Herpes simplex virus type 1 ICP0 induces CD83 degradation in mature dendritic cells independent of its E3 ubiquitin ligase function

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Mature dendritic cells (mDCs) are the most potent antigen-presenting cells known today, as they are the only antigen-presenting cells able to induce naïve T-cells. Therefore, they play a crucial role during the induction of effective antiviral immune responses. Interestingly, the surface molecule CD83 expressed on mDCs is targeted by several viruses. As CD83 has been shown to exert co-stimulatory functions on mDCs, its downmodulation represents a viral immune escape mechanism. Mechanistically, it has been shown that herpes simplex virus type 1 infection leads to proteasomal degradation of CD83, resulting in a strongly diminished T-cell stimulatory capacity of the infected mDC. Previous data suggest that the viral immediate-early protein ICP0 (infected-cell protein 0) plays an important role in this process. In the present study, we showed that ICP0 is sufficient to induce CD83 degradation in the absence of any other viral factor. However, the mechanism of ICP0-mediated CD83 degradation is not yet understood. Here, we provide evidence that ubiquitination of lysine residues is, despite the published E3 ubiquitin ligase activity of ICP0, not necessary for CD83 degradation. This finding was underlined by the observation that expression of an ICP0 mutant lacking the E3 ubiquitin ligase domain in mDCs still induced CD83 degradation. Finally, inhibition of E1 activating enzyme using the specific inhibitor 4-(5-nitro-furan-2-ylmethylene)-3,5-dioxo-pyrazolidin-1-yl-benzoic acid ethyl ester did not prevent CD83 degradation. Taken together, our data provide strong evidence that ICP0 alone induces CD83 degradation independent of its E3 ubiquitin ligase function and of the ubiquitin machinery.

INTRODUCTION

Dendritic cells (DCs) are the most potent antigen-presenting cells with the unique ability to induce effective primary immune responses. DCs exist in an immature and a mature state. As immature DCs, they reside in almost all peripheral tissues until they encounter and take up antigen, which leads to their maturation. As a consequence, DCs start to migrate along a chemokine gradient towards the draining lymph nodes where they finally present antigen to resident T-cells. These T-cells become activated and are directed to the sites of infection. During maturation, the expression of MHC class II as well as of co-stimulatory molecules, such as CD40, CD80 and CD86, is upregulated (Banchereau & Steinman, 1998). In addition to the above-mentioned proteins, the expression of the surface molecule CD83 is also strongly induced during maturation, and therefore CD83 is considered one of the best markers of mature DCs (mDCs) (Zhou & Tedder, 1996). Nevertheless, other cell types such as activated B- and T-cells, as well as regulatory T-cells, also express CD83 (Wolenski et al., 2003).

In addition to the membrane-bound form (mCD83), a soluble form of CD83 (sCD83) also exists (Hock et al., 2001, 2004). This soluble form has been shown to ameliorate the symptoms of experimental autoimmune encephalomyelitis, a model for the early inflammatory phase of human multiple sclerosis, in both a prophylactic and a therapeutic setting (Zinser et al., 2004). Moreover, sCD83 has been reported to prevent graft rejection in several murine and rat transplantation models (Bock et al., 2013; Ge et al., 2010; Lan et al., 2010). However, the precise molecular pathways have still to be elucidated.

Several reports have been published regarding the biological function of the mCD83 molecule. Fujimoto et al. (2002) demonstrated that CD83-deficient (CD83\(^{-/-}\)) mice have a block in CD4\(^{+}\) single-positive thymocyte development that results in a selective reduction in peripheral CD4\(^{+}\) T-cells. Whilst WT thymocytes and bone-marrow stem cells that were transferred to CD83\(^{-/-}\) mice were not able to differentiate into mature CD4\(^{+}\) T-cells, CD83\(^{-/-}\) thymocytes and stem cells developed normally in WT mice, demonstrating that expression of CD83 on thymic
epithelial cells influences CD4+ T-cell development in the thymus.

