-axis as mean ± SD derived from five different donors.

Nonidet P-40 and proteinase inhibitors (Sigma-Aldrich). After 1 h on ice, cells were centrifuged for an additional hour at 13,000 × g at 4°C to remove the insoluble fraction. The supernatant was stored at −20°C before use. After SDS-PAGE, the protein was transferred onto a polyvinylidene difluoride membrane and either stained with polyclonal rabbit anti-phospho-STAT3 (Cell Signaling Technology) or the following anti-human mAb: c-FLIPL (clone NF6) (24), Bcl-2 (clone 7; BD Biosciences, Heidelberg, Germany), or Bcl-xL (clone 44; BD Biosciences). Thereafter, blots were stripped and restained with anti-human β-actin (clone AC-15) (Abcam). In all cases the staining with primary Ab was followed by staining with HRP-coupled secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1/10,000 dilution and detection by ECL (Pierce, Rockford, IL).

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HCMV infection of DCs

Immature DCs and DCs exposed to LPS with or without IL-10 for 2 days were infected with an endothelial-cell adapted strain of virus at a multiplicity of infection of 1. The cells were then incubated for 3 days in DC
medium before centrifugation onto glass slides and drying. Slides were subsequently rehydrated in PBS, fixed in a 50:50 mix of acetone to methanol at −20°C and washed thrice in PBS. Immunohistochemistry of infected and uninfected cells was then performed using a monoclonal anti-IE Ab (clone E13; Argene, Varilhes, France) and FITC-labeled secondary Abs (Southern Biotechnology).

Ag uptake
DCs were incubated with FITC-dextran (10 μg/ml) for 1 h at either 37°C or 4°C. Subsequently, cells were washed three times and then analyzed by flow cytometry. Uptake of dextran was determined by measuring the difference between the results at 37°C and 4°C. FITC-dextran was provided by Sigma-Aldrich.

RNA sample preparation
Cells (10⁷) were lysed with 300 μl of MagnaPure lysis buffer (Roche Applied Science, Mannheim, Germany) and samples were frozen at −70°C. After thawing, lysates were mixed and transferred into MagnaPure sample cartridges and mRNA was isolated with a MagnaPure LC device using mRNA standard protocol for cells. The elution volume was set to 50 μl. One aliquot of 8.2 μl RNA was reverse transcribed using avian myeloblastosis virus and oligo(dT) as primer (first strand cDNA synthesis kit Roche Applied Science) according to the manufacturer’s protocol in a thermocycler. After termination of cDNA synthesis, the reaction for RT-PCR; Roche Applied Science) according to the manufacturer’s protocol, the eloblastosis virus and oligo(dT) as primer (first strand cDNA synthesis kit Roche Applied Science) according to the manufacturer’s protocol. mRNA was isolated with a MagnaPure-LC device. After thawing, lysates were mixed and transferred into MagnaPure sample cartridges and mRNA was isolated with a MagnaPure LC device with the LightCycler FastStart DNA Sybr green I kit (Roche Applied Science) according to the manufacturer’s protocol. RNA input was normalized by the average expression of the housekeeping genes encoding β-actin and cyclophilin B. Copy number was calculated from a virtual standard curve, obtained by plotting a known input concent-

LightCycler PCR
Target sequences were amplified using LightCycler Primer sets (Search-LC, Heidelberg, Germany) with the LightCycler FastStart DNA Sybr green I kit (Roche Applied Science) according to the manufacturer’s protocol. RNA input was normalized by the average expression of the housekeeping genes encoding β-actin and cyclophilin B. Copy number was calculated from a virtual standard curve, obtained by plotting a known input concent-

### Results

#### Activation of STAT3 in cmvIL-10-treated DCs

In our study, we analyzed the action of cmvIL-10 on three different DC stages: immature DCs, LPS-exposed DCs, or mature DCs. For comparison, we included hIL-10 and ebvIL-10 in our analysis. In the first series of experiments activation of STAT3, a key mediator of the IL-10 response (25), was determined in immature DCs (Fig. 1). Western blot analysis revealed that cmvIL-10 triggered strong STAT3 activation in DCs although it shares only 27% amino acid sequence identity with hIL-10. In contrast, we could not detect phosphorylated STAT3 in immature DCs treated with ebvIL-10 for either 15 min (Fig. 1) or 2 h (data not shown). Moreover, tration of a plasmid to the PCR-cycle number at which the detected fluorescence intensity reached a fixed value. The data of two independent analyses for each sample and parameter were averaged as adjusted transcripts per microtiter cDNA or as ratios to control values.

