



Characterization of Antigen-Presenting Cell Subsets in Human Liver-Draining Lymph Nodes

Patrick P. C. Boor¹, Brenda M. Bosma¹, Khe T. C. Tran², Luc J. W. van der Laan², Hanneke Hagens², Jan N. M. IJzermans², Herold J. Metselaar¹ and Jaap Kwekkeboom^{1*}

¹ Department of Gastroenterology and Hepatology, Erasmus MC–University Medical Centre, Rotterdam, Netherlands,

² Department of Surgery, Erasmus MC–University Medical Centre, Rotterdam, Netherlands

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*Correspondence:

Jaap Kwekkeboom
j.kwekkeboom@erasmusmc.nl

Specialty section:

This article was submitted to
Immunological Tolerance and
Regulation,
a section of the journal
Frontiers in Immunology

Received: 18 September 2018

Accepted: 19 February 2019

Published: 14 March 2019

Citation:

Boor PPC, Bosma BM, Tran KTC, van der Laan LJW, Hagens H, IJzermans JNM, Metselaar HJ and Kwekkeboom J (2019) Characterization of Antigen-Presenting Cell Subsets in Human Liver-Draining Lymph Nodes. *Front. Immunol.* 10:441. doi: 10.3389/fimmu.2019.00441

T-cell immunity in the liver is tightly regulated to prevent chronic liver inflammation in response to antigens and toxins derived from food and intestinal bacterial flora. Since the main sites of T cell activation in response to foreign components entering solid tissues are the draining lymph nodes (LN), we aimed to study whether Antigen-Presenting Cell (APC) subsets in human liver lymph-draining LN show features that may contribute to the immunologically tolerant liver environment. Healthy liver LN, iliac LN, spleen and liver perfusates were obtained from multi-organ donors, while diseased liver LN were collected from explanted patient livers. Inguinal LN were obtained from kidney transplant recipients. Mononuclear cells were isolated from fresh tissues, and immunophenotypic and functional characteristics of APC subsets were studied using flowcytometry and *in vivo* cultures. Healthy liver-draining LN contained significantly lower relative numbers of CD1c⁺ conventional dendritic cells (cDC2), plasmacytoid DC (PDC), and CD14⁺CD163⁺DC-SIGN⁺ macrophages (MF) compared to inguinal LN. Compared to spleen, both types of LN contained low relative numbers of CD141^{hi} cDC1. Both cDC subsets in liver LN showed a more activated/mature immunophenotype than those in inguinal LN, iliacal LN, spleen and liver tissue. Despite their more mature status, cDC2 isolated from hepatic LN displayed similar cytokine production capacity (IL-10, IL-12, and IL-6) and allogeneic T cell stimulatory capacity as their counterparts from spleen. Liver LN from patients with inflammatory liver diseases showed a further reduction of cDC1, but had increased relative numbers of PDC and MF. In steady state conditions human liver LN contain relatively low numbers of cDC2, PDC, and macrophages, and relative numbers of cDC1 in liver LN decline during liver inflammation. The paucity of cDC in liver LN may contribute to immune tolerance in the liver environment.

Keywords: myeloid dendritic cell, plasmacytoid dendritic cell, macrophage, human liver, immunological tolerance

INTRODUCTION

The liver is continuously exposed to food components and bacterial products that enter the liver from the gastrointestinal tract via the portal vein. To prevent continuous liver inflammation in response to these gut-derived components, local immune responses in the liver are tightly regulated. Therefore, the liver environment favors induction of peripheral immune tolerance rather than immunity (1).

It has been hypothesized that dendritic cells (DC) are key players in maintaining the fine balance between T-cell responsiveness and unresponsiveness in the liver (2). Conventional DC (cDC) are the most highly specialized antigen-presenting cells (APC) and they play a critical role in the initiation and direction of T-cell responses (3, 4). Immature cDC are located throughout most body tissues and are specialized in the uptake and processing of antigens. Under steady state conditions there is a continuous migration of antigen-loaden cDC from non-lymphoid tissues through the lymphatics toward the draining lymph nodes (LN), where they present antigens that they acquired in their tissue of origin to T cells, and this process is accelerated in response to inflammatory signals. During migration to LN, cDC complete their maturation and acquire potent T cell stimulatory capacity by upregulation of MHC and co-stimulatory molecules (5). In addition to migratory cDC, LN contain resident cDC which emerge from circulating precursors and spend their entire lives within LN, where they acquire antigens from non-lymphoid tissue that in soluble form via lymphatic drainage enter LN, and present them to T cells (6).

Human cDC can be subdivided into two distinct ontogeneic lineages: type 1 cDC (cDC1) which express high levels of CD141 (BDCA3) and the necrotic cell receptor CLEC9a, and type 2 cDC (cDC2) that express CD1c (BDCA1) (7–9). Both cDC subsets are potent stimulators of CD4⁺ T-cell responses (10), but differ in stimulatory capacity of CD8⁺ T cells. cDC1 have a superior ability to cross-present antigens from necrotic cells to CD8⁺ T cells compared to cDC2 (11–14). The cross-presenting capacity of cDC2 is still unclear, although recent data suggest that they can cross-present soluble antigens (15).

Next to cDC, plasmacytoid DC (PDC) are a separate DC lineage. PDC are unique in rapidly producing massive amounts of type I interferon upon recognition of viral nucleotides or self-DNA-protein complexes by their Toll-Like receptors (TLR). Although PDC have weaker antigen presenting capacity than cDC, they can mature upon stimulation and prime CD4⁺ and CD8⁺ T cell responses (9, 16). On the other hand they can contribute to immunological tolerance by their capacity to generate CD4⁺ and CD8⁺ regulatory T cells (Treg) from naïve CD4⁺ or CD8⁺ T cells, respectively (17, 18).

Considerable efforts have been done to study whether DC in liver tissue possess unique characteristics that may contribute to immunological tolerance in the liver, but contradictory conclusions have been reached. On the one hand, mouse liver MDC were reported to have an immature phenotype and to be functionally compromised (19–22). On the other hand, however, other studies found no functional differences between distinct DC subsets in mouse liver and spleen, but reported differences in DC subset composition between both tissues (23, 24). In comparison to blood, human liver was shown to contain more cDC1 (25). In addition, human liver cDC2 produce higher amounts of the anti-inflammatory cytokine IL-10 compared to blood or skin MDC, although also being able to produce pro-inflammatory cytokines upon TLR-stimulation (26–28).

T cell activation by DCs is generally not initiated in parenchymal tissue, but in specific secondary lymphoid organs like the spleen or tissue-draining LN. In humans, major liver

lymph vessels leave the liver at the hilus, and liver-derived lymph drains to LN situated at the hilus and along the hepatic artery and portal vein (29). Experimental animal studies have shown that cDC that migrate from the liver are trapped in liver-draining LN (30–32), and that T-cell activation to antigens delivered into the liver mainly occurs in these LN (32, 33). In humans, T cells specific for HBV antigens have been found to be enriched in liver-draining LN and not in the liver itself (34).

Despite the probable importance of hepatic LN in the regulation of T cell responses to antigens in the liver, very little is known about the composition and functions of APC in human liver lymph-draining LN. We therefore asked whether APC in liver LN differ from their counterparts in LN located at sites of the body where predominantly immunogenic responses are required, such as inguinal LN to which lymph from the legs is drained and which should be able to react to external pathogens upon disturbances of the skin barrier. The aim of this study was therefore to compare subset composition, phenotype and functions of the main known human APC subsets in human liver-draining LN with their counterparts in skin and muscle-draining inguinal LN. In addition, we compared APC subsets in liver LN with those in another secondary lymphoid tissue, i.e., spleen, and with those in human liver tissue, which contains cDC that migrate to liver LN.