Interestingly, overexpression of CD83 on naïve effector T-cells results in a regulatory phenotype (Reinwald et al., 2008). Transgenic overexpression of CD83 (CD83tg) under the control of a MHC class I promoter on follicular and marginal-zone B-cells has differential consequences. Whilst B-cell receptor signalling was reduced in all subsets, CD83tg marginal-zone B-cells secreted more IL-10 upon TLR4 stimulation, and CD83tg follicular B-cells showed enhanced cell death (Uhde et al., 2013). Knockdown experiments of mCD83 by small interfering RNA technology in DCs led to a significantly reduced in vitro stimulatory capacity of these DCs, thereby suggesting a co-stimulatory function for mDCs (Aerts-Toegaert et al., 2007; Prechtel et al., 2007).

Considering the functional importance of CD83, it is not surprising that different viruses target CD83 upon infection of mDCs (Kruse et al., 2000; Morrow et al., 2003; Sénéchal et al., 2004). The herpesvirus family comprises several highly successful human pathogens. Examples are herpes simplex viruses types 1 (HSV-1) and 2 (HSV-2), varicella-zoster virus (VZV), human cytomegalovirus (HCMV), Epstein–Barr virus and Kaposi’s sarcoma-associated herpesvirus. Common to all herpesviruses is a tripartite replication cycle that is strictly regulated and subdivided into an immediate-early (IE), early (E) and late (L) phase. Moreover, herpesviruses establish lifelong persistence in their host (Whiteley & Roizman, 2001).

It has been reported that infection of mDCs with VZV results in loss of CD83 (Morrow et al., 2003), but the mechanism leading to this loss of cell-surface expression has not been elucidated so far. In contrast, Sénéchal et al. (2004) postulated that HCMV infection of mDCs induced the shedding of a sCD83 molecule into the supernatant shortly after infection. Subsequently, this sCD83 molecule led to a reduction in DC-mediated T-cell stimulation (Sénéchal et al., 2004). In sharp contrast to this postulated HCMV-mediated shedding mechanism, HSV-1 induces the degradation of the CD83 molecule via the cellular proteasome in an infected-cell protein 0 (ICP0)-dependent manner (Kruse et al., 2000; Kummer et al., 2007). Consistent with the small interfering RNA experiments of Prechtel et al. (2007), the loss of CD83 from the cell surface upon infection with HSV-1 results in inhibition of T-cell proliferation and thus in a reduced immune response of the host (Kruse et al., 2000).

ICP0 is one of the most important IE proteins of HSV-1. It stimulates the initiation of lytic infection and is responsible for reactivation of the viral genome, and therefore ICP0 is a key regulator of lytic and latent infections (Everett, 2000; Everett et al., 2004; Hagglund & Roizman, 2004). Beside the activation of gene expression (Everett et al., 1991), ICP0 can function as an ubiquitin E3 ligase. This activity is mediated by its so-called RING finger domain (Boutell et al., 2002; Van Sant et al., 2001). It enables ICP0 to induce proteasome-dependent degradation of several cellular proteins including the centromere proteins CENP-C (Lomonte & Everett, 1999) and CENP-A (Lomonte et al., 2001), as well as the catalytic subunit of the DNA protein kinase (Lees-Miller et al., 1996; Parkinson et al., 1999). Furthermore, ICP0 mediates proteasomal degradation of the promyelocytic leukaemia (PML) protein (Everett et al., 2006; Maul & Everett, 1994) and the SUMO-modified Sp100 protein (Gu & Roizman, 2003), which results in the disruption of ND10 domains (Everett et al., 1998).

