Cell Microbiol. Author manuscript; available in PMC 2014 March 15.

Published in final edited form as:
Cell Microbiol. 2013 May ; 15(5): 809–822. doi:10.1111/cmi.12074.

Major histocompatibility complex class-II molecules promote targeting of human immunodeficiency virus type 1 virions in late endosomes by enhancing internalization of nascent particles from the plasma membrane

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Summary

Productive assembly of human immunodeficiency virus type 1 (HIV-1) takes place, primarily, at the plasma membrane. However, depending on the cell types, a significant proportion of nascent virus particles are internalized and routed to late endosomes. We previously reported that expression of human leucocyte antigen (HLA)-DR promoted a redistribution of Gag in late endosomes and an increased detection of mature virions in these compartments in HeLa and human embryonic kidney 293T model cell lines. Although this redistribution of Gag resulted in a marked decrease of HIV-1 release, the underlying mechanism remained undefined. Here, we provide evidence that expression of HLA-DR at the cell surface induces a redistribution of mature Gag products into late endosomes by enhancing nascent HIV-1 particle internalization from the plasma membrane through a process that relies on the presence of intact HLA-DR α and β-chain cytosolic tails. These findings raise the possibility that major histocompatibility complex class-II molecules might influence endocytic events at the plasma membrane and as a result promote endocytosis of progeny HIV-1 particles.

Introduction

Assembly of retrovirus is a complex sequential process that involves convergence of viral structural components and genomic RNA at specialized membrane microdomains. The human immunodeficiency virus type 1 (HIV-1) $Pr55^{gag}$ polyprotein precursor (Gag) is the major virion structural protein and its expression alone is sufficient to generate virus-like

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Supporting information
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particles (VLPs) (Sundquist and Krausslich, 2012). $Pr55^{gag}$ consists of four major domains, matrix (MA or p17), capsid (CA or p24), nucleocapsid (NC or p7) and the late domain, p6. The N-terminal myristoylated MA mediates Gag targeting and membrane binding, whereas CA and NC promote Gag multimerization. In addition, NC contains zinc-finger motifs involved in specific recognition of viral RNA for packaging of the genome. The p6 domain through its PTAP and YP(X)nL/LXXLF motifs recruits several components of the endosomal sorting complex required for transport (ESCRT) machinery, normally involved in cytokinesis and the biogenesis of multivesicular bodies (MVB), to facilitate efficient scission of HIV-1 particles from the plasma membrane (PM) (von Schwedler *et al.*, 2003; Strack *et al.*, 2003).

Human immunodeficiency virus type 1 Gag-mediated assembly and budding has long been considered to take place at the PM at least in T lymphocytes as well as in most model cell lines such as human embryonic kidney (HEK) 293, HeLa and T cell lines (Sundquist and Krausslich, 2012). However, this notion has been challenged by several reports as in these model cells a substantial proportion of Gag was also found to localize in intracellular compartments that express late endosome (LE) or MVB markers (Sherer *et al.*, 2003; Dong *et al.*, 2005; Grigorov *et al.*, 2006; Perlman and Resh, 2006). Furthermore, this intracellular localization of Gag was particularly pronounced in macrophages where very large numbers of mature and budding viral particles are found to accumulate in intracellular vacuolar structures believed to correspond to LE/MVB (Raposo *et al.*, 2002; Pelchen-Matthews *et al.*, 2003). However, these structures were ultimately shown to be deep invagination of the PM still connected to the surface through narrow channels (Deneka *et al.*, 2007; Welsch *et al.*, 2007; Bennett *et al.*, 2009). Studies aimed at defining the itinerary that newly synthesized Gag follows before reaching its cell surface or LE/MVB location have led to different models of Gag-mediated assembly. Indeed, Gag was proposed to be first inserted into late endosomal membranes and either retained or transported further to the PM, depending on the trafficking properties of these compartments (Raposo *et al.*, 2002; Pelchen-Matthews *et al.*, 2003; Sherer *et al.*, 2003; Lindwasser and Resh, 2004; Ono and Freed, 2004; Dong *et al.*, 2005; Grigorov *et al.*, 2006; Perlman and Resh, 2006; Joshi *et al.*, 2009). Alternatively, we (Finzi *et al.*, 2007) and others (Rudner *et al.*, 2005; Harila *et al.*, 2006; 2007; Jouvenet *et al.*, 2006; Neil *et al.*, 2006; Ivanchenko *et al.*, 2009) have provided evidence indicating that newly synthesized Gag is first targeted to the PM where viral assembly and release occur; however, a significant proportion of viral particles that are presumably inefficiently released in the extracellular milieu are internalized and routed to LE/MVB compartments. The extent of viral particle internalization appears dependent on the cell type as well as on viral factors. For instance, the Vpu accessory protein has been found to counteract the antiviral activity of Tetherin (also called BST2, CD317 and HM1.24), a host restriction factor that strongly inhibits HIV-1 release (Neil *et al.*, 2008; Van Damme *et al.*, 2008). Tetherin was found to cross-link nascent viral particles on host cell surface (Perez-Caballero *et al.*, 2009) and to mediate their internalization and targeting to endosomal compartments (Harila *et al.*, 2006; Neil *et al.*, 2006). Some cell types, such as HeLa cells, Jurkat T cells and primary macrophages constitutively express Tetherin, and as a result restrict the release of HIV-1 in a Vpu-dependent manner, whereas other cells, such as HEK 293T or SupT1 T cells do not

express Tetherin and consequently are permissive for HIV-1 release independently of Vpu (Neil *et al.*, 2007; 2008; Van Damme *et al.*, 2008).

Our investigation of host factors influencing HIV-1 Gag trafficking and release led to the finding that major histocompatibility complex (MHC) class-II molecules HLA (human leucocyte antigen)-DR induce a redistribution of Gag into LE/MVB and an augmented detection of mature virions in these compartments upon their stable expression in HeLa cells or transient expression in HEK 293T cells, thus resulting in a marked decrease of HIV-1 particle release (Finzi *et al.*, 2006). The classical heterodimeric MHC-II molecules HLA-DR, -DP and -DQ are composed of two glycosylated α and β chains involved in antigen presentation, and are expressed primarily in antigen-presenting cells, such as macrophages and dendritic cells, but also in activated T lymphocytes (Busch *et al.*, 2000; Baecher-Allan *et al.*, 2006). We previously showed that the effect of MHC-II on Gag localization was restricted to classical MHC-II molecules as HLA-DQ partially recapitulated the effect of HLA-DR, whereas non-classical MHC-II molecules such as HLA-DM and -DO did not have any effect on Gag accumulation in LE/MVB nor on HIV-1 particle release (Finzi *et al.*, 2006). Importantly, this process was found to strictly rely on the presence of the cytoplasmic tails of the HLA-DR α and β chains. In this report we further investigated the mechanism underlying the effect of HLA-DR on HIV-1 Gag relocalization and virus particle release. Our results suggest that expression of HLA-DR at the cell surface induces a redistribution of mature Gag products into LE/MVB by enhancing nascent HIV-1 particle internalization from the PM through a process that is dependent on the presence of intact HLA-DR α and β-chain cytosolic tails.