TISSUES, MATERIALS, AND METHODS

Collection of Lymph Nodes, Spleen, and Liver Perfusate

Spleen tissues were obtained from multi-organ donors during organ donation procedures for the purpose of donor HLA-typing, and residual tissue was used for research. Iliac LN were collected for research purposes from 6 multi-organ donors during organ donation procedures. Hepatic LN were resected along the hepatic artery and portal vein in the porta hepatis from donor livers and diseased patient explant livers during liver transplantation procedures. Liver leukocytes were collected during routine perfusion of liver grafts immediately before transplantation, as we described previously (27). Inguinal LN were obtained from kidney transplant recipients during kidney transplantation procedures, and are considered as skin/muscle draining LN. The Ethics Committee of the Erasmus MC approved collection of the tissues for research purposes, and informed consent of each patient was obtained.

Reagents

The following mAbs and reagents were used: IgG1-FITC, IgG1-PerCP-Cy5.5, IgG2a-APC, IgG1-PB, CD14-PE, CD14-PerCP, CD45-HorizonV500, streptavidin-PerCP, anti-IFN γ -FITC, 7AAD and Lineage cocktail-FITC (CD3, 16,19, 20,14, 56) from BD Biosciences, Erembodegem, Belgium; CD80-FITC from Beckman Coulter, Woerden, the Netherlands; anti-BDCA1-PE, anti-BDCA2-FITC, anti-BDCA2-biotin, anti-BDCA4-PE, anti-BDCA3-APC, anti-Clec9a-VioBlue, CD19 microbeads and anti-PE-microbeads from Miltenyi Biotec, Bergisch Gladbach, Germany; CD1a-PB, CD209 (DC-SIGN)-PECy7, CD206-eFluor450, CD40-APC and CD274

(PDL1)-PECy7 from eBioscience, Vienna, Austria; CD86-APC, CD86-PB, CD163-PerCP-Cy5.5, CD40-PerCP-Cy5.5, HLA DR-APC-Cy7 and CD209 (DC-SIGN)-APC from Biolegend, London, United Kingdom. PMA, ionomycin and brefeldin A are from Sigma-Aldrich, St Louis, MO. The fix&perm cell permeabilization kit was obtained from An der Grub, Vienna, Austria.

Isolation of Mononuclear Cells (MNC)

Lymph nodes and spleen tissues were cut into small pieces and treated with 500 $\mu\text{g/ml}$ collagenase type IV and 200 $\mu\text{g/ml}$ DNase I (both from Sigma Aldrich, Zwijndrecht, the Netherlands) at 37°C. After 20 min the collagenase reaction was stopped by adding fetal bovine serum and the tissue pieces were passed over a nylon mesh filter (100 μm pore diameter) to obtain single cell suspensions. To determine possible effects of collagenase on surface molecule expression, in preliminary experiments LN and spleen tissues were divided into two parts, and one part was treated with collagenase and the other part not. We observed that yields of MDC subsets were considerably increased upon isolations with collagenase, and therefore all data presented in the paper are derived from cells isolated by collagenase treatment, except data on liver cells because these were obtained using single cells collected from liver perfusates. MNC were obtained from single cell suspensions of tissue by Ficoll Paque (GE Healthcare Biosciences AB, Uppsala, Sweden) density gradient centrifugation. Cell yield and viability was determined by counting with trypan blue.

Flowcytometry

Flowcytometric analysis was used to immunophenotype splenic, lymph node-, and liver APC. MNC were labeled with different antibody combinations. Appropriate isotype matched control antibodies were used to determine gates. APC subsets were determined within a MNC gate after exclusion of CD45⁻ non-hematopoietic cells and 7AAD⁺ dead cells. cDC subsets were distinguished on basis of CD11c, CD1c, and CD141 expression. PDC were defined as BDCA2⁺BDCA4⁺ cells, and monocytes/macrophages as CD14⁺. At least 500,000 events were acquired using a FACSCanto II flowcytometer and analyzed using FACSDiva 6.1 software (BD Biosciences) And Flowjo software (version 10.2, BD Biosciences).

Purification of cDC2 and PDC

cDC2 were enriched from hepatic LN and spleen MNC by incubation with CD19, CD3, and CD15 microbeads and depletion of B and T cells and granulocytes by separation over a Large Depletion column using a MidiMACS separation device (Miltenyi Biotec, Bergisch Gladbach, Germany). Subsequently, the non-adherent cells were labeled with CD1c-PE, CD14-PerCP and CD20-Pacific Blue antibodies, and cDC2 were isolated by flowcytometric cell sorting by first excluding CD14⁺ and CD20⁺ cells, and subsequent selection of CD1c⁺ cells. Purity of the isolated cDC2 as determined by flowcytometry was typically >95%. To isolate PDC, LN MNC were labeled with anti-PE-conjugated anti-BDCA-4 antibody and anti-PE microbeads

(Miltenyi Biotec) and purified by two round of separation over MS columns.

Cytokine Production and T-Cell Stimulatory Capacity of DC

Purified cDC2 from hepatic LN and spleen from the same donors were cultured at a concentration of 2×10^4 cells/200 μl in round bottom 96-well culture plates in RPMI1640 supplemented with 10% FCS (Hyclone, Logan, UT, USA), penicillin (100 U/ml), streptomycin (100 U/ml) and GM-CSF (500 U/ml; Leucomax, Novartis Pharma, Arnhem, the Netherlands) and stimulated with either 20 $\mu\text{g/ml}$ synthetic double-stranded RNA (polyriboinosinic-polyribocytidylic acid; poly I:C; Sigma-Aldrich, St. Louis, MO) and 1,000 U/ml IFN- γ or CD40L-transfected J558 plasmacytoma cells (2×10^4 cells/well) and 1,000 U/ml IFN- γ for 24 h and 37°C. After 24 h, supernatants were harvested and levels of IL-12, IL-6, and IL-10 were determined by standard enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (eBioscience, Vienna, Austria).

The T cell stimulatory capacity of cDC2 or PDC was assessed by co-culturing purified MDC or PDC at different concentrations (10, 5, 2.5, 1.25×10^3 cells/200 μl) in round bottom 96-well culture plates in RPMI1640 supplemented with 10% FCS, penicillin and streptomycin with 1.5×10^5 CD3⁺ T cells isolated by magnetic selection using the pan T-cell isolation kit (Miltenyi Biotec) from blood of a healthy volunteer. In all experiments T cells from the same individual were used. After 5 days, T cell proliferation was assessed by measuring the incorporation of [³H]-thymidine (Radiochemical Center, Amersham, Little Chalfont, UK). 0.5 μCi was added per well and cultures were harvested 18 h later.

To determine their capacity of induce T-helper 1 responses, 1×10^5 CD45RA⁺CD45RO⁻CD3⁺ naïve T cells obtained by flowcytometric cell sorting from blood of a healthy volunteer, were co-cultured with 1×10^4 cDC2 isolated from hepatic LN or spleen. After 7 days T cells were re-stimulated with PMA (40 ng/ml) and ionomycin (1 $\mu\text{g/ml}$) for 6 h. During the last 5 h of restimulation brefeldin A (5 $\mu\text{g/ml}$) was added to inhibit protein transport processes. Intracellular IFN- γ expression was determined by using the Fix&perm cell permeabilization kit according to the manufacturer's instructions.