The most common mechanism for targeting proteins to the proteasome is the ubiquitination process, which consists of three steps. In brief, ubiquitin is activated by the ubiquitin-activating enzyme E1 in an ATP-dependent manner and is then transferred to a ubiquitin conjugation enzyme, E2. With the help of E3, a ubiquitin-protein ligase, the activated ubiquitin moiety is then attached to a lysine residue on the target protein. Multiple cycles of ubiquitin conjugation lead to the formation of a polyubiquitin chain, which targets the protein substrate to the proteasome for degradation (Glickman & Ciechanover, 2002). However, there is increasing evidence suggesting an additional ubiquitin-independent proteasomal degradation. The HCMV tegument protein pp71, for example, induces ubiquitin-independent degradation of the retinoblastoma (Rb) protein and hDaxx via the cellular proteasome (Hwang & Kalejta, 2007; Kalejta & Shenk, 2003; Winkler et al., 2013).

When we previously reported the ICP0-induced proteasomal degradation of CD83 upon HSV-1 infection, we hypothesized that ICP0, as an E3 ubiquitin ligase, might either directly or indirectly ubiquitinate CD83 and thereby target CD83 to the cellular degradation machinery (Kummer et al., 2007). This was an intriguing conclusion at that time and thus we performed additional experiments to prove or disprove this hypothesis. In the present study, we have shown that ICP0, independent of its E3 ubiquitin ligase activity, is sufficient to induce CD83 degradation, suggesting a ubiquitin-independent proteasomal degradation of CD83.

RESULTS AND DISCUSSION

HSV-1 infection induces proteasomal degradation of CD83 in mDCs

We reported previously that, during HSV-1 infection of mDCs, CD83 is degraded by the cellular proteasome and that ICP0 plays a crucial role in this process (Kruse et al., 2000; Kummer et al., 2007). As ICP0 possesses E3 ubiquitin ligase activity, we hypothesized that CD83 is ubiquitinated by ICP0 and degraded via the proteasome. Therefore, Western blot analyses were performed in order to determine the intracellular concentrations of CD83 after HSV-1 infection in the presence and absence of the proteasome inhibitor MG-132. Mature DCs were mock infected or HSV-1 infected and treated with or without 10 μM MG-132. At 16 h post-infection (p.i.) cells were harvested and

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CD83 expression was analysed by Western blotting (Fig. 1a). HSV-1-infected samples in the absence of MG-132 showed strongly reduced levels of CD83 in comparison with mock-infected cells (Fig. 1a, top panel), whereas the addition of MG-132 largely prevented CD83 downmodulation. The membrane was reprobed with an ICP0-specific antibody to monitor infection efficiency (Fig. 1a, middle panel), and to prove that there is no significant difference in ICP0 expression in MG-132-treated HSV-1-infected samples compared with untreated HSV-1-infected mDCs. In addition, a β-actin-specific antibody was used to verify equal loading (Fig. 1a, bottom panel). To demonstrate that this inhibition was not an MG-132-specific effect, epoxomicin, an alternative and highly specific proteasome inhibitor, was additionally used. Thus, mDCs were infected and treated with the proteasome inhibitors MG-132 (10 μM) or epoxomicin (5 μM) as described above. After 24 h, the cells were harvested and analysed for their CD83 surface expression by FACS. Whilst CD83 surface expression was significantly reduced by HSV-1 infection compared with mock controls, both MG-132 and epoxomicin prevented CD83 degradation (Fig. 1b). These data confirmed previous data that HSV-1-mediated CD83 degradation can be specifically prevented by inhibition of the proteasome.

**ICP0 is sufficient to induce proteasomal CD83 degradation**

Using co-transfection experiments in human embryonic kidney 293T (HEK293T) cells, we demonstrated previously that the HSV-1 IE protein ICP0 plays an important role in the virus-induced CD83 degradation (Kummer et al., 2007). In order to investigate whether ICP0 alone is also sufficient to induce CD83 degradation in mDCs, cells were electroporated with 4 μg GFP-tagged ICP0 expression plasmid or with 4 μg empty vector as a control, using a Lonza Nucleofector. After 16 h of incubation, the cells were harvested and flow cytometric analyses for CD83 surface expression were performed (Fig. 2). ICP0-positive mDCs (Fig. 2; open bars) showed clearly reduced CD83 levels compared with mDCs electroporated with an empty vector (Fig. 2; filled bars). Inhibition of the cellular proteasome by addition of MG-132 again prevented CD83 degradation to a large extent. In order to analyse the specificity of ICP0-induced CD83 downmodulation, we additionally investigated whether ICP0 had an effect on the surface expression of CD86, which was also upregulated on mDCs (Fig. 2). In contrast to CD83, CD86 expression was not influenced by ICP0. These data clearly demonstrated that ICP0-mediated degradation of CD83 in mDCs is specific, as other surface molecules such as CD86 were not affected. Moreover,