Results

Inhibition of endocytosis inhibits HLA-DR-mediated accumulation of Gag in LE/MVB

Human leucocyte antigen-DR could mediate a redistribution of Gag by enhancing internalization of nascent virions or assembling complexes from the PM or, alternatively, by promoting direct targeting of Gag to LE/MVB followed by assembly and budding into these compartments. To distinguish between these two possibilities, we inhibited internalization processes from the PM with a dominant negative (DN) mutant of dynamin (Dyn K44A), which is known to interfere with clathrin- and caveolae-dependent endocytosis (Grant and Donaldson, 2009). As expected, the DN Dyn mutant reduced transferrin receptor (TfR) endocytosis as measured by flow cytometry (Fig. S1). HEK 293T cells were co-transfected with plasmids encoding Dyn (K44A) or Dyn wt along with an HIV-1 HxBc2 proviral construct and either an HLA-DR-expressing plasmid (pBud DR) or an empty control vector (pBud). Forty-eight hours post transfection, cells were immunostained with polyclonal anti-p24 and monoclonal anti-HLA-DR Abs and analysed by fluorescence microscopy. In agreement with our previous report (Finzi *et al.*, 2006), 80–90% of Gag-positive cells presented a diffuse Gag staining in the absence of HLA-DR (Fig. 1A column i and Fig. 1B). In contrast, upon HLA-DR expression, a marked redistribution of Gag within large intracellular vesicles was observed in approximately 60% of Gag-expressing cells (Fig. 1A column ii and Fig. 1B). These compartments were positive for HLA-DR (Fig. 1A column ii, merge) and as previously reported, positive for LE/MVB markers CD63, Lamp1 and

lysobisphosphatidic acid (data not shown) (Finzi *et al.*, 2006). Interestingly, upon coexpression of HLA-DR and Dyn (K44A), a significant proportion of HLA-DR-positive cells displayed a drastic reduction of the intracellular punctuate Gag staining; instead, these cells exhibited patches of Gag and HLA-DR at the PM, in addition to the typical diffuse Gag staining, (Fig. 1A column iv). Indeed, quantitative analysis of the different Gag localization patterns revealed that while the percentage of cells that displayed solely an intracellular punctuate phenotype was reduced by ~ 1.5-fold in the presence of Dyn (K44A) (from ~60% in presence of Dyn wt to ~ 40% in presence of the DN mutant), the percentage of cells that exhibited a diffuse/patch Gag staining pattern increased by ~ 1.5-fold [from ~ 40% in presence of Dyn wt to ~ 60% in presence of Dyn (K44A)]. These results suggest that dynamin-dependent endocytosis might be required for intracellular accumulation of Gag in presence of HLA-DR.

To assess whether this relocation of Gag to the PM was sufficient to rescue the defect of viral release imposed by HLA-DR, we coexpressed HxBc2 and HLA-DR in HEK 293T cells in presence of Dyn wt or Dyn (K44A) as described above and analysed viral particle release efficiency by pulse-chase labelling and immunoprecipitation experiments (Fig. 1C and D). Despite an increased Gag localization at the PM in presence of Dyn (K44A), we did not observe a rescue of the virus particle release defect imposed by HLA-DR (Fig. 1D). In fact, HLA-DR still decreased HIV-1 particle release by two- to threefold both in the presence of Dyn wt or DN Dyn (K44A).

Gag late-budding domain is required for HLA-DR-mediated Gag accumulation in LE/MVB

The HIV-1 Gag precursor contains short amino-acid motifs, including a PTAP motif, which is involved in the recruitment of components of the ESCRT machinery. The engagement of this machinery by Gag is required to promote the last step of progeny virion morphogenesis, which involves the scission of virion and cellular membranes (Sundquist and Krausslich, 2012). The intracellular accumulation of fully mature virions mediated by HLA-DR (Finzi *et al.*, 2006) represents a phenotype that is in sharp contrast with Gag late (L) domain defects, which result in accumulation of immature viruses tethered to the PM. Thus, given that intracellular accumulation of Gag and mature virions in the presence of HLA-DR appears to arise by endocytosis, the resulting decrease in virus particle release would occur subsequently to that imposed by L-domain defects. Therefore, we next tested whether recruitment of the ESCRT machinery by Gag or scission of progeny virions from cellular membranes was required for endocytosis of Gag by HEK 293T cells in presence of HLA-DR. To this end, HEK 293T cells were transfected with the HxBc2 proviral construct or its L-domain defective (PTAP−) counterpart, together with empty or HLA-DR encoding plasmids. Two days later, Gag localization was analysed by immunofluorescence microscopy using polyclonal anti-p24 Abs. While HLA-DR expression resulted in the accumulation of WT Gag in large intracellular punctuate structures (Fig. 1A column ii), this HLA-DR-mediated Gag redistribution could not be detected in cells producing PTAP-defective virus particles (Fig. 2). In fact, the distribution of PTAP-defective Gag in presence or absence of HLA-DR was very similar and comparable to the WT Gag control. Therefore, these results support the notion that HLA-DR enhances endocytosis of virus particles that underwent the final scission step from the PM or, alternatively, that the L-domain of Gag is

the region responsive to HLA-DR-mediated relocalization of Gag and mature virions to intracellular compartments.

HLA-DR inhibits the release of divergent retroviral-like particles

To evaluate whether the effect of HLA-DR on HIV-1 particle release involves specific viral structural determinants, we tested the release of HIV-1 and murine leukaemia virus (MLV) VLPs in presence or absence of HLA-DR. To this end, we used an HIV-1 Gag-Pol-Tat-Rev expressing construct (pPAX2) or a MLV Gag-Pol packaging construct (pCIG3N) that we co-transfected with HLA-DR or an empty plasmid. As shown for full infectious HIV-1 particles (Fig. 1C), the release of HIV-1-derived VLPs was decreased by approximately twofold upon HLA-DR expression (Fig. 3). Interestingly, HLA-DR had also the capacity to reduce the release of MLV-derived VLPs (Fig. 3). This inhibition of HIV-1 and MLV VLP release by HLA-DR did not result in a detectable accumulation of mature Gag in cell lysates. In fact, while a decrease of mature cell-associated Gag species (HIV p24; MLV p30) was observed in the context of constructs encoding VLPs, unprocessed Gag levels (HIV p55; MLV p60) were not as much affected, suggesting that some cell-associated mature Gag might be targeted for degradation as a result of their endocytosis in presence of HLA-DR (Fig. 3A). Therefore, HLA-DR-mediated inhibition of virus particle release does not appear to require specific HIV-1 virion structural proteins as a similar inhibitory effect can be observed with VLPs derived from MLV, a widely divergent retrovirus.