Statistical Analysis

The Mann-Whitney *U*-test was used to test differences between tissues from different patients, and the Wilcoxon signed rank test was used for analysis of differences between paired tissues of the same donors. A $p < 0.05$ was considered significant. GraphPad Prism 5 software was used to perform the statistical tests.

RESULTS

Characterization of Conventional Dendritic Cell Subsets in Lymphoid Organs and Liver

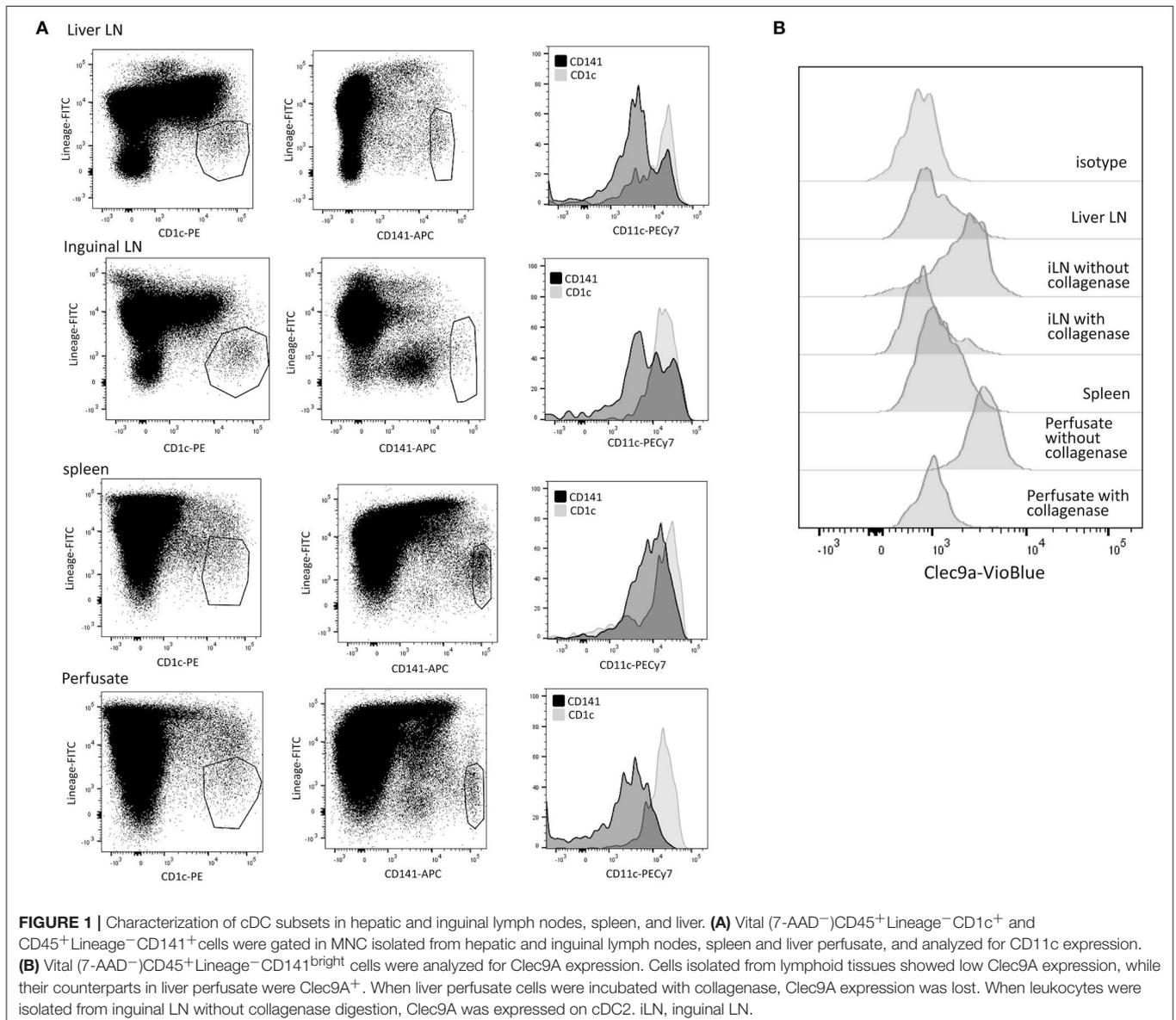
To characterize DC subsets in the different tissues, MNC were isolated from freshly resected hepatic LN, inguinal LN, spleen, and liver graft perfusates, and analyzed for expression

of CD45, CD11c, CD1c, CD141, and lineage markers (CD3, CD14, CD16, CD19, CD20, and CD56). Since leukocytes in liver graft perfusates accurately represent leukocytes present in liver tissue (27, 28, 35–37) we will refer to liver perfusate DC as liver DC. First, we analyzed CD11c expression on CD45⁺Lineage⁻CD1c⁺ and CD45⁺Lineage⁻CD141⁺ cells. We observed that lineage⁻CD1c⁺ cells express high levels of CD11c, while part of lin⁻CD141^{hi} cells were CD11c^{dim} in the different tissues (**Figure 1A**). In accordance with previous publications on cDC subsets in human tissues (9, 14), we concluded that CD141^{hi} cDC1 have variable CD11c expression. Therefore defined cDC1 as lin⁻CD141^{hi} cells and cDC2 as lin⁻CD11c⁺CD1c⁺ cells. Lin⁻CD141^{hi} cells from liver perfusate also expressed Clec9A, identifying them as bona fide cDC1 (13, 14). However, Clec9A expression was reduced or absent on cDC1 in lymphoid tissues (**Figure 1B**). This appeared to be due to the collagenase digestion used to isolate single cells from lymphoid tissues, which was

not required for liver perfusate. Incubation of liver-derived leukocytes with collagenase resulted in loss of Clec9A expression on liver-derived cDC1, while isolation of single cells from LN without collagenase digestion resulted in cDC1 with clear Clec9A expression (**Figure 1B**). In none of the tissues cDC1 expressed CD1a, CD206, or DC-SIGN (data not shown), indicating that both types of LN, as well as spleen and liver, contain a homogeneous population of cDC1. In contrast, in all examined tissues 10–20% of cDC2 expressed CD1a and a small proportion expressed CD206 (data not shown), suggesting that a minority of cDC1 may represent migratory DC (38).