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**Fig. 1.** CD83 degradation upon HSV-1 infection of mDCs can be prevented by inhibition of the proteasome. (a) Mature DCs were mock infected or HSV-1 infected and treated with 10 μM MG-132 1 h.p.i. or were left untreated. Cells were harvested after 16 h, separated using SDS-PAGE and analysed for CD83 expression using Western blotting. The upper panel illustrates the total amount of the highly glycosylated CD83 molecule in mock-infected and HSV-1-infected cells without and with proteasome inhibitor treatment. The membrane was reprobed with an ICP0-specific antibody as an infection control (middle panel), and detection of β-actin served as loading control (lower panel). (b) Mature DCs were mock infected or HSV-1 infected and treated with 10 μM MG-132 or 5 μM epoxomicin 1 h.p.i. or were left untreated. CD83 surface expression was analysed by flow cytometry at 16 h.p.i. Mock-infected controls were set to 100% and the relative surface expression is shown. The experiments were performed at least five times with cells from different donors. ***P<0.001 (significant change); NS, non-significant changes (P>0.05).
we showed here for the first time that ICP0 is sufficient to induce CD83 degradation in mDCs in the absence of any other viral factor.

**Lysine residues are dispensable for ICP0-induced CD83 degradation**

Su et al. (2009) reported that murine CD83 expressed on CD4+ T-lymphocytes is downmodulated by GRAIL (gene related to anergy in lymphocytes). Like ICP0, GRAIL also possesses an E3 ubiquitin ligase activity, mediated by its RING finger domain (Anandasabapathy et al., 2003; Su et al., 2009), and ubiquitinates murine CD83, thereby marking it for proteasomal degradation. In addition, this group identified lysines 168 and 183 in the cytoplasmic domain of murine CD83 as essential for CD83 downmodulation (Su et al., 2009), whereas lysine 192 was dispensable for CD83 degradation.

Thus, to determine whether ICP0 also induces CD83 degradation via the ubiquitination of specific lysine residues, we next analysed different variants of CD83 containing specific lysine mutations in their cytoplasmic domain. As endogenous CD83 of mDCs would mask possible effects on CD83 mutants, we performed co-transfection experiments using HEK293T cells. In the first step, 1 μg WT CD83 expression plasmid was co-transfected together with an ICP0-GFP expression plasmid in HEK293T cells. To verify the specificity of ICP0-induced CD83 degradation, a CD86 expression plasmid was co-transfected in parallel, which was cloned into the same vector as CD83, together with an ICP0 expression plasmid as described above. Co-transfection of a CD83 or a CD86 expression plasmid together with an empty vector served as a control. After 24 h of incubation, the cells were harvested and analysed for surface expression of CD83 and CD86 using flow cytometry (Fig. 3a). The presence of ICP0 led to reduced CD83 surface levels compared with cells transfected with an empty vector (Fig. 3a, left). In sharp contrast, CD86 surface expression was not influenced at all by ICP0 (Fig. 3a, right). In addition, total protein levels of CD83 and CD86 in presence or absence of ICP0 were analysed using Western blotting (Fig. 3b, upper panels). These data verified that CD83 was specifically downmodulated by ICP0 whereas CD86 protein levels were not affected. The membrane was reprobed with an ICP0-specific antibody to control transfection efficiency (Fig. 3b, middle panels). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as a loading control (Fig. 3b, lower panels). These data clearly confirmed the findings from the electroporation experiments in mDCs described above.
Therefore, these results underline that HEK293T cells represent an adequate model system to investigate the effect of ICP0 on CD83 degradation.

