Dendritic cell deficiencies persist seven months after SARS-CoV-2 infection

Running title: DC deficiency after SARS-CoV-2 infection

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ABSTRACT

Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV)-2 infection induces an exacerbated inflammation driven by innate immunity components. Dendritic cells (DCs) play a key role in the defense against viral infections, for instance plasmacytoid DCs (pDCs), have the capacity to produce vast amounts of interferon-alpha (IFN-α). In COVID-19 there is a deficit in DC numbers and IFN-α production, which has been associated with disease severity. In this work, we described that in addition to the DC deficiency, several DC activation and homing markers were altered in acute COVID-19 patients, which were associated with multiple inflammatory markers. Remarkably, previously hospitalized and non-hospitalized patients remained with decreased numbers of CD1c+ myeloid DCs and pDCs seven months after SARS-CoV-2 infection. Moreover, the expression of DC markers such as CD86 and CD4 were only restored in previously non-hospitalized patients, while no restoration of integrin β7 and indoleamine 2,3-dyoxigenase (IDO) levels were observed. These findings contribute to a better understanding of the immunological sequelae of COVID-19.

INTRODUCTION

Coronavirus disease 19 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and may progress with mild symptoms or asymptomatically in most of the individuals, while others experience an acute respiratory distress syndrome (ARDS) and poorer prognosis, including death [1]. Disease severity depends on the balance between host immune response, viral replication and tissue and organ damage. In severe COVID-19 there is a deregulation of this response, characterized by an hyper-inflammation driven by innate immunity, characterized by very high levels of cytokines and pro-inflammatory biomarkers, also known as cytokine storm [2,3].

One of the innate immune cell types that may play a pivotal role in the response against SARS-CoV-2 are the dendritic cells (DCs). There are two main DC types, conventional or myeloid DCs (mDCs) which include CD1c+, CD16+ and CD141+ mDC subsets, and plasmacytoid dendritic cells (pDCs). In general, DCs participate in antigen presentation, cytokine production, control of inflammatory responses, tolerance induction, immune cell recruitment, and viral dissemination. However, the role of these cells in response to acute SARS-CoV-2 infection and the recovery in convalescent subjects is not fully characterized. Some studies have shown a decrease of DC numbers in response to infection in peripheral blood [4] and also an association with disease severity [5]. This deficiency seems to be due to the migration of some DC subsets, such as CD1c, to the lung [6], and probably to other inflammatory foci. pDCs also seems to play a key role in COVID-19 [7]. pDCs are the main type I interferon (IFN-I) producers, with 1000-fold production compared to other immune cell types [8]. IFN-I is known to have an essential role in viral infections [9].

Significantly, pDCs depletion has been associated with poor COVID-19 prognosis [10]. Moreover, critical patients showed a highly impaired IFN-I response [7] associated with high viral load and aggravated inflammatory response [11].

The recovery of DC defects after COVID-19 could be crucial, since the normalization of the innate immune system after the acute insult would mean the system's readiness to respond to new viral and bacterial challenges. However, the recovery of DC cell numbers and function after COVID-19 is unknown. This recovery is also important in the sense that a variable proportion of people who have overcome COVID-19 show clinical sequelae [12] which relation with innate immune defects needs to be clarified. Thus, the aim of the study was to analyze DC defects associated with SARS-CoV-2 infection, COVID-19 severity and whether these defects were restored after a median of seven months after the resolution of the infection.

RESULTS

Patients with acute SARS-CoV-2 infection show a considerable decrease in DC percentages and TLR9-dependent IFN-α production

In order to investigate the effect of SARS-CoV-2 infection on the innate immune system, we first analyzed the percentages of total DCs and the different subsets in acute SARS-CoV-2 infected patients (COVID-19 patients) compared with age and sex matched healthy donors (HD). Specifically, we measured mDCs (CD123- CD11c+), including CD1c+, CD16+ and CD141+ mDC subsets, and pDCs (CD123+ CD11c-) (Supplementary Fig. 1a). Our results showed that acute COVID-19 patients exhibited a significant decrease in the percentages of total mDCs mainly due to CD1c+ mDCs decreased in comparison with HD. Meanwhile CD16+ and CD141+ mDCs remained at similar levels of HD (Fig. 1a). Remarkably, the percentage of pDCs in acute COVID-19 patients was considerably diminished with respect to HD (Fig. 1b left). Then, we calculated the ratio mDC/pDC in the different subjects, which was much lower in HD that in COVID-19 patients (Supplementary Fig. 2a). Additionally, based on previously published results[13], the following pDC subsets were analyzed: P1-pDC (CD86-PD-L1+), P2-pDC (CD86+PD-L1+) and P3-pDC (CD86+PD-L1-). Here, a lower percentage of P2- and P3-pDCs was observed in acute COVID-19 patients than in HD (Supplementary Fig. 2b). pDCs are known to be the main producers of IFN-α [8]. Therefore, to study their function in SARS-CoV-2 infection, we stimulated peripheral blood mononuclear cells (PBMCs) with CpG oligodeoxynucleotides class A (CpG)-A, a Toll-like receptor (TLR)-9 dependent stimulation, and we analyzed IFN-α production. We found that IFN-α production