Liver LN Contain Relatively Low Numbers of cDC2

To compare relative numbers of cDC subsets in the different tissues, we quantified proportions of lineage⁻CD141^{bright} cDC1

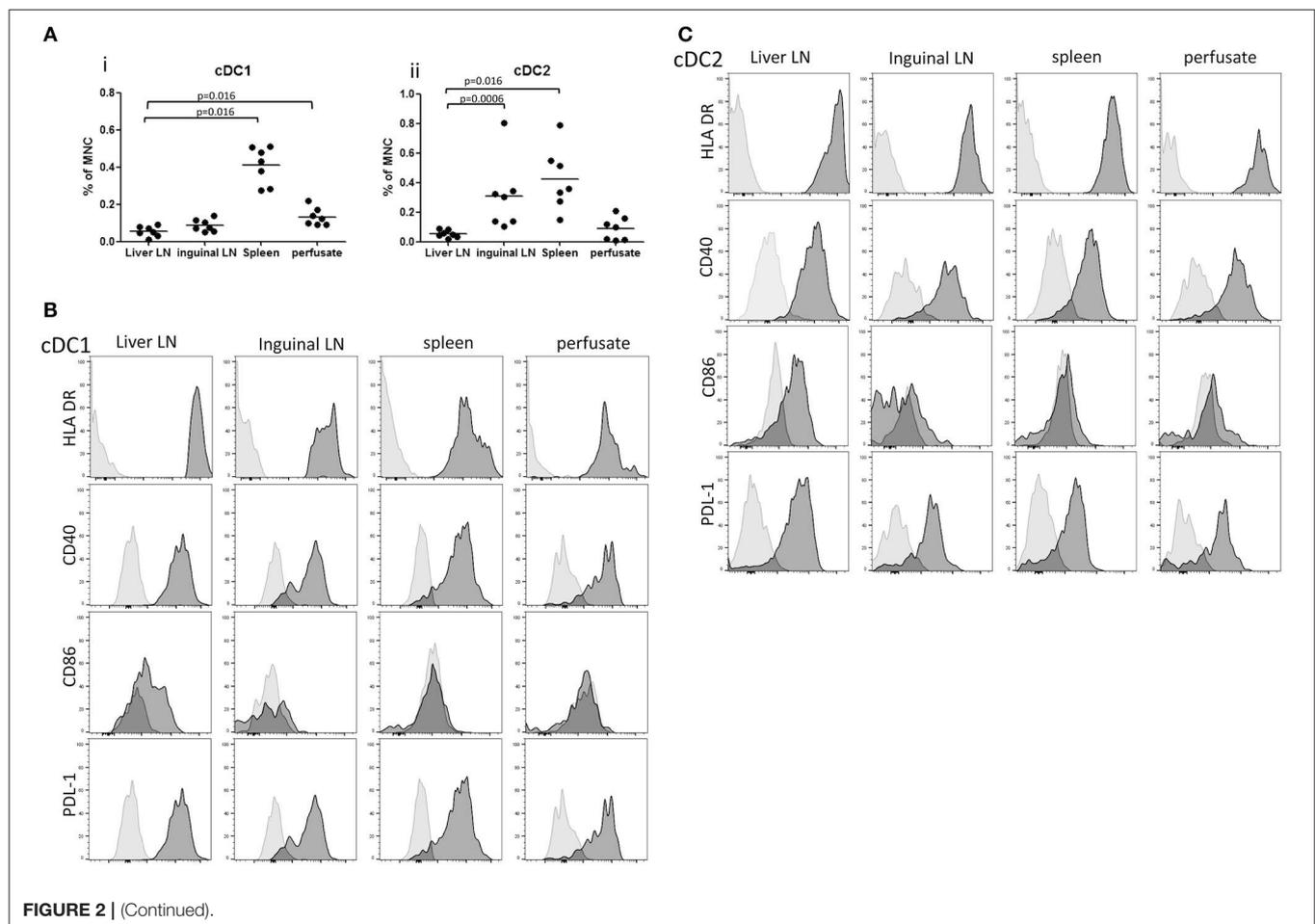


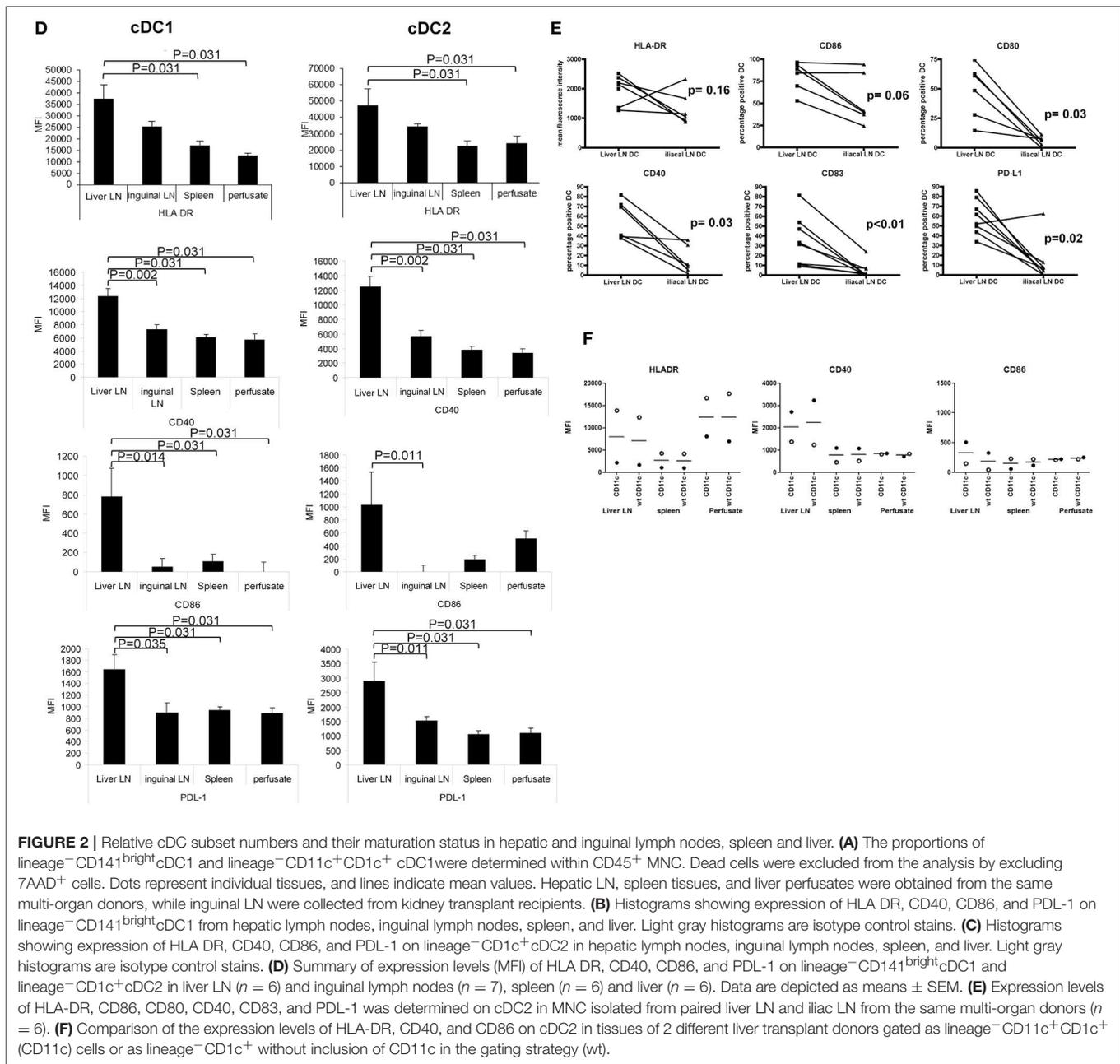
and lineage⁻CD11c⁺CD1c⁺ cDC2 within CD45⁺ cells. Of all tissues, spleen contained the highest proportions of both cDC subsets (**Figure 2A**). Interestingly, hepatic LN contained 6 times lower proportions of cDC1 compared to inguinal LN (0.053% vs. 0.31%; $p = 0.0006$), while both types of LN contained small but similar proportions of cDC2. These data demonstrate a selective reduction of the relative numbers of the cDC2 subset in human liver LN.