HLA-DR enhances the internalization of mature Gag products towards LE/MVB compartments

We next directly evaluated whether HLA-DR-mediated redistribution of HIV-1 Gag in LE/MVB resulted from an internalization process from the PM. To do so, we analysed newly synthesized Gag trafficking in the presence or absence of HLA-DR using a procedure that we developed, which combines a subcellular fractionation method that efficiently separates PM from LE/MVB and pulse-chase radiolabelling (Finzi *et al.*, 2007). Briefly, HEK 293T cells were transfected with the HxBc2 proviral construct together with empty or HLA-DR-expressing vectors. Forty-eight hours post transfection, cells were metabolically labelled with [^{35}S] methionine-cysteine for approximately 10 min and chased for various times prior to cell lysis and subcellular fractionation by optiprep gradient centrifugation. As shown in Fig. S2, expression of HLA-DR did not affect the location of PM-enriched fractions, which were found at the top of the gradient in the presence or absence of HLA-DR. Gag-related products in optiprep fractions were detected by immunoprecipitation using monoclonal anti-p24 Abs. As previously described (Finzi *et al.*, 2007), after 10 min of labelling (time 0), Gag-related products were primarily detected in PM-enriched fractions. However, after 30 min of chase, mature Gag products started to gradually accumulate in fractions enriched in LE/MVB markers (compare fractions 1–6 and 13–14; Fig. 4A, DR−), reflecting progressive internalization of mature and/or maturing viral particles from the PM towards LE/MVB. Interestingly, in HLA-DR-expressing cells, which displayed a marked reduction of virus particle release (Fig. 4B), accumulation of mature Gag products in LE/MVB-enriched fractions started to be detected as early as the end of the pulse-labelling period (time 0) (compare fractions 1–6 and 13–14; Fig. 4A, DR+). Indeed, quantification of the relative amounts of p25 and p24 (p25/p24) mature products in PM- and LE/MVB-

associated fractions at the 30 min chase point indicated that nearly 50% of mature Gag was LE/MVB-associated in presence of HLA-DR compared to only 25% in its absence (Fig. 4C). Altogether, these results strongly suggest that indeed HLA-DR promotes internalization of mature Gag products from the PM towards LE/MVB.

HLA-DR cell-surface expression is necessary to promote internalization of nascent HIV-1 particles

We next evaluated whether HLA-DR needs to be specifically expressed at the cell surface in order to relocate Gag into LE/MVB and inhibit HIV-1 particle release. To address this question, HLA-DR cell-surface expression was modulated through the use of the membrane-associated RING-CH 8 (MARCH-8) E3 ubiquitin (Ub) ligase, an E3 ubiquitin ligase homologous to the K5 viral proteins first identified in Kaposi's sarcoma-associated herpes virus and murine γ herpes virus 68 (Goto *et al.*, 2003; Ohmura-Hoshino *et al.*, 2006a). Indeed, in human and murine immature dendritic cells, cell-surface MHC-II molecules are downregulated through polyubiquitination of the β chain and this modification is required for efficient endocytosis and sorting of MHC-II molecules into luminal vesicles of MVB (van Niel *et al.*, 2006; Shin *et al.*, 2006). We first analysed whether MARCH-8 expression could downregulate HLA-DR cell-surface expression in our experimental system. Cells were transfected with a plasmid encoding HLA-DR together with increasing concentrations of MARCH-8-expressing plasmid (pCS2 MARCH-8myc6) or empty control vector. Forty-eight hours later, cells were either stained for cell-surface HLA-DR or permeabilized and stained for HLA-DR and further analysed by flow cytometry. The data of Fig. 5A reveal that HLA-DR cell-surface expression was significantly decreased in a dose-dependent manner upon MARCH-8 coexpression. In contrast, the levels of HLA-DR detected after cell permeabilization were less affected by the presence of the E3 ligase (note that due to the permeabilization step, the staining efficiency of HLA-DR cannot be compared to non-permeabilized cells). These results indeed confirmed that MARCH-8 induced a cell-surface downregulation of HLA-DR molecules. In fact, consistent with previous data (Ohmura-Hoshino *et al.*, 2006b), we found that HLA-DR accumulated in CD63+ intracellular compartments upon expression of MARCH-8 (data not shown). We then analysed the effect of downregulating cell-surface HLA-DR on Gag localization and virus particle release as described above. Figure 5B reveals that expression of MARCH-8 completely abolished the effect of HLA-DR on Gag intracellular localization, suggesting that cell-surface expression of HLA-DR molecules is required to enhance Gag internalization in intracellular compartments. Indeed, analysis of Gag trafficking in presence of HLA-DR revealed that coexpression of MARCH-8 inhibited HLA-DR-mediated enhancement of mature Gag products internalization (Fig. S3A). Quantification of the total amounts of mature Gag products (p25/p24) associated to high-density fractions after 30 min of chase showed that HLA-DR expression did not induce HIV-1 Gag accumulation into LE/MVB upon coexpression with MARCH-8 (Fig. S3C). Finally, consistent with the results obtained on Gag localization and trafficking, MARCH-8 coexpression abolished the negative effect of HLA-DR on HIV-1 particle release (Figs 5C, D and S3B). Importantly, the levels of HLA-DR detected upon coexpression of MARCH-8 were similar to the control (Fig. 5C, bottom panel), thus indicating that the lack of effect of HLA-DR in presence of the E3 ligase is not due to decreased expression. Altogether, these results suggest that expression of HLA-DR at

the cell surface is necessary to promote internalization of nascent HIV-1 particles from the PM. This data also implies that intracellular HLA-DR accumulation alone is not sufficient to restrict virus particle release.

The cytosolic tails of the HLA-DR α and β chains are required to promote the inhibition of HIV-1 release

We next analysed the domains of HLA-DR that were responsible for the inhibition of HIV-1 release. As our previous results showed that HLA-DR-mediated inhibition of HIV-1 particle release could not be observed with HLA-DR molecules lacking the α and β-chain cytoplasmic tails nor with structurally related but functionally distinct molecules such as HLA-DM or HLA-DO (Finzi *et al.*, 2006), we engineered a series of chimeric molecules harbouring the extracellular and transmembrane (TM) domains of HLA-DM or -DO and the cytosolic domains of DR (Fig. 6A). All chimeric constructs were adequately expressed upon transfection in HEK 293T cells as demonstrated by their detection at the cell surface (DR, DM-DR) or after cell permeabilization in the case of DO-DR as it is well established that HLA-DO luminal domain is misfolded and does not egress from the endoplasmic reticulum (ER) unless HLA-DM is present (Liljedahl *et al.*, 1996; Deshaies *et al.*, 2005) (Fig. 6B). Fusion of the cytosolic domains of the α and β chains of HLA-DR to the TM and extracellular domains of the corresponding chains of HLA-DO (DO-DR) did not inhibit the release of HIV-1 Gag VLPs (Fig. 6C, compare lane 3 with lane 1; quantification in Fig. 6D), consistent with the known property of HLA-DO to be localized mainly in the ER and not at the PM (Liljedahl *et al.*, 1996; Deshaies *et al.*, 2005) (Fig. 6B). In contrast, fusion of the cytosolic tails of the α and β chains of HLA-DR to the TM and extracellular domains of the corresponding chains of HLA-DM restored inhibition of HIV-1 particle, indicating that the cytoplasmic tails of the α and β chains of HLA-DR are indeed necessary and perhaps sufficient to mediate the inhibition of HIV-1 Gag particle release (Fig. 6C, compare lanes 4 and 1 with lanes 2 and 1; quantification in Fig. 6D).

Discussion

Human leucocyte antigen-DR could induce a redistribution of HIV-1 Gag in LE/MVB either by increasing Gag internalization from the PM or by promoting a direct targeting of Gag to LE/MVB. If the first possibility could hypothetically be supported by the fact that a significant pool of MHC-II traffic to LE/MVB via the cell surface (Dugast *et al.*, 2005; McCormick *et al.*, 2005), the second possibility could conceivably result from HLA-DR ability to induce the formation of intracellular compartments reminiscent of LE/MVB (Calafat *et al.*, 1994), which would indeed provide additional internal membrane platforms for Gag assembly.