**Apoposis**

DCs were treated as indicated for 2 days before being harvested and the degree of apoptosis was determined by staining with annexin V-FITC and cyttofluorimetric analysis.

**T cell stimulation**

Allogeneic T cells were isolated from PBMC by removal of CD14+ monocytes by Ab-coupled magnetic beads (Miltenyi Biotec). APCs and B cells were depleted with Dynabeads reactive to human IgG (Dynal Biotec, Hamburg, Germany). DCs were left untreated or treated with LPS and hIL-10, cmvIL-10, or ebvIL-10 for 2 days. DCs were then washed four times with PBS before being mixed with allogeneic T cells at a ratio of 1:50. After 2 days expression of DC-SIGN (Fig. 3), ICAM-1 (B) on DCs was measured by flow cytometry. The mean fluorescence intensity is given on the y-axis. The results shown are representative of three separate experiments with cells derived from different donors.

### Table 1. Susceptibility to HCMV infection of DCs treated with LPS alone or in combination with IL-10

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage of Susceptibility (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>17 (±10)</td>
</tr>
<tr>
<td>LPS with hIL-10</td>
<td>85 (±15)</td>
</tr>
<tr>
<td>LPS with cmvIL-10</td>
<td>87 (±6)</td>
</tr>
<tr>
<td>LPS with ebvIL-10</td>
<td>35 (±9)</td>
</tr>
</tbody>
</table>

* Immature DCs were exposed for 2 days to LPS without or with IL-10 as indicated.

* Cells were infected with an endothelial cell-adapted strain of HCMV (MOI = 1). Degree of HCMV infection is given relative to untreated immature DCs (100%). Results are derived from four individual experiments with DCs from different donors (± SD).
phosphorylation of STAT3 associated with cmvIL-10 could not be abrogated by preincubating DCs with ebvIL-10 (data not shown). Thus, cmvIL-10 strongly induces the IL-10 signaling pathway in DCs.

Enhanced expression of DC-SIGN on cmvIL-10-treated DCs

DC-SIGN is a lectin expressed on the cell surface of immature DCs and functions as a receptor for several viruses (13), including HCMV (26). We investigated, therefore, whether cmvIL-10 modulates the expression of this important molecule. Both cmvIL-10 and hIL-10 but not ebvIL-10 enhanced the density of DC-SIGN on immature DCs (Fig. 2). In contrast, previously matured DCs did not show significantly increased levels of DC-SIGN upon treatment with cmvIL-10 (Fig. 2B). Expression of CD86, MHC class I and B7-H1, a putative negative regulator of T cells, was not affected by IL-10 alone either in immature or mature DCs (data not shown).

We now assessed the influence of cmvIL-10 on DC-SIGN expression during DC maturation. For this purpose, immature DCs were activated with LPS, a potent maturation stimulus, in the presence or absence of varying quantities of IL-10. As shown in Fig. 3, the expression of DC-SIGN was decreased on immature DCs treated with LPS alone. In the presence of cmvIL-10 or hIL-10, however, immature DCs exposed to LPS up-regulated DC-SIGN. The maximal effect of cmvIL-10 and hIL-10 on DC-SIGN occurred at a concentration of 25 ng/ml whereas ebvIL-10 did not significantly influence DC-SIGN expression even at concentrations up to 200 ng/ml (data not shown). In comparison, ICAM-1 up-regulation induced by LPS was efficiently blocked in the presence of cmvIL-10 or hIL-10. Interestingly, the expression of DC-SIGN correlated with susceptibility of DCs to HCMV infection (Table I). After LPS-induced maturation, DCs became less susceptible to HCMV infection relative to immature DCs. When simultaneously exposed to cmvIL-10 or hIL-10, LPS-treated DCs retained their susceptibility to infection. In contrast, ebvIL-10 had only a minor effect on the degree of HCMV infection. These results suggest that cmvIL-10 could enhance HCMV-infection by up-regulating DC-SIGN.