Both cDC Subsets in Liver LN Are Highly Activated

To compare the maturation status of both cDC subsets in the different tissues, we analyzed their expression of HLA-DR, the co-stimulatory molecules CD40 and CD86, and the co-inhibitory molecule PDL-1. Because we had no more than 8 fluorochrome channels available on our FACS, we omitted CD11c from our gating strategy, and defined cDC2 cells for this analysis as lineage⁻CD1c⁺. Nevertheless, all cDC2 identified by this gating strategy were HLA-DR⁺, like cDC1 (**Figures 2B,C**). Expression levels of HLA-DR on both cDC subsets was higher in liver LN compared to spleen and liver (**Figures 2B–D**). Even more interestingly, in hepatic

LN both cDC subsets expressed significantly higher levels of CD40, CD86 and PDL-1 compared to their counterparts in inguinal LN, spleen and liver, indicating that both cDC subsets in hepatic LN are in a more activated/mature status. To exclude that the observed differences in maturation status were confounded by possible differences between multi-organ donors (from which hepatic LN, spleens and liver perfusates were collected) and kidney transplant recipients (from which inguinal LN were obtained), paired comparisons of expression of HLA-DR, CD40, CD80, CD83, CD86, and PDL-1 on cDC2 in hepatic and iliac LN derived from the same multi-organ donors were performed. The data derived from these measurements confirm that cDC2 in hepatic LN have a more mature/activated immunophenotype compared to their counterparts in non-liver-draining LN (**Figure 2E**). Finally, we analyzed HLA-DR, CD40, and CD86 expression on cDC2 in 2 additional sets of tissues using antibody panels in which we replaced PD-L1 antibody by CD11c antibody, allowing for gating of cDC2 as lineage⁻CD11c⁺CD1c⁺ cells. This gating strategy yielded similar results for expression levels of HLA-DR and co-stimulatory molecules as the gating strategy without CD11c (**Figure 2F**).



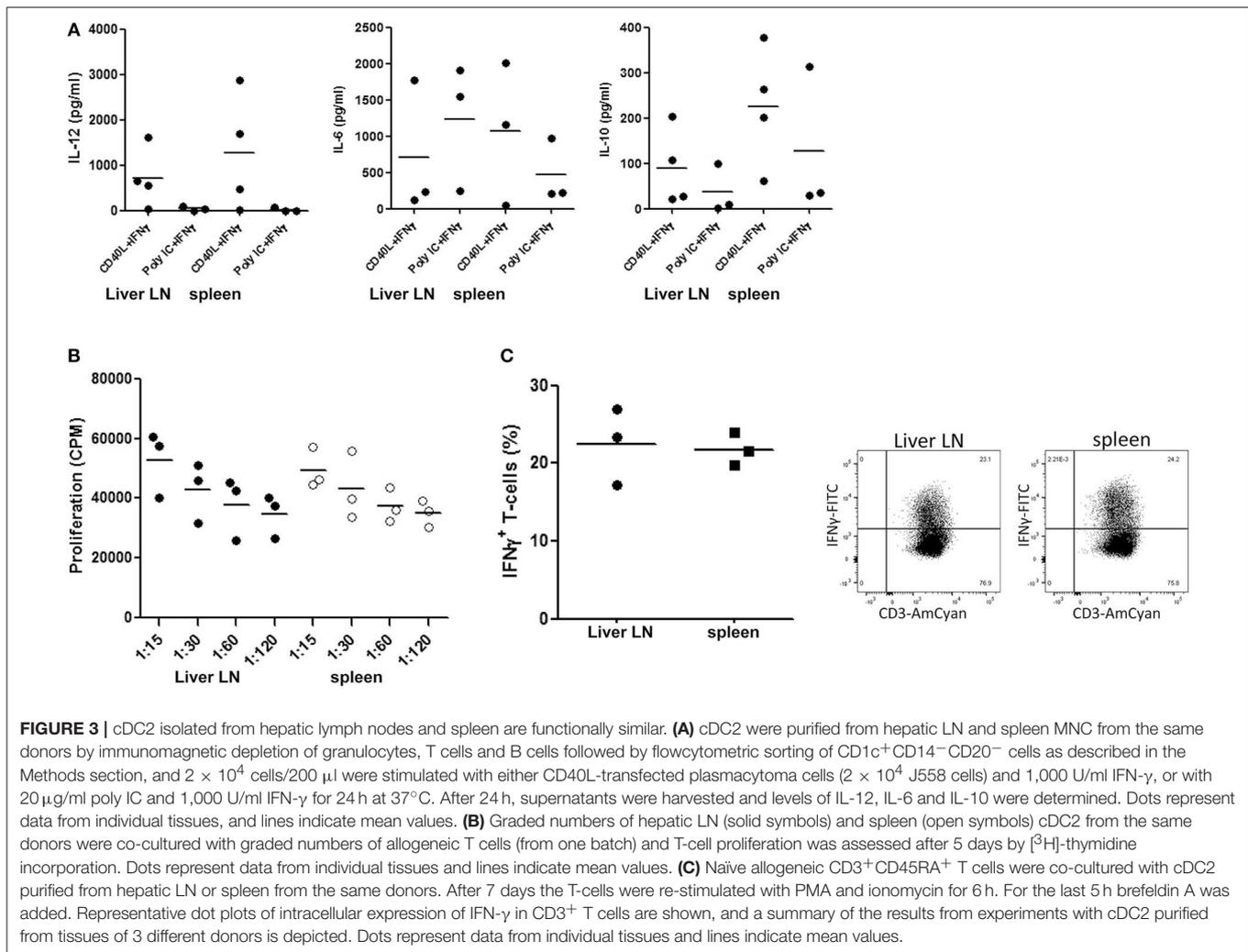


cDC2 in Hepatic Lymph Nodes Are Functionally Similar to Those in Spleen

We compared the functional properties of the most prominent cDC-subset present in hepatic LN, i.e., the cDC2, with those of its counterpart in spleen. cDC2 were purified from hepatic LN and spleen MNC obtained from the same donors and we analyzed their cytokine production upon *ex vivo* stimulation with CD40L plus IFN- γ or TLR3-agonist poly I:C plus IFN- γ . Since synergistic stimulation via TLR or CD40 with IFN- γ is required to induce maximal IL-12 production by human cDC2 (39), we added IFN- γ in all conditions. The

cells secreted the pro-inflammatory cytokines IL-12 and IL-6 as well as the immune-regulatory cytokine IL-10 without apparent differences between those derived from hepatic LN and spleen (**Figure 3A**).

In addition, we compared their T-cell stimulatory capacity. Despite their more mature immunophenotype, cDC2 from liver LN demonstrated a comparable capacity to stimulate proliferation of allogeneic T-cells as their counterparts isolated from spleen (**Figure 3B**). Moreover, cDC2 from hepatic LN and spleen induced IFN- γ production in naïve allogeneic T-cells (**Figure 3C**) to a comparable extent.