in acute COVID-19 was much lower than in HD (Fig. 1b right). To clarify if the decreased IFN-α production was due to a diminished percentage of pDCs, we performed a correlation analysis and we found that the IFN-α production was positively associated with the percentage of pDCs in both acute COVID-19 patients and HD (Fig. 1c). In conclusion, patients with acute SARS-CoV-2 infection exhibit a deficit in DC numbers and also decreased TLR9-dependent IFN-α production.

Acute SARS-CoV-2 infected patients show an altered pattern of DC activation markers

Afterwards, we analyzed the expression of DC activation markers in acute COVID-19 patients and HD. We measured the expression of homing receptors ((integrin-β7 (β7) and C-C chemokine receptor type 7 (CCR7)), co-stimulatory molecules (CD86 and CD4), and markers of immune tolerance and suppression ((Indoleamine 2,3-dioxygenase (IDO) and Programmed Death-ligand 1 (PD-L1)) (Supplementary Fig. 1b). Most of the DC subpopulations, presented lower percentage of β7, specially total mDCs, CD1c+ mDCs and pDCs and a higher percentage of CCR7+ DCs in acute COVID-19 patients compared with HD (Table 1). We also found lower percentage of CD86+ cells in acute patients in CD1c+ and CD16+ mDCs and pDCs. No differences were in CD4+ DC levels (Table 1). Lastly, acute COVID-19 patients showed higher percentage of IDO+ cells within CD1c+ and CD16+ mDCs compared with HD, while a lower percentage PD-L1+ was seen within pDCs (Table 1). These results are indicative of alterations in different homing and activation patterns of DCs in response to SARS-CoV-2 infection.

IFN-α production is associated with COVID-19 severity

The next step of this study was to investigate whether DC numbers and their function might be different in acute COVID-19 depending on disease severity. Therefore, we classified acute COVID-19 patients in two groups: severe ((high oxygen support requirement and Intensive Care Unit (ICU) admission or death)) and mild (low oxygen requirement and no ICU admission) (Supplementary Table 1). Our results did not show any significant difference in the percentage of mDCs and subpopulations and pDCs between severe and mild COVID-19 patients (Fig. 2a). However, we found increased levels of total CCR7+ mDCs and PD-L1+ CD141+ mDCs in severe patients (Supplementary Fig. 3). Importantly, we did find a considerable decrease in TLR9-dependent IFN-α production in severe subjects compared to mild patients (Fig. 2b). In summary, acute SARS-CoV-2 infected patients with severe symptoms exhibit a lower capacity to produce IFN-α than patients with mild symptoms.

DC parameters are differentially associated to inflammation markers in mild and severe acute SARS-CoV-2 infected patients

Then, DC numbers and activation markers were correlated to multiple inflammatory marker levels, including clinical biomarkers ((C-reactive protein (CRP), D-dimers and lactate dehydrogenase (LDH)), pro-inflammatory cytokines ((tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8, IL1-β, macrophage inflammatory protein (MIP1)-α, MIP1-β, interferon inducible protein (IP)-10 and interferon (IFN)-γ) and soluble (sCD25)), and neutrophil numbers. These correlations were done in the overall group of patients during acute

infection and also dividing in both severe and mild COVID-19. In the overall population, we observed correlations of dendritic cell subset levels with different pro-inflammatory cytokines and clinical biomarker levels (Supplementary Fig. 4). Interestingly, we observed a different correlation pattern in severe and mild patients and of note, more associations were found in mild patients (Fig. 3). On one hand, regarding COVID-19 patients with mild symptoms, the percentages of DC subpopulations were inversely correlated with D-dimers, IL-6, IL-8, sCD25 levels and neutrophil numbers, while they were positively correlated with TNF-α, IL-1β, MIP-1α, MIP1-β and IFN-γ levels, with the exception of CD16+ mDCs that were negatively correlated with most of the inflammatory parameters. It is remarkable, that the percentage of pDCs showed a strong inverse correlation with D-dimer levels and neutrophil numbers. Focusing on DC homing and activation markers, regarding the expression of β7 in DCs, inverse associations prevailed, highlighting the strong correlations found in CD16+ β7+ mDCs with D-dimers and in β7+ pDCs with IL1-β. In contrast, the expression of CD86 and IDO in DCs was predominantly positively associated to several inflammatory markers, mainly in the case of CD141+ mDCs and pDCs (Fig. 3a). On the other hand, in severe COVID-19, many associations were lost (e.g. IDO expression) and others were opposite (e.g. CD86), comparing with mild patients. For instance, remarkably, the DC percentages and the expression of β7 and CD86, the associations found with inflammatory marker levels showed an opposite trend. (Fig. 3b). Therefore, we conclude that DC levels and activation markers are associated to the inflammatory status of acute SARS-CoV-2 infected subjects, with a differential profile between patients with severe symptoms compared to those with mild symptoms.