The cytoplasmic domain of human CD83 contains three lysine residues at its C terminus that are analogous to those present in murine CD83. Therefore, to investigate whether any of these corresponding lysine residues are essential for CD83 degradation, we constructed a CD83 expression plasmid whereby lysine residues at positions 177, 192 and 201 were changed to arginine residues (designated K177/192/201R). This CD83 K177/192/201R expression plasmid was co-transfected into HEK293T cells together with an ICP0 expression plasmid as described above. At 24 h post-transfection, the cells were harvested, the lysates were separated by SDS-PAGE and CD83-specific Western blot analyses were performed (Fig. 4, top panels). As a loading control, the membrane was reprobed with a GAPDH-specific antibody (Fig. 4, bottom panels). The ICP0 expression levels are shown in the middle panels (Fig. 4).

In the presence of ICP0, CD83 K177/192/201R was degraded to a similar degree as the WT CD83. Thus, in sharp contrast to the results reported by Su et al. (2009) regarding GRAIL-mediated CD83 degradation, none of the here mutated lysine residues was essential for ICP0-dependent CD83 degradation. Manifold reasons might account for these differences. First, Su et al. (2009) looked at murine CD4+ T-cells, whereas here we examined human mDCs. Moreover, it is generally difficult to compare effects resulting from viral infections with a physiological process such as T-cell activation.

After showing that the lysine residues in the cytoplasmic domain were not essential, we next investigated whether the cytoplasmic CD83 domain as a whole was required for the ICP0-induced degradation. Therefore, we generated a CD83 mutant lacking the entire cytoplasmic CD83 domain (designated CD83 ET Stop). Co-transfection experiments were performed as described above, and CD83 expression levels were analysed using Western blotting. Surprisingly, also in this case, the mutated CD83 molecule (CD83 ET Stop) was degraded in the presence of ICP0 (Fig. 4, top row). These data strongly indicated that the cytoplasmic domain of CD83 is not required for ICP0-mediated CD83 degradation.

Recently, it has been demonstrated that even a single lysine residue can be sufficient to allow ubiquitination and degradation of a target protein. Moreover, even the position of this lysine was reported not to be important (Setz et al., 2013). Therefore, next we mutated all lysines present in the CD83 protein to arginines (designated CD83 K→R) to investigate whether a single lysine residue present in the entire CD83 protein might be sufficient and/or essential for CD83 degradation. Expression plasmids for WT CD83 or the mutated CD83 K→R were transfected together with an ICP0 expression plasmid or with an empty vector as described above. Unexpectedly, this K→R mutant was also degraded to a similar extent as the WT protein in presence of ICP0 (Fig. 4, top panels). Taken together, these data strongly suggested that CD83 degradation is completely independent of any lysine residue present in the entire CD83 protein.

E3 ubiquitin ligase activity of ICP0 is not required to induce CD83 degradation

It is well known that proteins targeted to proteasomal degradation can be ubiquitinated at residues other than lysines. Ubiquitination has also been shown to take place upon cysteine, serine, threonine and N-terminal methionine residues (Breitschopf et al., 1998). However, mutating all these residues inside the CD83 protein would result in a completely different protein. Therefore, we decided to look at this question from a different angle and hypothesized that, if the E3 ubiquitin ligase activity of ICP0 catalyses the ubiquitination of CD83 and thereby targets the protein to proteasomal degradation, an ICP0 mutant lacking this E3 ubiquitin ligase activity should not induce CD83 degradation. Thus, we next performed co-transfection experiments with an ICP0 RING finger deletion mutant (ICP0-FXE) which consequently has no E3 ubiquitin ligase activity (Boutilier et al., 2002; Lorick et al., 1999). CD83 WT and CD83 K→R expression plasmids were co-transfected either with ICP0 or with ICP0-FXE as described above. Again, both CD83 WT and CD83 K→R were clearly reduced in presence of WT ICP0 (Fig. 5a, b, top panels). Furthermore, co-transfection of ICP0-FXE also resulted in a clear reduction in CD83 levels (both WT and K→R), although...
less pronounced in the case of WT ICP0 compared with the control vector. A Western blot analysis using an ICP0-specific antibody showed that these variations were not due to lower expression levels of ICP0-FXE (Fig. 5a, b, middle panel). In our previous study, we also used this ICP0-FXE mutant, lacking the E3 ubiquitin ligase domain, and also observed only a slight CD83 degradation in comparison with WT ICP0. Considering these data, at that time we consequently hypothesized that, due to the lack of the E3 ubiquitin ligase domain, CD83 was no longer ubiquitinated and thus was not degraded (Kummer et al., 2007). However, the new data, presented here, and the fact that CD83 degradation also takes place in the complete absence of any lysine residues (see Fig. 5), indicates that the RING finger domain is not essentially involved in CD83 degradation.