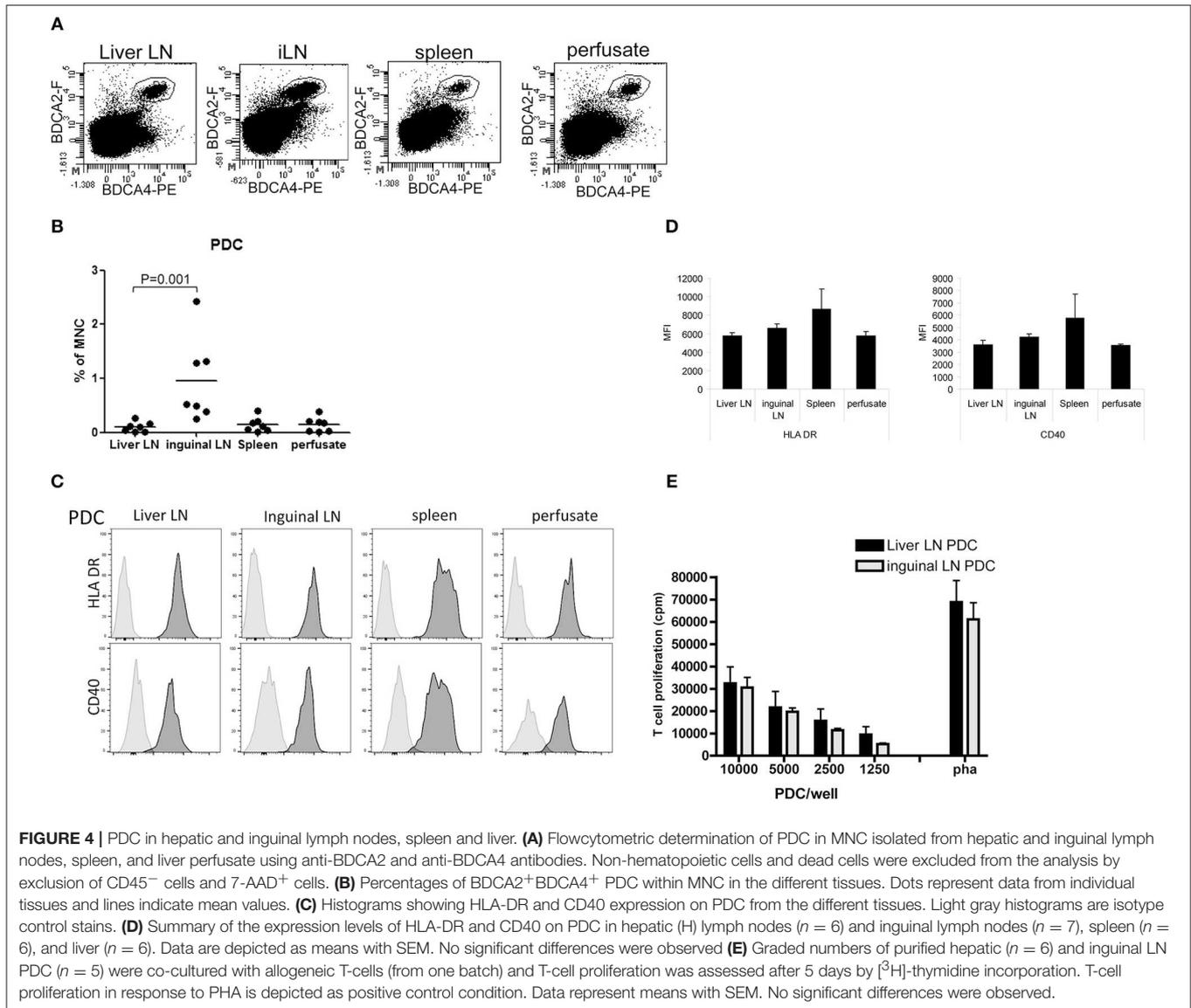
Liver LN Contain Relatively Low Numbers of Plasmacytoid Dendritic Cells

Relative PDC numbers within CD45⁺ MNC of the different tissues were determined using anti-BDCA2 and anti-BDCA4 antibodies (**Figure 4A**). Hepatic LN contained on average 0.13% PDC, which is comparable to the relative numbers of PDC in spleen (0.14%) and liver (0.19%), but ~ 7 times lower than PDC numbers in inguinal LN (0.95%; **Figure 4B**). The maturation status of PDC was assessed by determination of the expression of HLA-DR, CD40, CD86, and PDL-1. In none of the examined tissues CD86 expression was detected on PDC, and PDL-1 expression was very low (data not shown). CD40 and HLA-DR expression on PDC did not differ between the tissues (**Figures 4C,D**). In addition, PDC isolated from liver LN and inguinal LN showed comparable *ex vivo* allogeneic T-cell stimulatory capacity (**Figure 4E**).

Liver LN Contain Relatively Low Numbers of CD14⁺DC-SIGN⁺Macrophages

In addition to the two MDC-subsets and PDC, we detected CD14⁺ cells in spleen and liver ($5.0 \pm 0.5\%$ and $11.3 \pm$

2.8% of total CD45⁺MNC, respectively). In contrast, these cells were rare in both types of LN, but hepatic LN contained even 2-fold lower relative numbers compared to inguinal LN ($0.23 \pm 0.06\%$ and $0.53 \pm 0.03\%$ of CD45⁺MNC, respectively; **Figures 5A,B**). CD14⁺ cells in both types of LN expressed much higher levels of DC-SIGN compared to those in spleen and liver (**Figures 5C,D**). In preliminary experiments in which we compared MNC isolations from LN or spleen with or without collagenase treatment, we observed that DC-SIGN expression was affected by collagenase digestion of the tissue (**Figure 5B**). Therefore, the expression levels of DC-SIGN on CD14⁺ cells isolated after collagenase digestion depicted in **Figure 5D** are probably under-estimations, except for liver cells which were always obtained as single cells present in liver perfusates and therefore not treated with collagenase. In all examined tissues the majority of CD14⁺ cells expressed CD163 (**Figure 5E**), but not CD206 (data not shown), suggesting that these cells represent macrophages (38, 40). Liver CD14⁺ cells represent most probably Kupffer cells, which have been shown to express low levels of DC-SIGN (41). The majority of CD14⁺DC-SIGN⁺CD206⁻ cells in LN have been shown to represent subcapsular macrophages (42,



43), while about one third expressed CD169 in addition to DC-SIGN (data not shown) and may therefore represent medullary sinus macrophages (43, 44). No CD14⁺CD169⁺ cells were found in spleen and liver. The activation status of the CD14⁺ cells was determined by the expression of HLA DR, CD80 and CD86. None of CD14⁺ cells in the examined tissues expressed CD80. Compared to CD14⁺ cells in inguinal LN, CD14⁺ cells in hepatic LN showed lower expression levels of HLA-DR and CD86, indicating a less activated status of these cells, although the presence of different macrophage sub-populations in the two types of LN cannot be excluded (Figures 5F,G).

