

# **Distinct Intracellular Trafficking of Hepatitis C Virus in Myeloid and Plasmacytoid Dendritic Cells**

**Mélanie Lambotin<sup>1,2</sup>, Thomas F. Baumert<sup>1,2,3\*</sup> and Heidi Barth<sup>1,2\*</sup>**

<sup>1</sup>Inserm U748, Strasbourg; <sup>2</sup>Université de Strasbourg; <sup>3</sup>Pôle Hépato-digestif, Hôpitaux Universitaires de Strasbourg, Strasbourg, France

\* Corresponding authors: Heidi Barth, Inserm U748, 3 Rue Koeberlé, 67000 Strasbourg, France, Phone: (+33) 3 68 85 37 09, E-mail: [barth@unistra.fr](mailto:barth@unistra.fr) and Thomas Baumert, Inserm U748, 3 Rue Koeberlé, 67000 Strasbourg, France, Phone: (+33) 3 68 85 37 03, E-mail: [Thomas.Baumert@unistra.fr](mailto:Thomas.Baumert@unistra.fr)

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1   **Abstract**

2   Dendritic cells (DCs) are of pivotal importance for the initiation of immune responses to  
3   control and eliminate viral infections. The molecular mechanisms of hepatitis C virus (HCV)  
4   antigen uptake and processing by blood DCs are poorly defined. Here we show that human  
5   blood DC subsets acquire HCV independent of the classical HCV entry factors. Following  
6   HCV uptake, human plasmacytoid and myeloid DC subsets deliver HCV antigen into distinct  
7   endocytotic compartments, which are dedicated to presentation to CD4+ or CD8+ T cells.  
8   Our findings support a model of HCV antigen processing and presentation in which DC  
9   subsets fulfil distinct functions.

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Dendritic cells (DCs) are crucial for the initiation of anti-viral immunity. As sentinels of the immune system, DCs capture viral antigen and present them to naive T cells, eliciting immunity. Two distinct pathways are known for the presentation of antigenic peptides on major histocompatibility complex (MHC) molecules. Exogenous antigens are processed and loaded on MHC II molecules to activate CD4<sup>+</sup> T cells. In contrast, endogenous antigens are presented by MHC I molecules and activate cytotoxic CD8<sup>+</sup> T cells. Additionally, some forms of exogenous antigen can gain access to the MHC I presentation pathway (19). This process, known as 'cross-presentation', is crucial in the initiation of antiviral immunity when DCs are not directly targeted by viruses, or when an infecting virus compromises DC function. Two major DC subsets exist in human: myeloid (mDCs) and plasmacytoid DCs (pDCs). Myeloid DCs are more efficient in priming T cell responses, while pDCs are the most potent producer of type I interferons (IFN- $\alpha/\beta$ ) upon viral infection (27).

Among the multiple blood-borne viruses, hepatitis C virus (HCV) has created a global health problem of substantial proportions. HCV is a major cause of chronic liver inflammation worldwide and the leading indication for liver transplantation in developed countries. Therapy for HCV-related chronic hepatitis is limited and a vaccine protecting against HCV infection is currently unavailable (26). In those individuals who clear the acute infection a robust and sustained CD4<sup>+</sup> and CD8<sup>+</sup> T cell response to multiple HCV epitopes is readily detectable in the blood suggesting efficient HCV-specific T cell priming by DCs. Although individuals who develop chronic infection can mount early, vigorous effector T cell responses, these responses are transient or narrowly focused (17). Recent *in vitro* studies indicated that HCV is a weak inducer of IFN- $\alpha$  and impairs TLR-9 mediated IFN- $\alpha$  production in plasmacytoid DCs (6, 12, 18) suggesting that HCV attenuates the early innate immune response. Although several *ex-vivo* studies demonstrated impaired DC-mediated cytokine production and maturation in patients with chronic HCV infection (reviewed in (11)) there is little evidence for global immune dysfunction in these patients. In this context, with a view to mapping the contribution of the DC in initiating a HCV-specific cellular response, we decided to undertake

an investigation into a poorly understood aspect of DC biology, namely the pathways through which human blood DC subsets internalize circulating HCV, and the subsequent intracellular antigen trafficking following HCV uptake by human blood DC subsets.