CD1c+ mDC and pDC levels and IFNα production are not normalized seven months after SARS-CoV-2 infection

Apart from COVID-19 patients in acute phase, we also studied patients after seven months of SARS-CoV-2 infection (median 208 interquartile range [IQR] [189 – 230]) days after symptoms' onset, Supplementary Table 1). Some of these patients were hospitalized during acute infection (Hosp 6M), while others were not (No Hosp 6M). We analyzed the percentages of DC subpopulations in these two groups and compared with HD's levels. First, we observed a higher percentage of total mDCs on previously hospitalized patients compared with HD (Fig. 4a). Regarding mDC subpopulations, while the percentages of CD141+ and CD16+ were not altered, the percentage of CD1c+ mDCs remained lower in patients after seven months compared with HD (Fig. 4b-d). Remarkably, the percentage of pDCs also persisted very low and was not restored seven months after the infection in these both groups (Fig. 4e), confirmed by the mDC/pDC ratio (Supplementary Fig. 5a). Moreover, the percentage of P1-pDCs (CD86- PD-L1+) was only reduced in previously hospitalized patients comparing with HD, unlike P2- (CD86+ PD-L1+) and P3-pDCs (CD86+ PD-L1-), that were decreased in both hospitalized and non-hospitalized ones (Supplementary Figure 5b). Next, to corroborate that our results were reproducible applying a paired analysis, we studied DC kinetic in a subgroup of subjects with available paired samples, analyzing the percentages of DC subpopulations in the acute phase, 6-8 months later and comparing them with HD. Even though the sample size was lower because of the sample availability, these results reproduced the analysis with unpaired samples (Supplementary Fig. 6).

When we measured the TLR9-dependent IFN-α production, we found that hospitalized patients seven months after the infection showed a lower IFNα production than HD, unlike non-hospitalized patients, which display a similar production comparing with HD (Figure 4f). Here, we conclude that the deficit of CD1c+ mDCs and pDCs is maintained seven months after SARS-CoV-2 infection independently of whether the patients were or not previously hospitalized, and that IFNα production is not restored in previously hospitalized patients seven months after infection.

Some DC activation markers are not normalized in previously hospitalized patients seven months after SARS-CoV-2 infection

Afterwards, we measured the DC activation and homing markers in previously hospitalized and non-hospitalized patients seven months after infection, and we compared them with the ones from HD. We observed that the expression of CD86 was lower in CD16+ and CD1c+ mDC subsets from hospitalized patients than in non-hospitalized ones and HD (Fig. 5a-b). Similar results were found in the expression of PD-L1 in total mDCs (Fig. 5c). Furthermore, hospitalized patients also showed lower levels of CD4 in total mDCs, CD1c+ and CD141+ mDCs and pDCs (Fig. 5d-g). In contrast, pDCs from hospitalized patients exhibited higher percentage of CCR7+ cells within pDCs compared with non-hospitalized ones and HD (Fig. 5h). In summary, these results show a recovery of some DC activation markers, mainly CD86 and CD4, only in previously non-hospitalized patients, while in more severe patients who required hospitalization, the defects in these markers persisted seven months after infection.

Some DC activation markers are not normalized neither in previously hospitalized nor in non-hospitalized patients seven months after SARS-CoV-2 infection

Importantly, when we focused on the expression of other DC activation markers, we observed a lower percentage of β7+ cells in all mDCs and pDCs from both hospitalized and non-hospitalized patients after seven months of infection compared to HD (Fig. 6a-e). The levels were also lower for IDO+ in total mDCs, CD1c+ and CD141+ mDCs and pDCs (Fig. 6f-i). Lastly, we also found that both hospitalized and non-hospitalized patients seven months after infection showed lower percentages of CCR7+ and CD4+ cells within CD16+ mDCs and PD-L1+ cells within pDCs compared to HD (Fig. 6j-l). In conclusion, we demonstrated that the alterations in integrin β7 and IDO, associated with migration and tolerance, are not restored to normal levels neither in previously hospitalized nor in non-hospitalized patients seven months after SARS-CoV-2 infection.