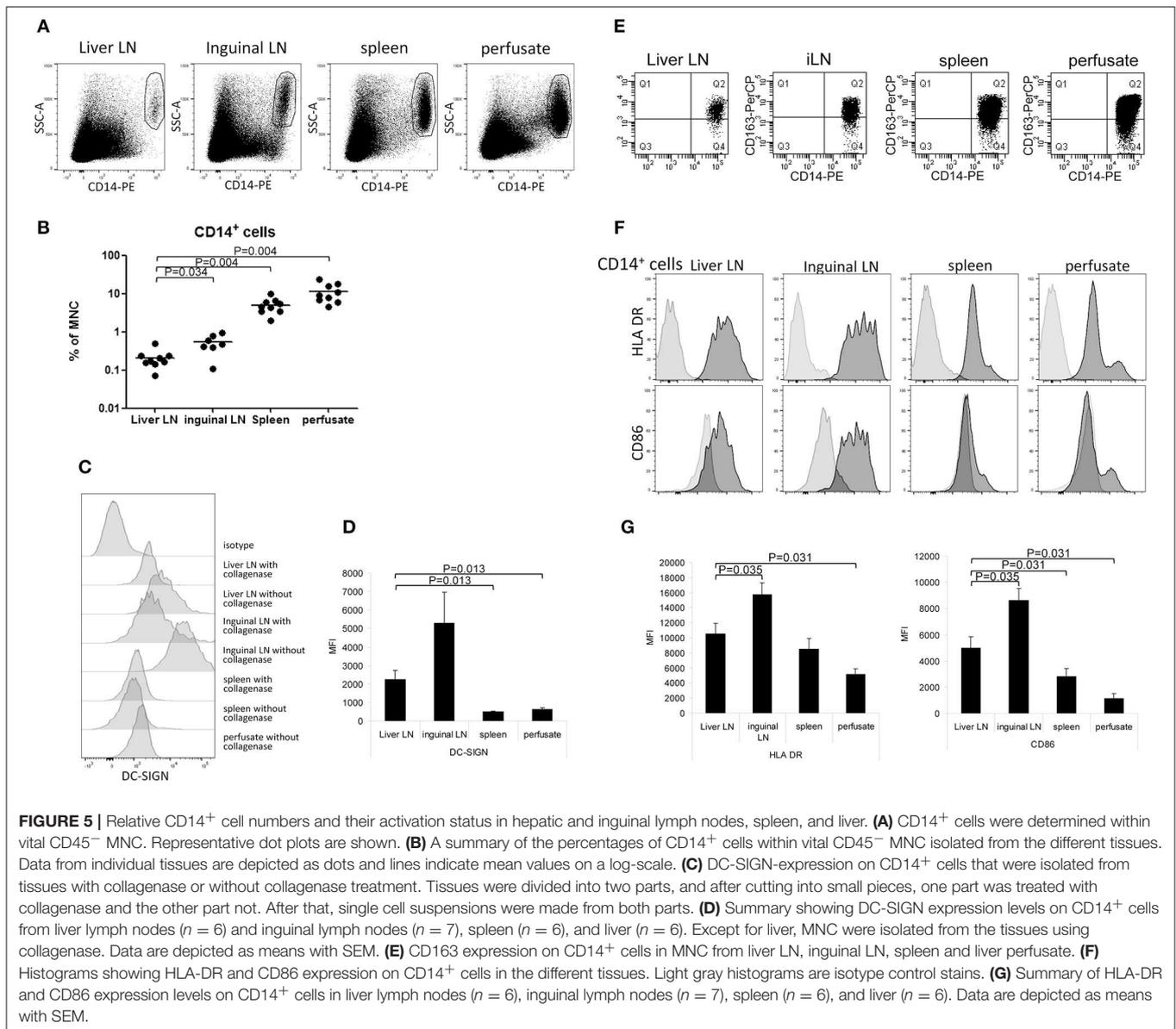
Decreased Numbers of cDC Subsets but Increased Numbers of Macrophages and PDC in Lymph Nodes of Diseased Livers

Finally, we studied whether inflammatory liver disease affects numbers of the different APC-subsets in hepatic LN. For this

purpose, we isolated MNC from freshly resected liver LN collected during liver transplant procedures from explant livers of patients with primary sclerosing cholangitis or auto-immune hepatitis, and compared relative numbers of the different APC subsets within CD45⁺ MNC with those in liver LN isolated from healthy livers of multi-organ donors. Interestingly, relative numbers of cDC1 were decreased, while relative numbers of macrophages and PDC were increased in hepatic LN of patients with inflammatory liver disease compared to hepatic LN from healthy liver (Figures 6A,B).

DISCUSSION

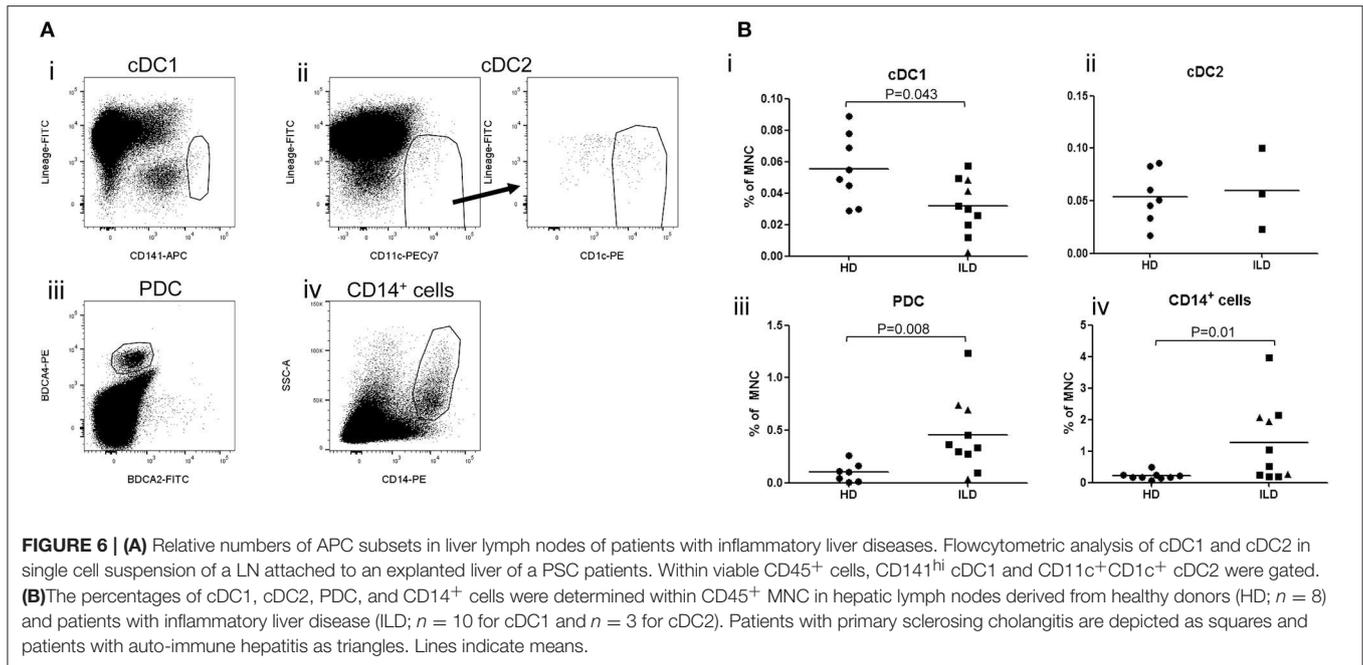
Previous studies have shown that human LN contain two main cDC subsets which correspond to the two main cDC subsets in blood: CD141^{bright} cDC1 and CD1c⁺cDC2 and (14, 38). The present study shows that the same cDC subsets are present in



liver LN. However, cDC2 are present at much lower relative numbers in hepatic LN compared to skin/muscle-draining inguinal LN and spleen, while cDC1 are a minor subset in both hepatic and inguinal LN. Like cDC1, cDC2 can stimulate both CD4⁺ and CD8⁺ T cells (10), but cDC2 can produce IL-12 while cDC1 cannot (39, 45). Since IL-12 is a main driver of T-helper 1 differentiation and cytotoxic CD8⁺ T cell responses, the reduced numbers of cDC2 in hepatic LN may contribute to the relatively low immunogenicity of the liver environment.