To elucidate pathways by which human blood DC subsets acquire HCV, we purified pDCs and mDCs from the blood of healthy subjects and analyzed the expression of putative HCV entry factors on the DC surface, such as the tetraspanin CD81, the scavenger receptor class B type I (SR-BI) and the tight-junction protein claudin-1 (CLDN1) (4). Freshly isolated pDCs (BDCA 2<sup>pos</sup>/CD11c<sup>neg</sup>/IL-3R<sup>high</sup>) and mDCs (BDCA 2<sup>neg</sup>/CD11c<sup>pos</sup>/IL-3R<sup>low</sup>) exhibited an immature phenotype as demonstrated by the absence of CD80 and low or moderate expression level of CD86 (Fig. 1A). Using mouse monoclonal anti-CD81 (JS-81; BD Biosciences), polyclonal rat anti-SR-BI (28) and rat anti-CLDN1 antibodies (9) which target the extracellular domains of the distinct HCV entry factors and flow cytometry, we found that different human blood DC subsets express distinct profiles of HCV entry factors. Although both DC subsets expressed low level of SR-BI, CD81 expression levels were significantly lower in pDCs compared to mDCs ( $p=0.023$ ). Cell surface expression levels of CLDN1 were lower on pDCs compared to mDCs but this difference did not reach statistical significance ( $p=0.144$ ) (Fig. 1B and C). The distinct profiles of HCV entry factors on the DC surface may lead to different interactions of mDCs and pDCs with HCV. To test this hypothesis, we incubated freshly isolated blood DC subsets with iodixanol purified cell-culture derived infectious HCV (HCVcc) produced in Huh7.5.1 cells by electroporation of the JFH1 genotype 2a genome (24). Produced virus was passaged several times to obtain high virus titer. Prior to the addition of HCVcc, blood DC subsets were incubated for 45 min with either anti-CD81, anti-SR-BI or anti-CLDN1 antibodies. These antibodies have been previously shown to inhibit HCVcc infection of Huh7.5.1 cells (9, 28). Following incubation with HCVcc for 2 h at 37°C, mDCs and pDCs were extensively washed and HCVcc acquisition quantified by real-time RT-PCR as described previously (16). In brief, total RNA was harvested using the RNeasy Mini kit (Qiagen). HCV RNA was amplified from 100 ng total RNA using SensiMix One-Step

Kit (Labgene scientific) and detected using Rotor-Gene 6000 (Corbett Life Science). The forward and reverse primer sequences were 5'-TCT GCG GAA CCG GTG AGT A-3' and 5'-GGG CAT AGA GTG GGT TTA TCC-3', respectively. The probe sequence was 5'-6FAM-AAA GGA CCC AGT CTT CCC GGC AA-TAMRA-3'. As shown in Figure 2A, HCV acquisition by both DC subsets did not markedly alter in the presence of antibodies targeting CD81, SR-BI or CLDN1 on the DC surface. We were surprised that SR-BI blocking had no effect on HCVcc acquisition by mDCs, since our previous studies indicated a prominent role for SR-BI in HCVcc uptake by monocyte-derived DCs (2). This finding may be explained by the very low expression level of SR-BI on both DC subsets when compared to *in vitro* generated monocyte-derived DCs (2). This observation is supported by Marukian and colleagues (13) who describe a more than 3 log lower expression level of SR-BI RNA in blood DC subsets as compared to *in vitro* generated monocyte-derived DCs. These results suggest that HCV entry factors, which play an important role in mediating HCV entry into hepatocytes (1), are not involved in mediating HCV acquisition by blood DC subsets and that other cell surface molecules, e.g. members of the C-type lectin family, may be involved in HCV acquisition. To address this possibility we analyzed cell-surface expression of DC-SIGN, which has been previously shown to bind recombinant HCV E2 protein (10). DC-SIGN was not detected on the cell surface of either DC subset (Fig. 1B). Our results confirm previously published results by other groups who also did not find DC-SIGN expression on freshly isolated blood DC subsets (15, 21). Additionally, blocking of DC-SIGN by anti-DC-SIGN antibodies (clone 120507, Abcam) did not inhibit HCVcc acquisition by mDCs and pDCs (Fig. 2B) suggesting that HCVcc acquisition by DC subsets does not depend on the C-type lectin DC-SIGN.