DISCUSION

The present study revealed that the deficits observed in CD1c+ mDCs and pDCs levels associated with altered homing and activation patterns in SARS-CoV-2 infected subjects in acute phase, were not restored beyond seven months after infection. Importantly, this long-term defects related to DC migration and tolerogenesis (integrin β7 and IDO expression) were present independently of whether or not the patients were previously hospitalized. In addition, hospitalized patients showed additional deficiencies related with DC activation.

pDCs are known to have an important role in the first line of defense against viral replication, which mainly resides in their capacity to produce IFN-I via TLR-7/8 stimulation [14]. In this study, we first observed that acute SARS-CoV-2 infected patients displayed a dramatic decrease in pDC levels and a considerable reduction of IFN-α production. The strong direct correlation between pDC levels and IFN-α production suggested that this cell type was the main producer of this cytokine as it happens in other viral infections [15]. In fact, SARS-CoV-2 is known to induce pDC activation, accompanied by a high production of IFN-I and other cytokines, which is critically depended on IRAK4 and UNC93B[16]. The observed reduction of IFN-α is in accordance with previous studies in animal models of SARS-CoV-1 infection, which associated this deficit with lethal pneumonia [17] and is also consistent with recently published data following transcriptomic approaches [11] and intracellular cytokine staining after TLR stimulation in SARS-CoV-2 infection [7]. Importantly, the low IFN-α production was the main parameter associated with disease severity, in agreement with previous studies [7,11], highlighting the potential use of this

291 measurement as an early biomarker of disease progression. The mechanisms
292 behind the attenuated IFN response have been related with viral antagonism of
293 STAT1 (Signal transducer and activator of transcription 1) phosphorylation [18]
294 and significantly, life-threatening ARDS in COVID-19 patients have been
295 associated with neutralizing auto-antibodies against IFN-I [19,20] and other inborn
296 errors of IFN-I immunity [21]. Furthermore, single cell RNA sequencing of antigen-
297 presenting cells revealed a lower expression of IFNAR1 and 2 in severe
298 COVID-19 patients, suggesting a defect in IFN-α signaling, and also a down-
299 regulation of IFN-stimulated genes in both moderate and severe patients[22]. All
300 these results support the essential role of IFN-I production in the first line of
301 defense in COVID-19 for avoiding disease progression and point out to early
302 immunotherapeutic strategies targeting this pathway. Remarkably, our results
303 showed that, seven months after SARS-CoV-2 infection, the IFNα production is
304 not completely restored to normal levels, but only in previously hospitalized
305 patients. This might be associated to the deficit in P1-pDCs found in
306 hospitalized patients but not in non-hospitalized ones, being this pDC subset
307 the main source of IFN-I[13]. Thus, our findings are indicative of a deficiency not
308 only in pDC numbers but also in their function seven months after SARS-CoV-2
309 infection in patients that were previously hospitalized.

310 Apart for IFN-I deficiency, one of the hallmarks of acute COVID-19 is the
311 detection in plasma of heightened levels of soluble pro-inflammatory cytokines
312 inducing a cytokine storm [23]. Here, we found multiple correlations between DC
313 numbers and activation markers with inflammatory marker and cytokine levels
314 in acute SARS-CoV-2 infected patients. It was remarkable that the lower
315 percentage of DCs was associated to higher levels of IL-6 and higher neutrophil

316 numbers. High levels of IL-6 in COVID-19 patients have been widely related to
317 a poorer disease progression[10]. Moreover, neutrophils have been described as
318 crucial drivers of hyperinflammation in COVID-19 [24]. It has to be also
319 underlined, that the percentage of DCs expressing integrin β7 was inversely
320 correlated to numerous inflammatory marker levels. These results suggest the
321 hypothesis that not DC *per se* but DC migration to inflammatory sites may
322 importantly contribute to the cytokine storm observed in SARS-CoV-2 infected
323 patients. Our results also showed that patients with distinct level of disease
324 severity displayed different associations of DC numbers and activation markers
325 with inflammation. Therefore, DCs might be important contributors to the high
326 inflammatory status characteristic of COVID-19 patients and this may dictate
327 subsequent clinical progression.

328 The decreased numbers of total mDCs, CD1c+ mDCs and pDCs found in acute
329 SARS-CoV-2 infected patients were in accordance with previous publications[5,6].
330 This fact might be explained by different mechanisms, including apoptosis due
331 to increased inflammatory mediators produced by abortive SARS-CoV-2
332 infection of myeloid cells [25]. Another non-exclusive explanation could be that
333 DCs migrate from peripheral blood to tissues or inflammatory sites, such as
334 CD1c+ mDCs preferential migration to the lungs in patients with severe COVID-
335 19 [6]. These defects were accompanied by alterations mainly found in activation,
336 migration and tolerogenic markers that importantly persisted seven months after
337 infection in previously hospitalized and also in non-hospitalized patients.
338 Especially persistent in the total and DC subsets was the decreased expression
339 of integrin β7. The expression of αEβ7 defines migration to antigen presentation
340 sites within lymph nodes [26] and α4β7 on mDCs and pDCs is indicative of