To investigate this in further detail, we next expressed the ICP0 WT and ICP0-FXE mutant in mDCs, and investigated their effects on endogenous CD83 expression. Mature DCs were electroporated with 4 μg of each ICP0 expression plasmid as described above. Electroporation of an empty vector was used as control. At 16 h after electroporation, the cells were harvested and ICP0-positive cells were analysed for their CD83 surface expression by flow cytometry (Fig. 6, left side). Both ICP0-positive cells (Fig. 6, open bars, left side) and ICP0-FXE-positive cells (Fig. 6, shaded bars, left side) showed a clear and comparable CD83 degradation compared with mDCs electroporated with an empty vector (Fig. 6, filled bars). Again, CD86 surface expression was not modulated by ICP0 WT or by ICP0-FXE. These data clearly demonstrated that the E3 ubiquitin ligase activity of ICP0 is not essential to induce CD83 degradation.

The ubiquitination machinery is not essential for ICP0-induced CD83 degradation

From the fact that the ICP0 E3 ubiquitin ligase activity is not required to induce CD83 degradation one cannot conclude that ubiquitination of CD83 per se, is not needed. However, there is an increasing body of evidence showing that various proteins can indeed be degraded via the cellular proteasome in a ubiquitin-independent manner. In this respect Kalejta and co-workers reported that the HCMV protein pp71 induces a ubiquitin-independent degradation of several cellular proteins including hDaxx (Hwang & Kalejta, 2007) as well as the Rb family of tumour suppressor proteins p105, p107 and p130 (Kalejta & Shenk, 2003).

Thus, next we investigated whether or not ubiquitination is required for HSV-1/ICP0-mediated CD83 degradation. Therefore, infection experiments were performed in the presence or absence of 4-[4-(5-nitro-furan-2-ylmethylene)-3,5-dioxo-pyrazolidin-1-yl]-benzoic acid ethyl ester (PYR-41), a specific inhibitor of the E1 activating enzyme of the ubiquitination cascade, which induces a complete block of the ubiquitination machinery (Yang et al., 2007). mDCs were mock infected or HSV-1 infected and at 5 h p.i. the mDCs were treated with 80 μM PYR-41 or were left untreated. After an additional 16 h, the cells were harvested and CD83 expression was analysed by Western blotting (see Fig. 7). Although there was an overall reduction in protein levels in the presence of PYR-41, there was still a highly significant (P<0.001) difference regarding CD83 expression.
levels between HSV-1-infected and mock-infected cells (Fig. 7a, top panel, and b). The membrane was reprobed with an ICP0-specific antibody to determine infection efficiency (Fig. 7a, middle panel) and with an anti-β-actin antibody to determine equal loading (Fig. 7a, bottom panel). These Western blot results clearly revealed that inhibition of ubiquitination did not prevent CD83 down-modulation.