Following virus capture, viral antigen is delivered into intracellular compartments which are dedicated to processing and presenting viral antigen to T cells (19). To investigate HCVcc uptake and trafficking in DC subsets, we monitored HCVcc uptake in mDCs and pDCs by anti-HCV E2 specific immunofluorescence and confocal laser scanning microscopy

1 using mouse anti-E2 monoclonal antibody (AP33, targeting epitope E2<sub>412-423</sub> (22)). To  
2 confirm specificity of HCVcc E2 protein staining, DC subsets were incubated with control  
3 iodixanol preparations obtained from culture supernatants of non-infected Huh7.5.1 cells,  
4 and subsequently stained with anti-E2 (AP33) antibody. HCVcc E2 protein was detectable in  
5 both DC subsets while no fluorescence signal was observed in DCs incubated with control  
6 iodixanol preparations (Fig. 2B). Furthermore, no fluorescence signal was detected in both  
7 DC subsets following incubation with HCVcc and subsequent labeling with irrelevant mouse  
8 IgG (Invitrogen) (data not shown). Finally, to verify the specificity of HCVcc E2-staining, DC  
9 subsets were co-stained with rabbit anti-core antibody (LifeSpan Biosciences) or irrelevant  
10 rabbit IgG (Bethyl Laboratories). Double staining demonstrated colocalization of HCVcc core  
11 and E2 protein in both DC subsets indicating uptake of specific components of the HCVcc  
12 particle (Fig. 2C). Quantification of HCVcc uptake by counting the average number of cells  
13 staining positive for HCVcc E2 protein per 300 cells, revealed that following a 3 h incubation  
14 with HCVcc, mDCs were significantly more efficient at HCVcc uptake than pDCs ( $p = 0.012$ )  
15 (Fig. 2D). This may be related to the intrinsically lower capability of pDCs in endocytosing  
16 antigens as compared to conventional DCs (8). To investigate whether HCVcc uptake is  
17 mediated by the HCV envelope glycoprotein E2, HCVcc were pre-incubated with human  
18 serum containing high-titer anti-E2 antibodies (20), that efficiently blocked HCVcc infection of  
19 Huh7.5.1 cells (24). As shown in Figure 2E, pre-incubation of HCVcc with HCV anti-E2  
20 containing serum markedly reduced HCVcc uptake by mDCs ( $40\% \pm 14.5\%$  inhibition of  
21 HCVcc uptake compared to human control serum,  $p = 0.02$ ) whereas a less potent effect was  
22 observed on pDCs ( $13.4\% \pm 10.3\%$  inhibition of HCVcc uptake compared to human control  
23 serum,  $p = 0.114$ ). These findings suggest that HCVcc uptake by DC subsets is partially  
24 mediated by HCVcc E2 protein interaction at the cell surface.

25  
26 Next, to gain a deeper insight into the mechanisms involved in HCVcc uptake by DC  
27 subsets, we studied HCVcc internalization by anti-E2 specific immunofluorescence following  
28 blockage of the HCV entry factors. Pre-incubation of mDCs and pDCs with polyclonal rabbit

1 anti-CD81 (Sigma-Aldrich), rat anti-SR-BI (28) or rat anti-CLDN1 antibodies (9) did not  
2 markedly inhibit HCVcc uptake (Fig. 2E). These results suggest that HCV cell entry factors  
3 which are crucial for viral uptake in hepatocytes do not support HCVcc uptake in DC subsets.  
4 One possible explanation may be the fact that DC subsets lack the expression of at least one  
5 HCV entry factor or that they do not express sufficient levels of HCV entry factors (Fig. 1B,  
6 (13)). Thus, future studies are required to identify cell-surface molecules on mDCs and pDCs  
7 triggering HCVcc endocytosis. However, we cannot rule out that HCV enters DC subsets via  
8 pinocytosis (14), a constitutive mechanism by which DCs acquire exogenous solute as part  
9 of their sentinel function.

10  
11 To localize internalized HCVcc particles within DC subsets, following HCV uptake, we  
12 co-stained mDCs and pDCs for the early endosome marker Rab5, the lysosomal-associated  
13 membrane protein 1 (Lamp1) and the transferrin receptor CD71, a marker for recycling  
14 endosomes. Within pDCs, HCVcc localized in organelles expressing the transferrin receptor  
15 CD71 - but not Rab5 or Lamp1, indicating that HCVcc antigen accumulates in recycling  
16 endosomes (Fig. 3A). In contrast, HCVcc co-stained with Lamp1 in mDCs - but not with  
17 Rab5 or CD71, suggesting localization of HCVcc antigen in lysosomal compartments (Fig.  
18 3A). This distinction in HCV antigen localization in the DC subsets was preserved at 1 and 3  
19 h after HCV uptake, indicating that differences observed in antigen localization are not due to  
20 differences in HCVcc uptake kinetics by mDCs and pDCs. Together, these data suggest that  
21 HCV antigen traffics within distinct intracellular compartments in pDCs and mDCs.