Two distinct groups of cmvIL-10-sensitive molecules in DCs

We further explored the effect of cmvIL-10 on molecules that are relevant for DC functions such as Ag presentation, costimulation, adhesion, and tolerance induction. In the presence of cmvIL-10, the LPS-induced up-regulation of classical MHC molecules (MHC class I and class II) was partially blocked, whereas the nonclassical MHC molecule HLA-DM was up-regulated (Fig. 4A). Moreover, the LPS-induced enhancement of costimulatory molecules (CD40, CD80, CD86, B7-H1, B7-DC) was prevented by cmvIL-10 and hIL-10 (Fig. 4B). B7-H1, which can inhibit T cell cytokine production, was also decreased on HUVECs exposed to cmvIL-10 or hIL-10 (data not shown). Interestingly, molecules relevant for cell
adhesion were either blocked (CD11c, CD18, ICAM-1) or enhanced (CD44, DC-SIGN) by IL-10 (Fig. 4C). In addition, we examined the influence of IL-10 on expression ofIDO, which regulates T cell proliferation and survival (27, 28). Cyt fluorometric and Western blot analyses revealed that LPS-exposed DCs up-regulate IDO in the presence of cmvIL-10 or hIL-10 (Fig. 5). The latter observation implies that cmvIL-10 and possibly other viruses regulate IDO in the presence of cmvIL-10 or hIL-10 (Fig. 5). The cmvIL-10-induced up-regulation of IDO on immature DCs treated with LPS and simultaneously with cmvIL-10 did not acquire full stimulatory capacity (Fig. 6C). In addition, we tested the immunosuppressive potential of the various IL-10 molecules in an autologous stimulation system (Fig. 6D). DCs treated with LPS in the presence of either hIL-10, cmvIL-10 or ebvIL-10 and pulsed with tetanus toxoid could not efficiently activate autologous Ag-specific T cells. Finally, production of important cytokines by LPS-treated immature DCs was affected by hIL-10, cmvIL-10, and to a lesser extent, ebvIL-10 (Fig. 7). The production of cellular IL-10 and IL-12 was suppressed whereas secretion of IL-6 was not significantly altered. Taken together, these findings demonstrate that cmvIL-10, hIL-10 and, to a lesser degree, ebvIL-10 interfere with important DC functions lost or acquired during maturation.

**Increased apoptosis of DCs exposed to cmvIL-10 in the presence of an inflammatory signal**

We explored in additional experiments whether cmvIL-10 affects DC survival after exposure to LPS. Unexpectedly, apoptosis of LPS-induced immature DCs increased in the presence of cmvIL-10 and hIL-10, whereas ebvIL-10 had no effect (Fig. 8A). Therefore, we analyzed the mRNA and protein expression of c-FLIP, Bcl-2, and Bcl-xL, which are important apoptosis-regulatory proteins. The c-FLIP protein is found in a short (c-FLIP S, 26 kDa) or long (c-FLIP L; 55 kDa) form. In DCs c-FLIP L is the predominant molecule whereas c-FLIP S is barely detectable (data not shown). Real time quantitative RT-PCR revealed that cmvIL-10 and hIL-10 reduced the maturation-associated increase of c-FLIP L mRNA (Fig. 8B). In addition, Western blot analysis (Fig. 8, C and D) showed that the LPS-induced up-regulation of antiapoptotic c-FLIP L and Bcl-xL protein was blocked by cmvIL-10 and hIL-10, whereas the expression of proapoptotic Bcl-2 was not affected.

**Table II.** Classification of IL-10-sensitive surface molecules on immature DCs

<table>
<thead>
<tr>
<th>Function</th>
<th>Group 1a</th>
<th>Group 2b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigen presentation</td>
<td>MHC class I, MHC class II</td>
<td>HLA-DM</td>
</tr>
<tr>
<td>Costimulation</td>
<td>CD40, CD80, CD86, CD150, B7-H1, B7-DC</td>
<td>ND</td>
</tr>
<tr>
<td>Adhesion</td>
<td>CD11c, CD18, ICAM-1</td>
<td>CD44, DC-SIGN</td>
</tr>
<tr>
<td>Activation/Maturation</td>
<td>CD25, CD83</td>
<td>ND</td>
</tr>
<tr>
<td>Tolerance</td>
<td>ND</td>
<td>IDO</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>CD95</td>
<td>ND</td>
</tr>
<tr>
<td>Complement regulation</td>
<td>CD55</td>
<td>ND</td>
</tr>
<tr>
<td>Other</td>
<td>CD150, CD200</td>
<td>ND</td>
</tr>
</tbody>
</table>

a hIL-10, cmvIL-10 and, to a lesser extent, ebvIL-10 blocked the LPS-triggered upregulation of most of the tested molecules in group 1.
b A small number of molecules on LPS-treated DCs in group 2 showed a different behavior and were enhanced by IL-10.
c ND, No data shown.