migration of these cells to gut [27]. Remarkably, SARS-CoV-2 has been shown to infect and productively replicate in human small intestinal organoids, increasing cytokine production and human angiotensin-converting enzyme 2 expression [28]. It has been also reported, that the disruption in gut barrier integrity contributes to COVID-19 severity (Giron et al., CROI 2021). Thus, the lower percentage of DCs expressing integrin β7 in peripheral blood might be a consequence of ongoing DC migration to the gut or other tissues or inflammatory sites up to seven months after infection. In fact, necropsy studies in SARS-CoV-2 infected patients have shown mononuclear inflammatory infiltrates in different organs [29]. Also prominent was the deficit in IDO expressing DCs seven months after infection. In contrast, IDO+ CD1c+ and CD16+mDC levels in acute infection were dramatically increased compared to HD. This is in agreement with other acute respiratory infections such influenza [30] and respiratory syncytial virus [31] in which IDO expression is increased in order to counteract excessive inflammation as happen after acute SARS-CoV-2 infection. However, in this infection, the tissue damage in low respiratory tract is prominent [32] and may persist at the long-term what may cause the exhaustion of IDO producing DCs and/or migration of these cells to inflammatory focus even after seven months after infection. Although these defects were present independently of whether or not the participants were previously at the hospital, hospitalized patients showed additional defects. These were, lower expression of the co-stimulatory molecule CD86, found in acute infection also by other authors [5,7,24] that persisted seven months after infection together with lower levels of CD4+ DCs. Low levels of activation molecules, such as CD86 have been related with a possible impairment in T cell and DCs response to the virus. Specifically, we

366 and others have found pDC hypo-responsiveness to HIV after CD4
367 downregulation in this cell type [15,33]. On the contrary, CCR7+ pDCs remained at
368 high levels even after seven months after infection indicating again ongoing
369 migration to lymph node or other inflammatory foci. In this line, the higher
370 expression of other chemokine receptors such as CCR1, CCR3 and CCR5 has
371 previously described in SARS-CoV-1 infected monocyte derived DCs [34].

372 It is unknown whether these defects in the DCs compartment will be reversible
373 after longer follow up or specific therapies may be needed for the normalization
374 of these defects. What is clear is that persisting symptoms and unexpected
375 substantial organ dysfunction are observed in an increasing number of patients
376 who have recovered from COVID-19 [12]. Actually, Huang C *et al.* recently
377 described that seven months after illness onset, 76% of the SARS-CoV-2
378 infected patients reported at least one symptom that persisted, being fatigue or
379 muscle weakness the most frequently reported symptoms [35]. In addition, many
380 of those previously hospitalized patients presented residual chest imaging
381 abnormalities, impaired pulmonary diffusion capacity and other extrapulmonary
382 manifestations as a low estimated glomerular filtration rate [35]. The immune
383 mechanisms that might be involved in the development of these persisting
384 symptoms are still unknown. However, it would be expected that seven months
385 after SARS-CoV-2 infection there is still an inflammatory response due to
386 persistent tissue damage or persistence presence of viral antigens in the
387 absence of viral replication which may cause these deficits in DC. In fact, it has
388 been reported that SARS-CoV-2 can persist in the intestines up to seven
389 months following symptoms resolution (Tokuyama et al., CROI 2021). Thus, we
390 postulate that the decrease in peripheral DCs numbers, along with the

391 alterations in DC homing and activation markers seven months after the
392 infection might be indicative of DC migration to inflammatory sites which may be
393 contributing to long-term symptoms, a phenomenon also known as long COVID.

394 One of the limitations of this study might be that, for a more precise
395 identification of pDCs, CD2+, CD5+ and AXL+ cells should have been
396 excluded[36]. Nevertheless, since these cell populations are barely represented
397 within PBMCs, the showed results correspond mainly to pDCs, although some
398 contamination with AS-DC cannot be excluded. The same happened with
399 CD123+ mDCs, which were not included in our gating strategy, however, the
400 levels of this subset was so low that did not change total mDC levels (data not
401 shown). Moreover, a limitation of this work might be that all patients included in
402 this study belong to the first wave of COVID-19 in Spain. It would have been
403 interesting to have access to tissue samples, however due to safety issues at
404 that moment of the pandemic it was not possible. At that time, different
405 experimental treatments with very limited but transitory immunosuppressive
406 effects were administered what may have affected the levels of immune
407 parameters. However, the agreement of our observations with other data in the
408 literature during acute infection and the persistence of these defects seven
409 months after infection minimized the potential bias of these treatments in our
410 results.

411 In summary, we have demonstrated that SARS-CoV-2 infected patients showed
412 a deficit in some DC subsets and alterations in DC homing and activation
413 markers, which are not restored more than seven months after the infection
414 independently of previous hospitalization. Our results suggest that there is an
415 ongoing inflammation which could be partially induced by DCs, these findings

might contribute to a better understanding of the immunological sequelae of COVID-19.