22  
23 The endosomes and lysosomes of antigen-presenting cells host the processing and  
24 assembly reactions that result in the display of peptides on MHC class II molecules. For  
25 mDCs, it is known that rapid presentation of exogenous antigens involves loading of peptides  
26 onto MHC class II molecules found in late endosomes and lysosomes (25). To further  
27 analyze the lysosomal compartment in mDCs that harbored HCVcc particles, lysosomal  
28 compartments were co-stained with anti-MHC class I and II, respectively. As shown in figure

3B, lysosomal compartments of mDCs colocalized with cytoplasmic pools of MHC class II, but not MHC class I, indicating that HCVcc particles appears to be processed in lysosomes that contains MHC class II molecules. Interestingly, in pDCs the HCVcc-containing, recycling-endosomal-compartment colocalized with MHC class I, but not with MHC class II (Fig. 3B). These findings suggests a mechanism by which HCVcc antigen is delivered to recycling endosomal compartments for processing and loading onto pre-synthesized pools of MHC class I molecules. Clearly, we cannot exclude the possibility that HCV antigen is also released from the endocytic compartment to the cytosol of the cell where it gains access to the classical pathway of MHC class I. However, Di Pucchio and colleagues (3) demonstrated that pDCs cross-present influenza-derived peptides on MHC class I molecules to CD8+ T cells by delivery of internalized virus to recycling endosomes. Thus, it is very likely that HCV-derived antigens are targeted to recycling endosomal compartments to ensure a rapid activation of the HCV-specific CD8+ T cell response by utilizing a storage pool of MHC class I molecules. However, it should be noted that our *in vitro* model, which uses freshly isolated blood DC subsets, probably does not fully represent the *in vivo* scenario. It is likely that DCs that are actively engaged in the immune response to HCV *in vivo* are phenotypically different from those used in this study. Furthermore, we have to point out that the majority of immunodominant CD4+ and CD8+ T cell epitopes derived from HCV non-structural proteins (5, 23). The mechanism of uptake and intracellular trafficking of HCV non-structural proteins remains to be determined.

In conclusion, our results demonstrate that HCV antigen traffics within distinct compartments of DC subpopulations. Our data implicate a model of HCV antigen processing and presentation in which mDCs and pDCs perform different functions in HCV-specific CD4+ and CD8+ T cell stimulation. Since resolution of HCV infection is characterized by a robust CD4+ T cell response which is essential for HCV-specific CD8+ T cell function (7), the distinct HCV antigen processing pathways in mDCs and pDCs may ensure the simultaneous initiation of rapid and effective HCV-specific CD4+ and CD8+ T cell responses. Further



1 investigation is needed to determine whether targeting viral antigen into distinct processing  
2 and presentation pathways may present an avenue for improving HCV vaccine delivery and  
3 aid immunotherapeutic approaches aimed at inducing strong and sustained CD4+ and CD8+  
4 T cell responses against HCV.

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10 31.

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## Figure legends

**Figure 1. Phenotypical characterization of mDCs and pDCs. (A)** Surface expression of BDCA2, CD11c, IL-3R, CD80 and CD86 on freshly isolated pDCs and mDCs was assessed by flow cytometry using PE-conjugated monoclonal antibodies (dark grey histograms) or isotype control antibodies (light grey histograms). **(B)** Expression of HCV entry factors CD81, SR-BI, CLDN1 and the C-type lectin DC-SIGN on pDCs and mDCs was analyzed by flow cytometry using anti-CD81, anti-SR-BI, anti-claudin-1, anti-DC-SIGN specific antibodies (open black histograms) and the respective isotype control (light grey histograms). **(C)** Expression levels of HCV entry factors on DC subsets are shown as mean fluorescence intensity (MFI) rate. The MFI rate corresponds to the MFI of cells labeled with specific antibody divided by the MFI of cells labeled with the matched isotype control. The means  $\pm$  SD of independent experiments with pDCs and mDCs from different donors are shown (for CD81, n=7 donors; for SR-BI, n=6 donors, for CLDN1, n=5 donors). Statistical analysis was carried out using the Mann-Whitney U-test. Tests of significance were two-sided, and a  $p$  value  $\leq 0.05$  was considered as significant (\*,  $p=0.023$  comparing CD81 expression level of mDCs and pDCs; n.s. = not significant).