**Impaired functional maturation of DCs exposed to cmvIL-10 in the presence of an inflammatory signal**

Having established that DC phenotype is profoundly altered by cmvIL-10 we wanted to investigate cmvIL-10-induced modulation of DC function. Immature DCs are able to capture Ag with high efficiency. With DC maturation this important function decreases. To test the effect of cmvIL-10 on Ag uptake we measured phagocytosis by using FITC-conjugated dextran (Fig. 6A). LPS-treated immature DCs showed a strongly reduced uptake of FITC-dextran relative to untreated immature DCs. In the presence of cmvIL-10 or hIL-10, however, the capacity for Ag uptake was partially restored whereas ebvIL-10 had only a weak effect. We furthermore analyzed the T cell stimulatory capacity of DCs. Proliferation of allogeneic T cells stimulated with DCs that had been treated with LPS in the presence of hIL-10, cmvIL-10, or ebvIL-10 was suppressed (Fig. 6B). Moreover, cytofluorometric analysis of the activation marker CD69 on T cells revealed that allogeneic DCs treated with LPS and simultaneously with cmvIL-10 did not acquire full stimulatory capacity (Fig. 6C). In addition, we tested the immunosuppressive potential of the various IL-10 molecules in an autologous stimulation system (Fig. 6D). DCs treated with LPS in the presence of either hIL-10, cmvIL-10 or ebvIL-10 and pulsed with tetanus toxoid could not efficiently activate autologous Ag-specific T cells. Finally, production of important cytokines by LPS-treated immature DCs was affected by hIL-10, cmvIL-10, and to a lesser extent, ebvIL-10 (Fig. 7). The production of cellular IL-10 and IL-12 was suppressed whereas secretion of IL-6 was not significantly altered. Taken together, these findings demonstrate that cmvIL-10, hIL-10 and, to a lesser degree, ebvIL-10 interfere with important DC functions lost or acquired during maturation.
These results demonstrate that cmvIL-10 and hIL-10 promote apoptotic cell death of DCs during LPS stimulation. Collectively, our data suggest that cmvIL-10 could help HCMV to evade the antiviral immune response by several distinct mechanisms despite its very low amino acid sequence identity with hIL-10.

**Discussion**

IL-10 is exploited by numerous pathogens to prevent their elimination from the host organisms (29). Some are able to induce cellular IL-10 whereas others, like HCMV and EBV, encode their own IL-10 homologues. The functional receptor for IL-10 is composed of two chains called IL-10R1 and IL-10R2 (18). Crystal structure analysis has shown that viral IL-10 homologues have evolved different means to engage IL-10R1 (30). These structural differences could result in altered signal transduction to the viral mimics. Indeed, we found that hIL-10 and cmvIL-10 induced a strong phosphorylation of STAT3, a key mediator of the IL-10 response (25). However, we could not detect activated STAT3 in ebvIL-10-treated immature DCs by using Western blot analysis. These differential signaling strengths mirror binding studies that have revealed a high IL-10R1 affinity of hIL-10 and cmvIL-10, and a comparatively low IL-10R1 affinity of ebvIL-10 (30).

Intriguingly, cmvIL-10 and hIL-10 but not ebvIL-10 up-regulate the expression of DC-SIGN on immature DCs and during LPS-induced DC maturation. It has been shown recently that DC-SIGN is important for DC infection with primary HCMV isolates (26). HCMV attached to DC-SIGN could misuse DCs as vehicles for transmission to permissive cells (26). Several other nonviral and...
viral pathogens have been discovered to target this C-type lectin (13), including Ebola virus (31, 32) and hepatitis C virus (33–35). Moreover, DC-SIGN plays a key role in the dissemination of HIV-1 (36, 37) and dengue virus (38, 39). These findings suggest that cmvIL-10 released during HCMV infection could facilitate propagation of important pathogens by increasing DC-SIGN expression. In contrast to immature DCs we did not detect any significant changes in expression of DC-SIGN or other surface molecules after treating mature DCs with IL-10. Resistance of mature DCs to IL-10 has also been reported recently (40) and could be due to the loss of IL-10R1 expression and reduced IL-10 binding (41).

Based on the reaction of immature DCs to an inflammatory signal (LPS) we could define two groups of IL-10-sensitive surface molecules (Table II). The majority of LPS-induced genes were repressed (group 1). A few functionally important IL-10-sensitive surface molecules, including HLA-DM, IDO, and DC-SIGN were increased by IL-10 (group 2). A similar dichotomy of IL-10-sensitive genes was also found recently in microarray experiments analyzing murine macrophages (42). Classical HLA molecules react to LPS and hIL-10 in a fashion typical of group 1 molecules (40, 41, 43, 44). We observed that cmvIL-10 also efficiently blocks LPS-induced up-regulation of HLA class I and class II molecules on immature DCs. In comparison, the effect of ebvIL-10 was weak. All costimulatory molecules tested also belong to group 1. B7-H1 has been associated with costimulatory (45) as well as with inhibitory (46) function. Nevertheless, B7-H1 responded to LPS and IL-10 like other costimulatory molecules and does not appear to be regulated differentially. Finally, the activation-induced up-regulation of most adhesion molecules (CD11c, CD18, ICAM-1) was repressed by IL-10. Taken together, cmvIL-10 could attenuate immunological synapse formation and T cell signaling by preventing LPS-induced enhancement of molecules involved in Ag presentation, costimulation, and adhesion (47).