MATERIALS AND METHODS

Study participants

Seventy one participants with confirmed detection of SARS-CoV-2 by reverse-transcription polymerase chain reaction (RT-PCR) were included. Out of these 71, 33 were hospitalized in acute phase of COVID-19 from March 25th to May 8th 2020, while 38 participants were recruited seven months after being diagnosed with COVID-19, from September 9th to November 26th 2020. These participants came from the COVID-19 patients' Cohort Virgen del Rocio University Hospital, Seville (Spain) and the COVID-19 Cohort IIS Galicia Sur (CohVID GS), Vigo (Spain). Twenty-seven healthy donors (HD), with cryopreserved pre-COVID-19 samples (May 12th to July 18th 2014) were included from the HD cohort, collection of samples of the Laboratory of HIV infection, Andalusian Health Public System Biobank, Seville (Spain) (C330024). Written or oral informed consent was obtained from all participants. The study was approved by the Ethics Committee of the Virgen del Rocio University Hospital (protocol code "pDCOVID"; internal code 0896-N-20). Hospitalized participants during the acute phase of infection were divided in Mild (n=17) or Severe (n=16), based on the highest grade of disease severity during course of hospitalization. Severe participants were those who required Intensive Care Unit admission, or having ≥6 points in the score on ordinal scale based on Beigel et al.[37] or death. Blood samples were collected at a median of 3 [interquartile range (IQR) 2 - 23] days after hospitalization and 14 [9 - 31] days after symptoms onset (Supplementary Table 1). The group of participants discharged after infection, included previously hospitalized (n=21) and previously non hospitalized subjects (n=17). The samples from these

participants were collected after a median of 201 [181 - 221] days after hospitalization and 208 [189 - 230] days after symptoms onset (Supplementary Table 1). COVID-19 participants in the different groups were age and sex matched with HDs' group (Supplementary Table 1).

Cell and plasma isolation

PBMCs from healthy donors and participants were isolated from fresh blood samples using BD Vacutainer® CPT™ Mononuclear Cell Preparation Tubes (with Sodium Heparin, BD Cat# 362780) in a density gradient centrifugation at the same day of blood collection. Afterwards, PBMCs were cryopreserved in freezing medium (90% of fetal bovine serum (FBS) + 10% dimethyl sulfoxide) in liquid nitrogen until further use. Plasma samples were obtained using BD Vacutainer™ PET EDTA Tubes centrifugation, aliquoted and cryopreserved at -80°C until further use.

Dendritic cell immunophenotyping

For dendritic cells (DCs) flow cytometry, PBMCs were centrifuged, pelleted and washed with Phosphate-buffered saline (PBS) and stained for 35 min at room temperature with LIVE/DEAD Fixable Aqua Dead Cell Stain (Life Technologies), BV421 CD86, BV650 CD11c, BV711 HLA-DR, BV786 CCR7 (CD197), FITC Lin-2 (CD3, CD14, CD19, CD20, CD56), BV605 CD16, PeCF594 PD-L1 (CD274), APC Integrin-β7 (BD Biosciences), PerCPCy5,5 CD4, APCCy7 CD1c, PeCy7 CD141 (BioLegend) and AF700 CD123 (R&D, San Diego, CA) antibodies. Then PBMCs were washed with Permeabilization Buffer 10X diluted 1:10 (eBioscience™), permeabilized by Fixation/Perm buffer (eBioscience™), and intracellularly stained with PE IDO (eBioscience, San Diego, CA, USA)

antibody. DCs were gated based on Lin-2 HLA-DR expression. Each subset (mDCs and pDCS) was gated based on CD123 and CD11c expression. mDCs subsets were gated by using CD16, CD1c and CD141 staining, for gating strategy see Supplementary Fig. 1. Flow cytometry analyses were performed on an LRS Fortessa flow cytometer using FACS Diva software (BD Biosciences). Data were analyzed using the FlowJo software (Treestar, Ashland, OR). At least $1x10^6$ events were acquired per sample.

Cell culture and IFN-α quantification

$1x10^6$ thawed PBMCs were incubated at 37 □C and 5% CO_2 during 18 hours in RPMI with 10% FBS without any stimuli or with 1 µM CpG-A (ODN 2216; InvivoGen). After incubation, cells were pelleted and the supernatants conserved for the subsequent quantification of IFN-α production at -80°C. The amount of IFN-α in cell culture supernatants was assessed by an IFN-α multisubtype enzyme-linked immunosorbent assay kit (PBL Interferon Source Cat# 41105) according to the manufacturer's instructions.