In this report we provide several lines of evidence indicating that HLA-DR promotes Gag redistribution in late endosomal compartments by enhancing internalization of nascent virus particles from the PM. First, inhibition of dynamin-dependent endocytosis using DN dynamin (K44A) resulted in a significant reduction of the HLA-DR-mediated redistribution of Gag within intracellular compartments (Fig. 1A and B). Notably, treatment of cells with this inhibitor of caveolae- and clathrin-dependent endocytosis was found to cause an

accumulation of Gag at or near the PM, suggesting that Gag molecules relocalizing to MVB in the presence of HLA-DR need to transit through the PM prior to undergoing a dynamin-dependent endocytosis step. Importantly, inhibition of PM to early endosome transport using DN mutant of dynamin did not restore HIV-1 viral particle release (Fig. 1C and D). Although this finding might indicate that HLA-DR-mediated endocytosis may be a downstream consequence of an activity that prevents the efficient release of HIV-1 particle in presence of HLA-DR, as was described for Tetherin-mediated restriction of retroviral particle release (Neil *et al.*, 2006), we cannot rule out the possibility that the block generated by Dyn (K44A) on the incompletely formed endosome may trap virus particles inside and as such may not allow them to access the extracellular medium. Second, using a subcellular fractionation method that separates PM from LE/MVB combined to pulse-chase labelling and immunoprecipitation (Finzi *et al.*, 2007), we monitored the traffic of newly synthesized Gag products in the presence or absence of HLA-DR. This approach was previously used to provide evidence that a significant pool of newly synthesized Gag products were internalized from the PM during assembly and release of HIV-1 from HEK 293T cells and indeed gradually accumulated in LE/MVB (Finzi *et al.*, 2007). Our results show that expression of HLA-DR accelerates the redistribution of newly synthesized Gag products from PM-associated fractions to LE/MVB-enriched fractions where they are primarily detected as mature forms (p25/p24) (Fig. 4). These findings, which support the results obtained with the DN Dyn (K44A) mutant, are consistent with the notion that HLA-DR enhances an internalization process that normally allows a pool of newly synthesized Gag to reach LE/MVB compartments in HEK 293T cells. Third, we provide evidence that this HLA-DR-mediated enhancement of Gag internalization involves virions that underwent a scission between viral and plasma membranes as the PTAP late-budding domain of Gag was found to be required for this process (Fig. 2). Although this finding could also be consistent with the late domain of HIV-1 Gag being the region responsive to HLA-DR-mediated relocalization of Gag, we consider it unlikely as HLA-DR could inhibit the release of MLV VLPs, which indeed consist of Gag that contain a PPXY late domain that is functionally distinct from the PTAP late domain of HIV-1 (Sundquist and Krausslich, 2012) (Fig. 3). Moreover, we found that HLA-DR did not affect the localization of an HIV-1 Gag mutant that preserved a functional late domain but could not multimerize because NC basic amino acids critical for Gag–Gag interactions were mutated (Cimarelli *et al.*, 2000) (data not shown), thus providing additional support for an effect of HLA-DR on the internalization of fully assembled and released virions.

A recent study examined the effect of HLA-DR on HIV-1 Gag distribution and release of infectious HIV-1 particles (Porter *et al.*, 2010). Despite observing similar effect of HLA-DR on Gag distribution and virus particle release, their results differed from ours in that they did not observe the requirement for intact HLA-DR α and β-chain cytoplasmic tails for the induction of Gag accumulation in intracellular compartments. Furthermore, in contrast to our initial results they found that HLA-DM, which is structurally related but functionally distinct from HLA-DR, could also induce a redistribution of Gag in intracellular compartment. These results led the authors to propose that transient overexpression of HLA-DR could induce a retention of Gag in intracellular compartments. This retention of Gag in intracellular compartments and the resulting reduction of virus particle release would be the

consequence of altered trafficking of Gag by overexpression of HLA-DR or other class-II antigen presentation pathway components. The data obtained in the present study are not consistent with such a non-specific intracellular retention model. First, we provide evidence that HLA-DR imposes its effect on Gag distribution at a post-assembly step, rather than early on following Gag neo-synthesis. In fact, if expression of HLA-DR was simply retaining Gag molecules en route for the cell surface, we would have detected this retention of Gag with late domain-defective Gag molecules or with Gag molecules that are unable to assemble because of mutation of NC basic amino acids. On the contrary, we found that these Gag mutants alleviated the effect of HLA-DR on Gag relocalization and viral particle release (Fig. 2). Second, our data showing that MARCH-8-mediated intracellular accumulation of HLA-DR in CD63+ compartment is not sufficient to induce Gag redistribution in LE/MVB are difficult to reconcile with an intracellular retention mechanism (Fig. 5). Last, our previous (Finzi *et al.*, 2006) and current results (Fig. 6) supporting a critical role of HLA-DR α and β-chain cytosolic tails in the HLA-DR-mediated inhibition of HIV-1 release argue against a non-specific effect resulting solely from protein overexpression. It is unclear why our results regarding the requirement for intact HLA-DR α and β-chain cytosolic tails for HLA-DR-mediated redistribution of Gag (Finzi *et al.*, 2006) differ from those of Porter *et al.* (2010). Perhaps, slight differences in the way the cytoplasmic tails of HLA-DR were truncated (complete truncation in our case) and our use of bicistronic constructs to express heterodimers rather than plasmids expressing individual α and β chains might potentially explain the discrepancies between our results.

How could HLA-DR mediate internalization of progeny virus particles from the cell surface? We observed that downregulation of cell-surface HLA-DR by overexpression of the MARCH-8 E3 ubiquitin ligase correlated with a strong reduction of HLA-DR-mediated Gag redistribution to intracellular compartments and a restoration of efficient virus particle release (Figs 5 and S3). This finding suggests that expression of HLA-DR at the cell surface might be required to mediate internalization of progeny virus particles. One possible model to explain this activity would be that HLA-DR molecules directly or indirectly induce a retention of progeny virions at the PM that leads to their endocytosis, a mechanism reminiscent of the Tetherin-mediated restriction of retroviral particles (Neil *et al.*, 2006; 2008; Perez-Caballero *et al.*, 2009). However, analysis of the domains of HLA-DR that are involved in inhibiting virus particle release reveals that the intracytoplasmic domain of the heterodimer molecule is necessary and perhaps sufficient to mediate this effect (Fig. 6). This finding makes it less likely that the extracellular domains of HLA-DR contribute to the retroviral release inhibitory activity of the heterodimer molecule, for instance through an interaction with a virion-associated cellular protein/polypeptide or a lipid component of the viral membrane, although we cannot rule out this possibility. As the effect of HLA-DR is observed on a widely divergent retrovirus (Fig. 3) and implicates the intracytoplasmic tails of the α and β chains, another possibility would be that HLA-DR mediates retro-viral particle endocytosis through a modification of the cellular environment. As eluded above, HLA-DR expression could induce the cell-surface expression of a Tetherin-like host factor. In that regard, we did not detect any induction of cell-surface Tetherin by HLA-DR in HEK 293T cells, which usually do not express the restriction factor (Neil *et al.*, 2008) and the inhibitory effect of HLA-DR on virus particle release was also observed with Vpu-

expressing proviral constructs (data not shown). These results rule out that Tetherin is a factor responsible for the effect of HLA-DR on HIV-1 particle release. Alternatively, expression of HLA-DR at the cell surface could directly modify endocytic processes at the PM via the α and β-chain cytoplasmic tails and as a result promote endocytosis of progeny HIV-1 particles. In that regard, the HLA-DR β-chain cytoplasmic tail was reported to be capable of transducing intracellular signals and shown also to play a critical role in the association of HLA-DR molecules with the cytoskeleton (Bouillon *et al.*, 2003; El Fakhry *et al.*, 2004). As HLA-DR expression was not found to increase the endocytosis of surface proteins such as the TfR and MHC-I molecules (data not shown), a general effect of HLA-DR on endocytosis appears excluded. Clearly, more studies will be required to fully understand the mechanism through which MHC-II molecules enhance the internalization of HIV-1 particles at the PM.