Expression of HLA-DM is increased by hIL-10, cmvIL-10, and to a lesser extent, ebvIL-10 on LPS-activated DCs. This nonclassical HLA molecule is part of an unusual extracellular presentation pathway that allows Ag processing and peptide loading outside immature DCs (48, 49). We observed that cmvIL-10 and hIL-10 also increase the expression of IDO on DCs during LPS-induced maturation. IDO plays an important role in the tryptophan catabolism and regulation of the adaptive immunity (27, 28). Production of IDO by human macrophages (50) or human DCs (51) causes depletion of tryptophan thereby suppressing T cell responses. It has been described that IDO-expressing human DCs act as specialized regulatory cells that could play a role in inducing tolerance in vivo (52). Thus, HCMV could subvert the antiviral T cell response through cmvIL-10-mediated enhancement of tolerogenic IDO on DCs. Indeed, previous studies have demonstrated that DCs pretreated with hIL-10 not only show decreased allostimulatory capacity but can also induce T cell anergy (40, 53). Moreover, these anergic cells are able to act as regulatory T lymphocytes that promote Ag-specific tolerance (54, 55). Thus, it is anticipated that DCs exposed to cmvIL-10 can induce T cell anergy and activate tolerogenic regulatory T cells that suppress antiviral T cell responses. In addition, our study revealed that hIL-10 and cmvIL-10 preserve the capacity of DCs for Ag uptake. During infection continuous uptake could enlarge the proportion of viral Ags presented by IL-10-exposed DCs and, therefore, skew tolerogenic activity toward antiviral T cells. These IL-10-mediated mechanisms could act in concert to attenuate memory T cell responses directed against viral Ags thereby delaying elimination of reactivated virus. Supporting this view we found a drastically reduced capacity of cmvIL-10 exposed DCs to activate T cells in allogeneic and autologous stimulation assays.

It has been recently demonstrated that increasing numbers of apoptotic DCs accumulate in the T cell area shortly after LPS-induced maturation (56). Gene expression analyses of mouse DCs indicate that maturation-associated DC apoptosis could be due to a rapid decrease in expression of antiapoptotic Bcl-2 (57, 58). We observed that hIL-10 and cmvIL-10 further enhanced apoptosis in LPS-exposed DCs. This effect was associated with decreased levels of c-FLIP L , an important apoptosis-regulatory protein that interferes with the activation of caspase-8 at the level of the death inducing signaling complex (59, 60). In addition, we detected decreased levels of antiapoptotic Bcl-x L in DCs stimulated with LPS in the presence of cmvIL-10 and hIL-10. Inhibition of c-FLIP L and Bcl-x L up-regulation could render DCs more susceptible to death receptor signaling. In this way, cmvIL-10 could contribute to the increased apoptosis detected after infection of immature DCs with HCMV (16).
In our comparative study we found that cmvIL-10 shapes phenotype, function, and survival of DCs more profoundly than ebvIL-10. For this reason it will be of interest to investigate whether the strong immunosuppressive potential of cmvIL-10 on DCs in vitro translates into efficient treatment of unwanted inflammatory conditions in the clinical setting. In contrast, cmvIL-10 could be a potential novel target for therapy of HCMV-associated disease in immunosuppressed patients.

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References

FIGURE 8. Increased DC apoptosis after LPS stimulation in the presence of cmvIL-10. Immature DCs were left untreated or treated with 1 μg/ml LPS for 2 days in the absence or presence of 25 ng/ml IL-10 as indicated. A, Apoptosis was assessed by staining cells with annexin V and subsequent cytofluorimetric analysis. The degree of apoptosis relative to untreated immature DCs is depicted. B, Levels of c-FLIP L mRNA were measured by quantitative real-time RT-PCR and are given relative to untreated immature DCs. c-FLIP L (C), Bcl-2 (D), and Bcl-xL (D) were detected by Western blot analysis. Expression of β-actin was determined simultaneously to control proper loading of SDS-PAGE gels. The results are derived from three individual experiments with DCs from different donors. Error bars represent SD. The probability was determined in comparison with DCs treated with LPS alone by using the paired Student’s t test (*, p < 0.05, **, p < 0.001).