Recent studies have reported alternative signals targeting proteins to the proteasome as well as possible intrinsic degradation signals making ubiquitination dispensable in specific cases (Schmidtke et al., 2013; Singh Gautam et al., 2012). Due to the observations that proteasomes have a longer evolutionary history than ubiquitin, the existence of ubiquitin-independent proteasomal degradation is quite feasible. Whilst proteasomes are found in all three biological kingdoms, in eukaryotes as well as in all archaea, the ubiquitin system is restricted to eukaryotes (reviewed by Erales & Coffino, 2014).

Although proteasome-dependent, ubiquitin-independent degradation has been described for some proteins, little is known regarding its precise mechanism. One potential mode of action has been described for the Rb protein. Rb degradation is mediated by the Mouse double minute 2 homologue (MDM2), which promotes interaction of Rb with the C8 subunit of the 20S proteasome and thus induces ubiquitin-independent degradation of Rb (Sdek et al., 2005). Accordingly, ICP0 may support the interaction of CD83 with the cellular proteasome via as-yet-unknown, intrinsic degradation signals. Therefore, additional cellular factors, which have to be activated by ICP0, may be involved in CD83 degradation. The fact that the cytoplasmic domain of CD83 is not necessary for the degradation implies that the potential interaction with ICP0 and possibly additional factors occurs before the protein reaches the membrane. This hypothesis is supported by preliminary data showing that CD83 was also degraded in presence of brefeldin A, a drug inhibiting the transport of proteins to the cell surface (data not shown). If this is true, CD83 already present on the membrane could be then degraded during re-internalization according to a postulated treadmill mechanism (Klein et al., 2005).

Taken together, in this study, we demonstrated that: (i) ICP0 is sufficient to induce CD83 degradation in mDCs; (ii) CD83 degradation does not depend on single lysine residues and furthermore is completely independent of any lysine residue present in the entire CD83 protein; (iii) CD83 is also degraded in mDCs when the ICP0-specific RING finger domain, encoding E3 ubiquitin ligase activity, is missing; and (iv) inhibition of the ubiquitination machinery by the addition of PYR-41 does not affect CD83 degradation. These data strongly suggest that ICP0 induces CD83 degradation in a ubiquitin-independent manner.

**METHODS**

**Virus strains, virus preparation and virus titration.** In these studies, the virus HSV-1/17 + CMV-EGFP/UL43 (HSV-1) was used. This virus contains the EGFP marker gene driven by the HCMV R promoter.
promoter and has been inserted into the gene for UL43, which has been described as a non-essential gene (Coffin et al., 1996; Samady et al., 2003). Virus stocks were prepared, and virus titres were determined as described previously (Sodeik et al., 1997).

**Generation of DCs.** PBMCs were isolated from different healthy donors by sedimentation with Lymphoprep (Nycomed Pharma) as described elsewhere (Kummer et al., 2007). Immature DCs were generated in the presence of 800 U granulocyte–macrophage colony-stimulating factor (GM-CSF) ml⁻¹ (CellGenix) and 250 U IL-4 ml⁻¹ (CellGenin). Maturation was induced by adding 10 ng TNF-α ml⁻¹ (Strathmann), 1 μg prostaglandin E2 ml⁻¹ (Sigma-Aldrich); 200 μU IL-1β ml⁻¹ (Strathmann), 40 U GM-CSF ml⁻¹, 1000 U IL-6 ml⁻¹ (Strathmann), and 250 U IL-4 ml⁻¹ to the medium. After an additional 2 days of cultivation, the DCs were mature. The maturation status was controlled by FACS, and mDCs were used for further experiments.

**Infection procedure.** mDCs were infected with the WT HSV-1 strain in a total volume of 300 μl infection medium containing pure RPMI 1640 and 20 mM HEPES at an m.o.i. of 1. The infection procedure was carried out for 1 h at 37 °C in a shaking heating block (Eppendorf) at 300 r.p.m. Afterwards, the cells were transferred to RPMI 1640 containing 1 % autologous serum, 10 mM HEPES, 2 mM l-glutamine, 100 U penicillin ml⁻¹, 100 μg streptomycin ml⁻¹, 40 U GM-CSF ml⁻¹ and 250 U IL-4 ml⁻¹ at a concentration of 0.5 × 10⁶ cells ml⁻¹.