Interestingly, both cDC subsets displayed a more mature phenotype with higher expression of co-stimulatory molecules in hepatic LN compared to inguinal LN, iliacal LN, spleen, and liver. This observation is in line with a previous study showing that rat liver lymph contains highly mature MDC (46). The enhanced maturation status of cDC in hepatic LN may be caused

by stimulation by bacterial products, such as endotoxins, that continuously enter the liver from the intestinal tract via the portal circulation. Such stimulation may have occurred in the liver before migration of cDC to the sentinel LN or in the LN. Nevertheless, cDC2 isolated from hepatic LN showed a comparable T-cell stimulatory capacity, cytokine production and capacity to induce IFN γ production in naïve T-cells compared to those isolated from spleen. We speculate that the enhanced expression of the co-inhibitory ligand PDL-1 on MDC in hepatic LN may compensate for the enhanced expression of co-stimulatory molecules in T-cell stimulation. The equal capacity of hepatic LN and splenic cDC2 to induce IFN γ -production in naïve T cells is probably associated with their similar capacity to produce IL-12. Our current data show that cDC2 in human liver LN are not functionally compromised, as has been suggested



previously for cDC in mouse liver (19, 20, 22). Accordingly, we have previously shown that cDC2 isolated from human liver tissue are able to produce pro-inflammatory cytokines and acquire T-cell stimulatory capacity upon TLR-activation (28).

We expected that liver inflammation would enhance migration of cDC to the draining LN, and thereby cause increased numbers of cDC in hepatic LN. Indeed, induction of inflammation in the skin by local injection of a TLR-9 agonist and GM-CSF into the scar of resected melanoma resulted in selectively increased numbers of cDC1 in sentinel lymph nodes (47). Moreover, inflammatory liver disease is associated with depletion of cDC1 from human liver, and it was hypothesized that they might have migrated to hepatic LN due to inflammatory stimuli (25). In contrast to our expectation, relative numbers of cDC1 were reduced in LN attached to inflamed livers compared to those in LN of healthy livers. We hypothesize that the decreased relative numbers of cDC1 in hepatic LN in these patients might be caused by activation-induced cell death or, alternatively, that liver cirrhosis impedes migration of MDC through lymphatics to the regional LN. It is tempting to speculate that the paucity of cDC1 in sentinel liver LN in end-stage liver diseases may lead to a further mitigation of T-cell responses to liver-derived antigens compared to healthy livers.

We confirm our previous finding that hepatic LN (48) contain much lower numbers of PDC than skin/muscle draining inguinal LN. However, we now show that PDC from both types of LN do not differ in terms of activation/maturation status and T-cell stimulatory capacity. Differences in migration route between MDC and PDC may explain the similar maturation status of PDC in hepatic and inguinal LN. PDC do not migrate via the lymph to LN, but migrate from the blood circulation into LN via the high endothelial venules, and are mainly attracted by

the CCR-7 ligands CCL19 and CCL21 (49, 50). Therefore, they are probably not exposed to maturation stimuli present in the liver environment, like cDC. PDC are important in anti-viral defense by production of IFN- α , amongst others against HCV (51, 52), but can also present antigens to T-cells. They are able to prime productive effector T-cell responses (9, 16), but can also induce differentiation of CD4⁺ as well as CD8⁺ Treg (17, 18). The circumstances in which the balance tips toward activation of effector T cells or Treg are not well-understood, and probably depend on the micro-environmental conditions in the tissue in which they reside. In addition to their capacity to directly activate T cells, PDC interact with cDC to enhance their T-cell stimulatory capacity (53, 54). Together, it is difficult to speculate on the immunological consequences of the low numbers of PDC in hepatic LN under steady state conditions. However, in contrast to cDC, numbers of PDC are increased in sentinel LN of inflamed livers with cholestatic or auto-immune diseases. Apparently, hepatic LN can “normalize” their PDC content during liver inflammation. We speculate that liver inflammation leads to increased production of CCL19 and CCL21 and/or other chemo-attractants in hepatic LN, that induce migration of PDC into LN.

In addition to cDC and PDC, also macrophages are able to present antigens to T cells although less efficiently (55). Liver LN were found to contain 2-fold lower numbers of CD14⁺ macrophages than in skin/muscle draining inguinal LN. Based on their immunophenotype, the majority of these cells are probably subcapsular macrophages, whose main function is to capture lymph-borne viral particles and present them to B-cells (56). However, they are also able to cross-present lymph-derived antigens to CD8⁺ T cells (57, 58), and promote activation of NK-cells, NKT-cells, PDC and $\gamma\delta$ -T-cells, possibly via production of type I IFN, IL12 and IL18 (59). In contrast to cDC, CD14⁺ cells

in hepatic LN showed a reduced maturation status compared to their counterparts in inguinal LN. The origin of the macrophages in liver LN is currently unknown. One possibility is that (a subset of) Kupffer cells migrate via lymph to hepatic LN, but in rat it has been shown that liver lymph does not contain Kupffer cells (30). Since macrophages are also present in inguinal LN, even in higher numbers, we favor the hypothesis that these macrophages derived from circulating monocytes. This hypothesis would also explain the relatively low activation status of these cells in liver LN, because they have, like PDC, not been exposed to endotoxins present in the liver. It can also explain the increase in CD14⁺ cells in lymph nodes during inflammatory conditions, since monocyte chemoattractant protein (MCP)-1 increases at the luminal surface of high endothelial venules during inflammation, which enhances the migration of CD14⁺ cells into LN (60).

The strength of this study is that we are the first to comprehensively characterize APC subsets in human liver lymph nodes. Limitations of our study are that: (1) We could not study the functional properties of cDC1 in liver LN, because it was impossible to isolate sufficient numbers of this rare subset; (2) Since the numbers of fluorochrome channels on our flowcytometer were limited to 8, we could not include CD11c in all phenotypic analysis of cDC2. However, main findings on the phenotypes of cDC2 were confirmed in measurements that included CD11c in the gating strategy.

In summary, we observed that in steady state conditions human liver LN contain relatively low numbers of cDC2, PDC, and subcapsular macrophages. Whereas, numbers of cDC1 further decrease during liver inflammation and cirrhosis, numbers of PDC and subcapsular macrophages increase,

suggesting important contributions of these APC cell subsets to liver immunity. Functional studies are required to reveal whether these APC cell subsets do play a role in regulating liver immunity. Importantly, we found that cDC2 in hepatic LN are not dysfunctional. The paucity of both main subsets of cDC subsets in liver LN, both during steady state conditions and during liver inflammation, may contribute to the immunologically tolerant liver environment.

AUTHOR CONTRIBUTIONS

PB contributed to study concept and design, acquisition, analysis and interpretation of data, statistical analysis, drafting of the manuscript, critical revision of the manuscript for important intellectual content. BB contributed to study concept and design, acquisition, analysis, and interpretation of data. KT and JI contributed to material support and critical revision of the manuscript for important intellectual content. LvdL contributed to critical revision of the manuscript for important intellectual content. HM obtained funding and contributed to critical revision of the manuscript for important intellectual content. HH contributed to material support. JK contributed to study concept and design, interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, and study supervision.

FUNDING

This work was partly funded by the Foundation for Liver and Intestinal Research, Rotterdam, Netherlands.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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