In conclusion, our results suggest that expression of HLA-DR at the cell surface induces a redistribution of mature Gag products into LE/MVB by enhancing nascent HIV-1 particle internalization from the PM through a non-virus specific process that is dependent on the presence of HLA-DR α and β-chain cytosolic tails. These findings raise the possibility that MHC-II molecules might influence endocytic events at the PM in MHC-II-expressing cells, such as macrophages and dendritic cells, and as a result promote endocytosis of progeny HIV-1 particles. Whether MHC-II molecules-mediated targeting of infectious viral particles in LE/MVB compartments modulates cell-to-cell viral transmission and/or promotes viral antigen presentation in antigen-presenting cells remains an important question that will need to be addressed in future studies.

Experimental procedures

Cells and plasmids

Human embryonic kidney 293T cells were obtained from the American Tissue Culture Collection (ATCC) and were maintained as described (Levesque *et al.*, 2003). The proviral construct encoding the HIV-1 infectious molecular clone, HxBc2 was previously described (Finzi *et al.*, 2006), while its derivative harbouring mutations in the PTAP late motif of Gag was obtained from Dr Heinrich Göttlinger (University of Massachusetts, Worcester) (Gottlinger *et al.*, 1991). The bicistronic expression plasmid encoding HLA-DR (pBud-DR) α and β chains and the empty control vector (pBud) were previously described (Finzi *et al.*, 2006). The plasmid constructs encoding chimera between HLA-DO and HLA-DR (DO-DR) or HLA-DM and HLA-DR (DM-DR) were generated by overlap PCR-based mutagenesis. The DO-DRα and DO-DRβ chimeric chains include the luminal and TM parts of DO fused to the cytoplasmic domains of the corresponding DR chains. The sequence around the α chain junction is TVLII/KGVRK. The sequence around the β chain junction is IQLR/NQKG. The two chimeric chains were cloned into the same pBud vector. The DM-DRα and DM-DRβ chimeric chains include the luminal and TM parts of DM fused to the cytoplasmic domains of the corresponding DR chains. The sequence around the α chain junction is IVLII/KGVRK. The sequence around the β chain junction is VISW/YFRN. The two chimeric chains were cloned into the same pBud vector. MLV Gag-Pol (pCIG3N) was described in Bock *et al.* (2000), while the plasmid encoding HIV-1 Gag-Pol-Tat-Rev

(pPAX2) was a kind gift of Dr D. Trono (University of Lausanne, Lausanne, Switzerland). The pCS2 MARCH-8myc6 construct and the empty vector (pCS2) (Rupp *et al.*, 1994) were kindly provided by Dr V. Steimle (University of Sherbrooke, Sherbrooke, Canada), while plasmids encoding Dyn wt as well as the DN mutant Dyn (K44A) were provided by Dr J.P. Gratton (IRCM, Montreal, Canada).

Antibodies, chemicals and other reagents

The following antibodies (Abs) were used: anti-p24 monoclonal Abs were isolated from supernatants of cultured hybridoma cells (catalogue no. HB-9725) obtained from the ATCC (Manassas, VA, USA); rabbit anti-p24 polyclonal Abs were obtained through the NIH AIDS Research and Reference Reagent Program (catalogue no. 4250); goat anti-p30 MLV polyclonal Abs were a kind gift of Dr P. Jolicoeur (IRCM, Montreal, Canada); L243 ($IgG2_a$) is a mouse monoclonal Ab that binds a specific HLA-DR conformational epitope dependent on the correct conformation of the α/β heterodimer (Panina-Bordignon *et al.*, 1992); DA6.147 (IgG_1) is a mouse monoclonal Ab that binds the cytoplasmic tail of HLA-DRα; Map.DM1 and Mags.DO5 are mouse monoclonal IgG1 Abs obtained from Dr L.K. Denzin (Memorial Sloan-Kettering Cancer Center, New York), which are specific for the luminal part of HLA-DM and HLA-DO respectively; rabbit polyclonal anti-actin Abs were purchased from Sigma-Aldrich, while anti-human CD71 (TfR) monoclonal antibodies (clone OKT9) were obtained from eBioscience.

Transfections and metabolic labelling

Human embryonic kidney 293T were transfected by the calcium phosphate method as described previously (Finzi *et al.*, 2006). All analyses were performed 48 h post transfection. For pulse-chase experiments, transfectants were starved for 30 min and then metabolically labelled with 1 mCi ml^{-1} [^{35}S] methionine-cysteine ([^{35}S] Protein Labeling mix, Perkin Elmer, Wellesley, MA, USA) in DMEM lacking methionine and cysteine and supplemented with 5% dialysed fetal bovine serum for short period of times (10–30 min). Labelled cells were then chased for different time intervals in DMEM containing excess of unlabelled methionine and cysteine. For regular metabolic labelling, transfectants were processed as described above except that cells were exposed to 1 mCi ml^{-1} [^{35}S] methionine-cysteine ([^{35}S] Protein Labeling mix) for 16 h prior to lysis and immunoprecipitation.

Subcellular fractionation, immunoprecipitation and virus particle release assay

The subcellular fractionation method as well as the analysis of released viral particles was previously described (Finzi *et al.*, 2007). Immunoprecipitations of subcellular fractions or lysates from whole cells and pelleted virus were performed as follows: samples were pre-cleared with mouse serum for 2 h at 4°C before being immunoprecipitated for 3 h at 4°C with mouse monoclonal anti-p24 Abs. Immunocomplexes were separated on a 12.5% SDS-PAGE glycine gel and analysed by autoradiography. Quantification of radioactive Gag-related bands was performed using a PhosphorImager equipped with the ImageQuant software 5.0. Virus particle release efficiency was calculated as a ratio of the p24 signal detected in the supernatant relative to the total Gag signal detected in cells (p55, p41 and p24/25) and in the supernatant (p24).

Immunostaining, microscopy and flow cytometry

Immunostaining was performed as described (Finzi *et al.*, 2006). Samples were examined by conventional epifluorescence micrographs on a Zeiss Cell Observer system (Zeiss, Toronto, ON, Canada) equipped with an Axiovert 200 M microscope using an 100× oil lens. Images were digitally deconvoluted with the Axio-Vision 4.1 software using the Nearest Neighbour deconvolution method. For some experiments, samples were analysed by confocal microscopy using a LSM710 laser scanning confocal microscope (Zeiss). Flow cytometry analysis was performed as described previously (Brunet *et al.*, 2000).