Cytokine quantification in plasma

Plasmas previously collected were used for the quantitative determination of cytokines. We used 3 different kits to quantify sCD25 by Human CD25/IL-2R alpha Quantikine ELISA Kit (R&D System, Cat# DR2A00), IP-10 by Human IP-10 ELISA Kit (CXCL10) (Abcam, Cat# ab173194) and IL-6, IL-8, IL-1β, TNF-α, IFN-γ, MIP-1α, MIP-1β by MILLIPLEX MAP Human High Sensitivity T Cell Panel (Merck Cat# HSTCMAG-28SK) according to the manufacturer's instructions.

Statistics

Statistical analyses were performed by using Statistical Package for the Social Sciences software (SPSS 25.0; SPSS, Inc., Chicago, IL) and R environment 4.0.3 (2020-10-10), using RStudio Version 1.3.959 as the work interface and GraphPad Prism, version 8.0 (GraphPad Software, Inc.). ROUT method was utilized to identify and discard outliers. Differences between conditions among different groups were analyzed by two-tailed Mann-Whitney U test. The Wilcoxon test was used to analyze paired samples. The Spearman test was used to analyze correlations between variables. All differences with a P value of < 0.05 were considered statistically significant.

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AUTHOR CONTRIBUTIONS

A.P.-G., J.V. and M.C.G.-C. performed the experiments, analyzed and interpreted the data and participated in writing of the manuscript. A.G.V., M.T.R., A.S.G., E.M.M. participated in data collection, data analysis and interpretation and performed experiments, M.R.J.-L., M.R.I.B. participated in manuscript data interpretation, I.R.-J., C.I., J.C.C. participated in data collection, C.S., C.R.-O., N.E., A.F.-V., M.C. participated in data collection and manuscript interpretation. L.F.L.-C. and E.P. participated in manuscript data analysis, patient and data collection, interpretation/ discussion of the results and coordination. E.R.-M., participated in data analysis and interpretation, writing,

conceived the idea and coordinate the project. A.P.-G., J.V. and M.C.G.-C. contributed equally to this work.

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CONFLICT OF INTEREST

The authors declare no competing interests.

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FIGURE LEGENDS

Figure 1. Patients with acute SARS-CoV-2 infection show a considerable decrease in DC percentages and TLR9-dependent IFN-α production

Bar graphs representing the percentage of total mDCs, CD1c+, CD141+ and CD16+ mDCs (A) and the percentage of pDCs and IFN-α production in response to CpG-A (B) in acute SARS-CoV-2 infected patients (acute) and healthy donors (HD). The median with the interquartile range is shown. (C) Correlation between the percentage of pDCs and IFN-α production in acute patients and HD. Each dot represents an individual. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Mann-Whitney U test was used for groups' comparisons and Spearman test for non-parametric correlations.

Figure 2. IFN-α production is associated with COVID-19 severity

Bar graphs representing the percentage of total mDCs, CD1c+, CD141+ and CD16+ mDCs subsets (A) and the percentage of pDCs and IFN-α production in response to CpG-A (B) in acute severe and mild SARS-CoV-2 infected patients. The median with the interquartile range is shown and each dot represents an individual. *p < 0.05. Mann-Whitney U test was used for groups' comparisons.

Figure 3. DC parameters are differentially associated to inflammatory markers in mild and severe acute SARS-CoV-2 infected patients

Heatmap graphs representing correlations between the percentages of DC subpopulations and the percentages of DCs expressing activation and homing

markers with inflammatory marker levels including CRP, D-dimer, LDH, TNF-α, IL-6, IL-8, IL1-β, MIP1-α, MIP1-β, IFN-γ, CD25, IP-10 and neutrophil numbers, in mild (A) and severe (B) SARS-CoV-2 infected patients. Blue color represents positive correlations and red color shows negative correlations. The intensity of the color indicates the R coefficient. The most relevant data are highlighted with black squares. *p < 0.05, **p < 0.01, ***p < 0.001. Spearman test was used for non-parametric correlations.

Figure 4. CD1c+ mDC and pDC levels and IFNα production are not normalized seven months after SARS-CoV-2 infection

Bar graphs representing the percentage of total mDCs, CD1c+, CD141+ and CD16+ mDCs, pDCs (A - E) and the IFNα production (F) in previously hospitalized (Hosp 7M) or previously non-hospitalized (No Hosp 7M) patients seven months after SARS-CoV-2 infection and in healthy donors (HD). The median with the interquartile range is shown and each dot represents an individual. **p < 0.01, ****p < 0.0001. Mann-Whitney U test was used for groups' comparisons.

Figure 5. Some DC activation markers are not normalized in previously hospitalized patients seven months after SARS-CoV-2 infection

Bar graphs representing the percentage of DC subpopulations expressing CD86 (A - B), PD-L1 (C), CD4 (D - G) and CCR7 (H) in previously hospitalized (Hosp 7M) or previously non-hospitalized (No Hosp 7M) patients 7 months after SARS-CoV-2 infection and in healthy donors (HD). The median with the

760 interquartile range is shown and each dot represents an individual. *p < 0.05,
761 **p < 0.01, ***p < 0.001, ****p < 0.0001. Mann-Whitney U test was used for
762 groups' comparisons.