**Figure 2. HCVcc acquisition by mDCs and pDCs. (A)** Role of HCV entry factors in HCVcc acquisition by pDCs and mDCs. DC subsets were pre-incubated with mouse anti-CD81 (5  $\mu$ g/ml), rat anti-SR-BI (dilution 1:50), rat anti-claudin 1 (dilution 1:50), anti-DC-SIGN antibodies (5 $\mu$ g/ml), irrelevant mouse IgG (5  $\mu$ g/ml) or rat IgG (dilution 1:50) for 45 min at 37°C. DC subsets were then incubated with HCVcc (~ MOI 0.01) for 2 h at 37°C. After extensive washing, the presence of HCVcc RNA was measured by real-time quantitative RT-PCR. As negative controls, DC subsets were incubated with PBS or control iodixanol preparations obtained from culture supernatants of non-infected Huh7.5.1 cells (NC, negative control). The data represent the mean  $\pm$  SD of triplicate samples from one experiment. The results shown are representative of three independent experiments using three different donors. **(B)** Visualization of HCVcc uptake into pDCs and mDCs. For HCVcc internalization,

DC subsets were incubated with iodixanol-gradient purified HCVcc (~ MOI 10) for 3 hours at 37°C. Following fixation and permeabilization, cells were stained for HCVcc E2 protein using mouse anti-E2 monoclonal antibody (AP33, in green) and the nucleus was stained with DAPI (in blue). Arrows indicate detectable HCVcc E2 protein. To confirm specificity of HCV E2 protein labeling, DC subsets were incubated with control iodixanol preparations obtained from culture supernatants of non-infected Huh7.5.1 cells and subsequently labeled with anti-E2 (AP33) antibody (NC, negative control). **(C)** For HCVcc double labeling, DC subsets were incubated with mouse anti-E2 (AP33, in green) and rabbit anti-core antibody (in red). Yellow colour in the overlay indicates co-localization of these two proteins in mDCs and pDCs. **(D)** HCVcc uptake by pDCs and mDCs was quantified by counting the number of cells that stained positive for E2 protein relative to the total number of cells (n=300). Results of pDCs and mDCs isolated from five different donors are shown. Statistical analysis was performed using Mann-Whitney U test. Tests of significance were two-sided, and a  $p$  value  $\leq 0.05$  was considered as significant. **(E)** Inhibition of HCVcc uptake in the presence of human anti-HCV serum (dilution 1:50) and antibodies targeting the HCV entry factors (rabbit anti-CD81, 6  $\mu$ g/ml; rat anti-SR-BI and rat anti-CLDN1, dilution 1:50). HCVcc uptake by DC subsets was analyzed as described in (D). Results are shown as percentage inhibition of HCVcc uptake compared to HCVcc uptake in the presence of the respective isotype controls (irrelevant IgG or human control serum) from pDCs and mDCs of four different donors.

**Figure 3. Distinct intracellular HCVcc trafficking in pDCs and mDCs.** **(A)** DC subsets were incubated for 3 hours at 37°C with HCVcc (~ MOI 10). Cells were then fixed and stained for HCV E2 protein (in green) and endocytotic compartments (in red), such as early endosomes (Rab5), recycling endosomes (CD71), and lysosomes (Lamp1). The overlay showing colocalization HCVcc E2 protein and endocytotic compartments is seen in yellow (indicated by arrows). Cell nuclei were revealed with DAPI staining in blue. In pDCs, HCVcc E2 protein is localized in recycling endosomes identified by CD71 but not in endocytotic compartments containing Rab5 or Lamp1. In contrast, HCVcc E2 protein colocalizes with

1 lysosome antigen Lamp1 in mDCs but not with CD71 or Rab5. Representative results from  
2 one of three independent experiments are shown. **(B)** Characterization of the endocytotic  
3 compartment containing HCVcc E2 protein in pDCs and mDCs. Plasmacytoid DCs were  
4 stained for recycling endosomes (CD71, in red) and MHC class I or II molecules (in green).  
5 The overlay image shows colocalization of CD71 with MHC I but not MHC II (indicated by  
6 arrows), indicating that recycling endosomes contain MHC class I molecules. Myeloid DCs  
7 were stained for lysosomes (Lamp1, in red) and MHC I or II (in green). The overlay image  
8 shows colocalization of lysosomes with MHC II but not MHC I (top panel), indicating that  
9 lysosomes contain MHC class II molecules. Data are representative of two independent  
10 experiments.