Internalization assay

Transfected cells were washed in PBS, resuspended in PBS containing anti-TfR Abs and incubated for 45 min at 4°C. Following washes in cold PBS, cells were incubated at 37°C in DMEM medium supplemented with 5% FBS for different time intervals. At each time point, cells were harvested, washed in cold PBS and stained with appropriate fluorochrome-coupled secondary Abs and pre-coupled anti-HLA-DR Abs (L243) for 30 min at 4°C. HLA-DR-positive cells were analysed for cell-surface TfR expression by flow cytometry.

Statistical analysis

Comparison between groups was performed with Student's *t*-test using the Sigmaplot 6.0 software. Data were expressed as means ± SD and *P*-values ≤ 0.05 were considered to be significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Pierre Guiot-Guillain, Mathieu Dubé and Mariana Bego for their scientific input and help in the preparation of the manuscript as well as Johanne Mercier for technical assistance. The HIV-1_{SF2} p24 rabbit antiserum was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. E. A. C. is recipient of the Canada Research Chair in Human Retrovirology. This work was supported by a grant from CIHR to E. A. C. and J. T.

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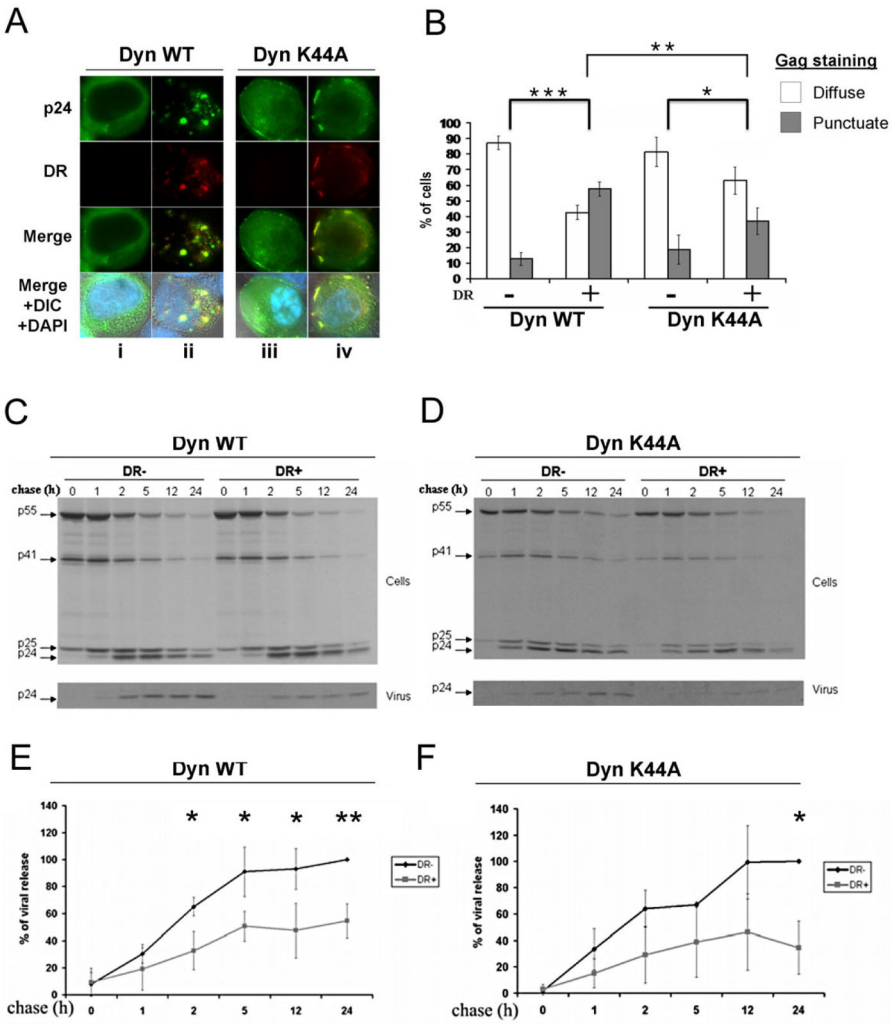

Fig. 1.

Effect of inhibition of endocytosis on HLA-DR-mediated Gag redistribution and reduction of viral particle release. HEK 293T cells were mock-transfected or co-transfected with plasmids encoding HLA-DR together with the HxBc2 provirus in presence of Dyn wt or Dyn K44A. Two days later, cells were immunostained using polyclonal anti-p24 and monoclonal L243 anti-HLA-DR Abs and analysed by epifluorescence microscopy (A). In the absence of HLA-DR (pBud, empty vector) (i), Gag displays a diffuse staining, whereas HLA-DR expression redirects Gag to large intracytoplasmic vesicles (punctuate staining) where it colocalizes with HLA-DR (ii). Dyn (K44A) expression induces a slight accumulation of Gag at the PM in addition to diffuse Gag staining (iii) and this effect was enhanced in the presence of HLA-DR (diffuse Gag staining and presence of Gag patches at the PM) (iv). Quantification of Gag-associated localization patterns (B). The number of cells displaying a diffuse (including patches at the PM) versus an intracellular punctuate Gag staining was evaluated in 200 cells per sample. Data shown represent the average of five independent experiments ± SD. Alternatively, cells were metabolically labelled with [^{35}S]Met/Cys for 30 min, and Gag-associated products in cell and virion lysates were immunoprecipitated with monoclonal anti-p24 Abs at different chase intervals in presence of

Dyn WT (C) or DN Dyn (K44A) (D). Data shown are representative of three independent experiments. Quantification of data presented in C and D respectively (E and F). Virus particle release (%) was calculated as the fraction of mature Gag signal (p24) detected in the supernatant relative to the total Gag signal (mature and immature Gag) detected in cells and the supernatant. Data represent the average ± SD of three independent experiments. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ by using Student's *t*-test. *P*-values ≥ 0.05 were considered to be not significant.

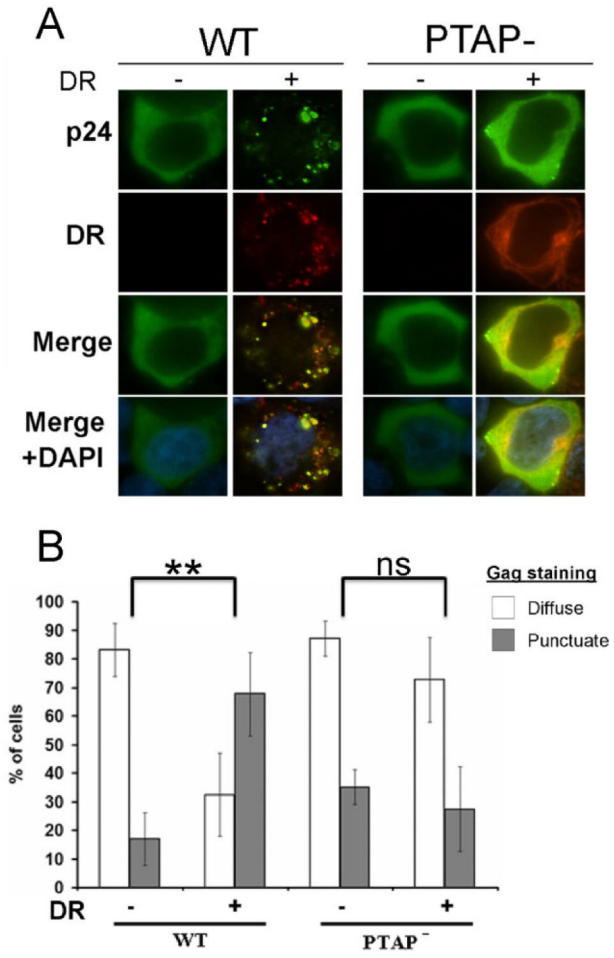

Fig. 2.