**Inhibitors.** The proteasome inhibitors MG-132 (Enzo Life Sciences) and epoxomicin (Sigma-Aldrich) were added at 1 h.p.i. at a concentration of 10 and 5 μM, respectively. PYR-41 (80 μM) was added at 5 h.p.i.

**Plasmids and constructs.** pEGFP-110wt (ICP0–GFP) and pEGFP-110FXE (ICP0–FXE), containing full-length ICP0 or the RING finger deletion mutant of ICP0, respectively, both fused to EGFP have been described previously (Lomonte & Everett, 1999). These constructs were used throughout the study. Furthermore, the pBS expression vector containing the phosphoglycerate kinase (pgk) promoter and terminator was used for the CD83 as well as the CD86 constructs. The coding sequence for CD83 WT, the CD83 ET Stop mutant lacking the cytoplasmic domain of CD83 and the lysine mutants (K177/192/201R and K⁻R) were synthesized and also cloned into this vector.

**Cell culture and transfection methods.** HEK293T cells were cultured in Dulbecco’s minimal essential medium (Lonza) supplemented with 10 % FCS, 2 mM l-glutamine, 100 U penicillin ml⁻¹, and 100 μg streptomycin ml⁻¹. HEK293T cells were co-transfected with 1 μg CD83 plasmids with 0.5 μg ICP0, ICP0-FXE or empty vector. Transfection of HEK293T cells was carried out with jetPRIME transfection reagent (Peqlab) according to the manufacturer’s recommendations. Cells were harvested at 24 h post-transfection.

**Electroporation of mDCs.** mDCs (2 × 10⁶) were electroporated with 4 μg ICP0–GFP plasmid DNA, 4 μg ICP0–FXE or 4 μg empty vector using an Amaxa Human Dendritic Cell Nucleofector kit (Lonza) and a Nucleofector I electroporation device, according to the manufacturer’s recommendations. After electroporation, the cuvette was immediately flushed with 500 μl warm RPMI 1640 and the mDCs were transferred to a 12-well tissue culture plate containing 600 μl pre-warmed RPMI 1640 supplemented with 2 % human AB serum, 500 U human recombinant IL-4 ml⁻¹ and 800 U human recombinant GM-CSF ml⁻¹.

**Flow cytometry.** The mature surface DC phenotype was analysed by FACS. Infected and transfected as well as electroporated cells were analysed for surface expression of CD83 and CD86 using mAbs (anti-CD83, clone 1B15ε; anti-CD86, clone IT2,2; BD Biosciences).

**Cell lysis and immunoblotting.** Cells (1 × 10⁶) were harvested after 16 or 24 h and solubilized in gel-loading buffer [50 mM Tris/HzCl (pH 6.8), 2 % SDS, 10 % glycerol, 10 % β-mercaptoethanol, 0.1 % bromphenol blue]. After separation using SDS-PAGE, the proteins were transferred to a nitrocellulose membrane, blocked with 5 % (w/v) dry milk and incubated with the primary antibody at 4 °C overnight. After incubation with the appropriate secondary HRP-labelled antibody, detection was performed using an ECL Western blotting substrate (Thermo Fisher Scientific). Quantification of Western blot results was carried out using Bio-1D software (Peqlab). The following primary antibodies were used: anti-CD83 (clone F-5; Santa Cruz Biotechnology), anti-β-actin (clone AC-74; Sigma-Aldrich), anti-ICP0 (clone 11060; Santa Cruz Biotechnology), anti-CD86 (clone HA5.2B7; Immunotech) and anti-GAPDH (clone 6C5; Millipore).

**Statistical methods.** Results are shown as means ± SD. Data were analysed using one-way ANOVA and Bonferroni’s multiple comparison post hoc test to determine the significance or variance in the experimental results obtained. Significance was accepted for P<0.05.

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