Intracellular relocalization of Gag induced by HLA-DR requires a functional Gag late domain. HEK 293T cells were transfected with HxBc2 (WT) or a variant lacking a functional PTAP motif (PTAP−), together with HLA-DR-expressing plasmid (DR+) or empty vector (DR−). Transfected cells were immunostained with rabbit polyclonal anti-p24 Abs and analysed by epifluorescence microscopy (A). Diffuse or punctuate Gag-associated staining patterns were quantified in 200 cells per sample (B). The data shown are means ± SD of three independent experiments. $^{**}P \leq 0.01$ by using Student's *t*-test. *P*-values ≥ 0.05 were considered to be not significant.

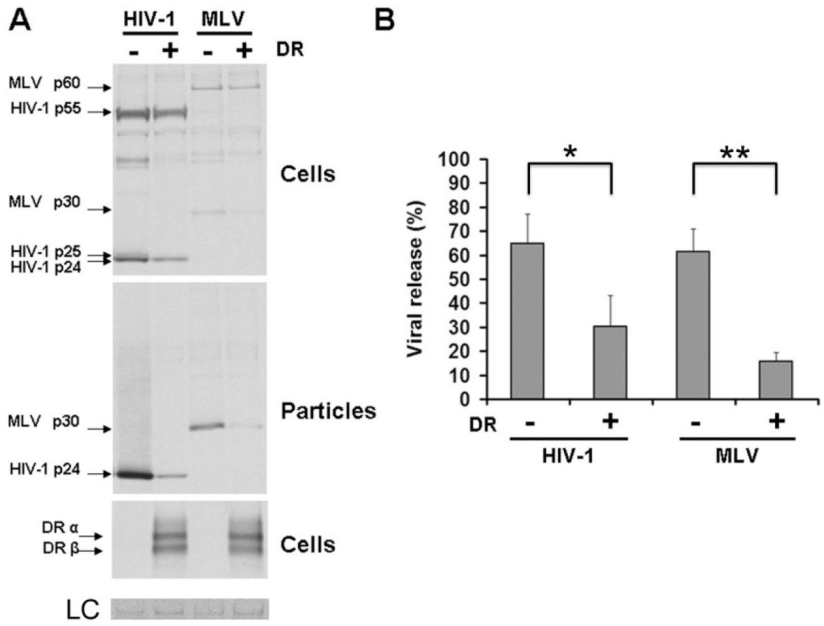

Fig. 3.

HLA-DR-induced inhibition of virus particle release is not restricted to HIV-1. HEK 293T cells were transfected with an empty (DR−) or HLA-DR (DR+) encoding plasmid together with a construct encoding HIV-1 Gag-Pol-Tat-Rev (PAX2) or MLV Gag-Pol (pCIG3N). (A) Following radiolabelling, lysates from cells and pelleted virus particles were immunoprecipitated with mouse monoclonal Abs against HIV-1 p24 or anti-p30 MLV polyclonal Abs. Cell lysates were also sequentially immunoprecipitated with mouse monoclonal anti-HLA-DR Abs. Immunocomplexes were resolved by PAGE. A non-specific cell-associated band observed after the immunoprecipitation was used as loading control (LC). Data shown are representative of three independent experiments (B). Virus particle release (%) was calculated as the fraction of mature Gag signal detected in the supernatant relative to the total Gag signal (mature and immature Gag) detected in cells and the supernatant. Data shown represent the average of three independent experiments ± SD. $*P \leq 0.05$, $**P \leq 0.01$, by using Student's *t*-test. *P*-values ≥ 0.05 were considered to be not significant.

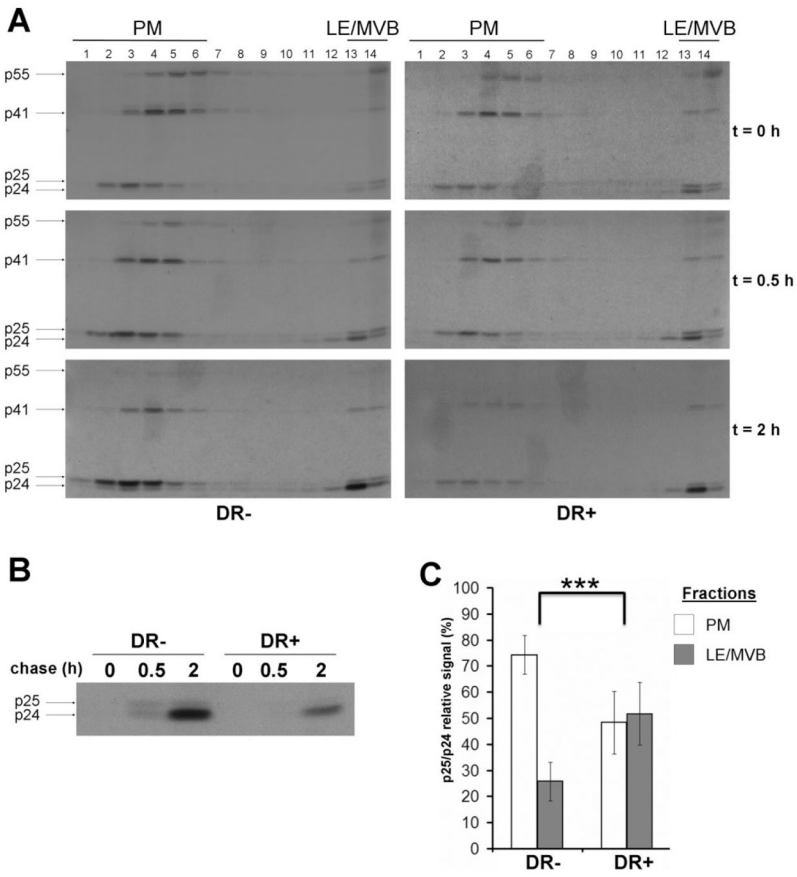

Fig. 4.

HLA-DR increases the internalization of mature Gag products from the PM towards LE/MVB. HEK 293T cells were transfected with the HxBc2 provirus together with empty (DR −) or HLA-DR (DR+) vectors. Two days after transfection, cells were metabolically labelled with [^{35}S]Met-Cys for 10 min and chased for various times prior to cell lysis and subcellular fractionation by optiprep gradient centrifugation. Fourteen fractions were collected from the top of the gradient and Gag-related products in each fraction were detected by immunoprecipitation using monoclonal anti-p24 Abs (A). HIV-1 particles released during the pulse-chase analysis were pelleted, lysed and immunoprecipitated as in A (B). (C) Quantification of the relative amounts of mature Gag products (p25 and p24) detected in PM- and LE/MVB-associated fractions (1–6 and 13–14 respectively) after 30 min of chase. Data from three independent experiments were quantified using a PhosphorImager equipped with an ImageQuant software 5.0 and are shown as means ± SD. ***$P \leq 0.001$ by using Student's *t*-test. *P*-values ≥ 0.05 were considered to be not significant.

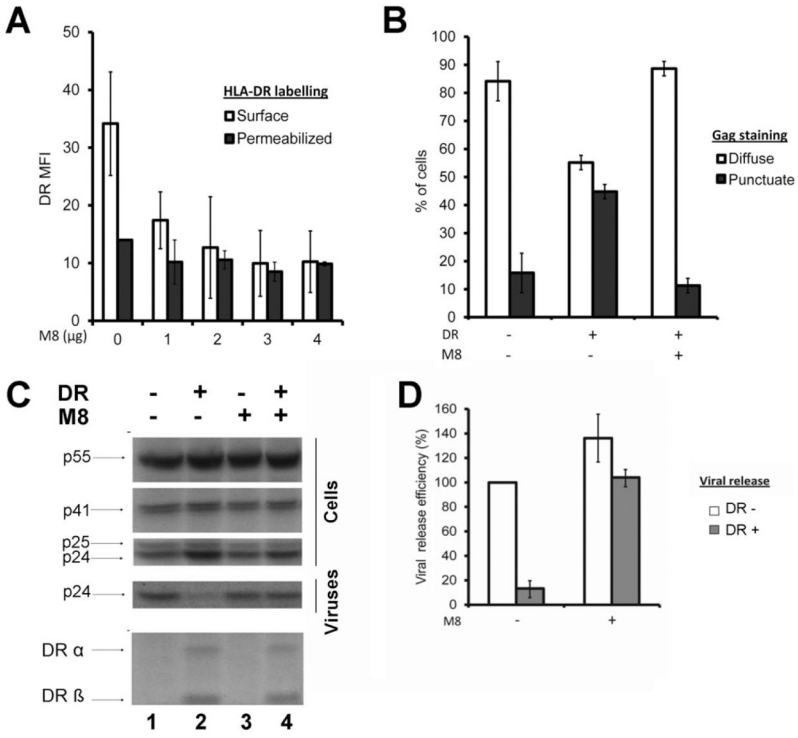

Fig. 5.

HLA-DR expression at the cell surface is required to affect Gag distribution and particle release. HEK 293T cells were transfected with HLA-DR or empty vector together with increasing amounts of a vector encoding the MARCH-8 (M8) E3 ligase (pCS2 MARCH-8myc6).

A. Transfected cells were either cell-surface labelled with monoclonal anti-HLA-DR Ab L243 (surface) or permeabilized with saponin before labelling with anti-HLA-DR Ab L243 to measure total HLA-DR expression (permeabilized). The graph depicts the absolute mean fluorescence intensity (MFI) values detected at the cell surface or after cell permeabilization and is representative of two independent experiments ± SD. The MFI value obtained with the isotype control was subtracted for each condition. Note that the MFI values obtained in permeabilized and non-permeabilized cells cannot be compared as the staining efficiency is different in the two conditions.

B. Gag localization was evaluated in cells co-transfected with the HxBc2 provirus and plasmids encoding HLA-DR or/and the MARCH-8 E3 ligase (M8). The number of cells displaying a diffuse versus a punctuate Gag staining was evaluated in 200 cells per sample. Data shown represent the average of three independent experiments ± SD.

C. Cells were metabolically labelled 48 h post transfection with [^{35}S]Met-Cys for 2 h. Lysates from cells and pelleted virus were immunoprecipitated with mouse monoclonal Abs against p24. Cell lysates were also sequentially immunoprecipitated with mouse monoclonal anti-HLA-DR Abs.

D. Virus particle release efficiency (%) was calculated as the ratio of mature Gag signal (p24) detected in the supernatant relative to the total Gag signal (mature and immature Gag) detected in cells and the supernatant. Virus release efficiency in the absence of HLA-DR and

MARCH-8 was set at 100%. The data shown are representative of two independent experiments ± SD.

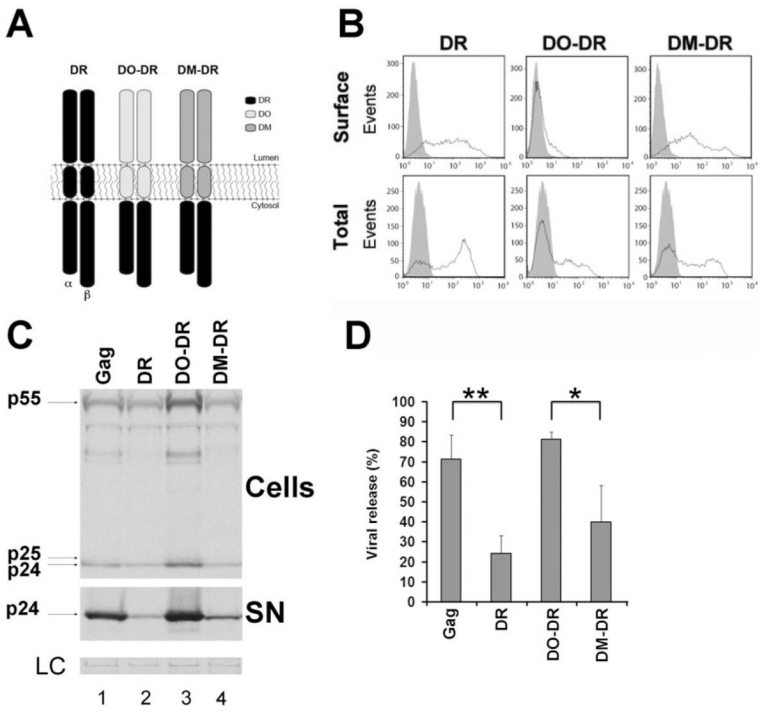

Fig. 6.

HLA-DR α and β-chain cytosolic tails are necessary to mediate inhibition of HIV-1 particle release. Schematic representation of HLA-DR chimeric proteins (A). HEK 293T cells were transfected with a construct encoding HIV-1 Gag-Pol-Tat-Rev (PAX2) together with an empty plasmid (DR-) or constructs encoding HLA-DR or HLA-DR chimeric proteins. Forty-eight hours post transfection, cells were either cell-surface labelled with specific monoclonal Abs (L243, for HLA-DR, Mags.DO5 for HLA-DO or Map.DM1 for HLA-DM) (surface) or permeabilized with saponin before labelling with the DA6.147 monoclonal Ab (binds the cytoplasmic tail of HLA-DRα) to measure total expression of all chimeric proteins (total) (B). Following radiolabelling, lysates from cells and pelleted virus particles (SN) were immunoprecipitated with mouse monoclonal Abs against HIV-1 p24. Immunocomplexes were resolved by PAGE. A non-specific cell-associated band observed after the immunoprecipitation was used as loading control (LC) (C). Data shown are representative of three independent experiments. Virus particle release (%) was calculated as the fraction of mature Gag signal (p24) detected in the supernatant relative to the total Gag signal (mature and immature Gag) detected in cells and the supernatant (D). The data shown are representative of three independent experiments ± SD. $*P \leq 0.05$, $**P \leq 0.01$, by using Student's *t*-test. *P*-values ≥ 0.05 were considered to be not significant.