763

764 **Figure 6. Some DC activation markers are not normalized neither in**
765 **previously hospitalized nor in non-hospitalized patients seven months**
766 **after SARS-CoV-2 infection**

767 Bar graphs representing the percentage of DC subpopulations expressing β7 (A
768 - E), IDO (F - I), CCR7 (J), CD4 (K) and PD-L1 (L) in previously hospitalized
769 (Hosp 7M) or previously non-hospitalized (No Hosp 7M) patients 7 months after
770 SARS-CoV-2 infection and in healthy donors (HD). The median with the
771 interquartile range is shown and each dot represents an individual. *p < 0.05,
772 **p < 0.01, ***p < 0.001, ****p < 0.0001. Mann-Whitney U test was used for
773 groups' comparisons.

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Table 1. Acute SARS-CoV-2 infected patients show an altered pattern of DC homing and activation markers

Activation markers	Dendritic Cells	Acute	HD	p
Beta7	**mDCs**	**5.7 [3.2-11.1]**	**22.1 [15.7-33.8]**	**<0.0001**
	CD1c+ mDCs	**43.8 [26.2-62.2]**	**62.0 [38.8-69.7]**	**0.0340**
	CD16+ mDCs	0.0 [0.0-0.0]	0.03 [0.00-0.0]	0.0851
	CD141+ mDCs	15.6 [9.9-28.2]	25.9 [18.8-33.3]	0.0547
	pDCs	**2.2 [0.5-3.3]**	**6.1 [3.2-10.4]**	**0.0004**
CCR7	**mDCs**	**3.1 [1.4-21.7]**	**0.9 [0.3-1.5]**	**<0.0001**
	CD1c+ mDCs	**18.2 [6.4-94.3]**	**4.5 [2.4-8.1]**	**<0.0001**
	CD16+ mDCs	**3.1 [0.3-14.4]**	**0.3 [0.1-0.5]**	**0.0015**
	CD141+ mDCs	**11.0 [3.1-18.5]**	**1.9 [0.6-4.6]**	**0.0005**
	pDCs	**0.9 [0.2-15.6]**	**0.0 [0.0-0.0]**	**<0.0001**
CD86	mDCs	64.9 [37.9-77.4]	57.7 [50.4-65.2]	0.4447
	CD1c+ mDCs	**5.5 [2.8-11.2]**	**12.8 [6.7-17.4]**	**0.0053**
	CD16+ mDCs	**95.8 [88.5-97.5]**	**98.1 [96.7-98.7]**	**0.0034**
	CD141+ mDCs	7.6 [0.0-22.3]	5.9 [2.6-13.6]	0.9075
	pDCs	**0.3 [0.0-0.9]**	**1.4 [0.4-2.2]**	**0.0024**
CD4	mDCs	8.8 [2.5-24.1]	5.6 [4.0-10.6]	0.3229
	CD1c+ mDCs	27.0 [7.7-61.5]	23.0 [15.7-51.1]	0.6457
	CD16+ mDCs	8.5 [2.2-16.5]	10.7 [6.6-14.6]	0.3194
	CD141+ mDCs	21.3 [10.6-51.1]	40.0 [21.1-47.7]	0.1109
	pDCs	57.3 [46.6-77.9]	70.5 [59.0-87.9]	0.0750
IDO	mDCs	1.3 [0.8-3.1]	2.0 [1.5-2.8]	0.1469
	CD1c+ mDCs	**9.1 [2.4-23.2]**	**2.2 [1.6-4.5]**	**0.0039**
	CD16+ mDCs	**6.8 [0.1-27.2]**	**0.4 [0.0-0.7]**	**0.0004**
	CD141+ mDCs	72.1 [56.9-85.7]	69.2 [59.4-79.5]	0.6460
	pDCs	0.0 [0.0-0.1]	0.1 [0.0-0.3]	0.2071
PDL1	mDCs	17.4 [5.1-34.4]	21.8 [10.1-41.3]	0.2531
	CD1c+ mDCs	0.9 [0.4-5.2]	0.6 [0.2-1.7]	0.1810
	CD16+ mDCs	28.6 [13.8-47.9]	21.0 [6.1-41.0]	0.2107
	CD141+ mDCs	0.0 [0.0-2.0]	0.9 [0.0-2.7]	0.2166
	pDCs	**0.8 [0.2-2.1]**	**5.6 [3.1-8.7]**	**<0.0001**

Percentages of dendritic cells positive for activation markers in acute SARS-CoV-2 infected patients (Acute) and healthy donors (HD) are presented. The median with interquartile ranges [IQR] is shown. Significant differences are indicated in bold.

Figure 1

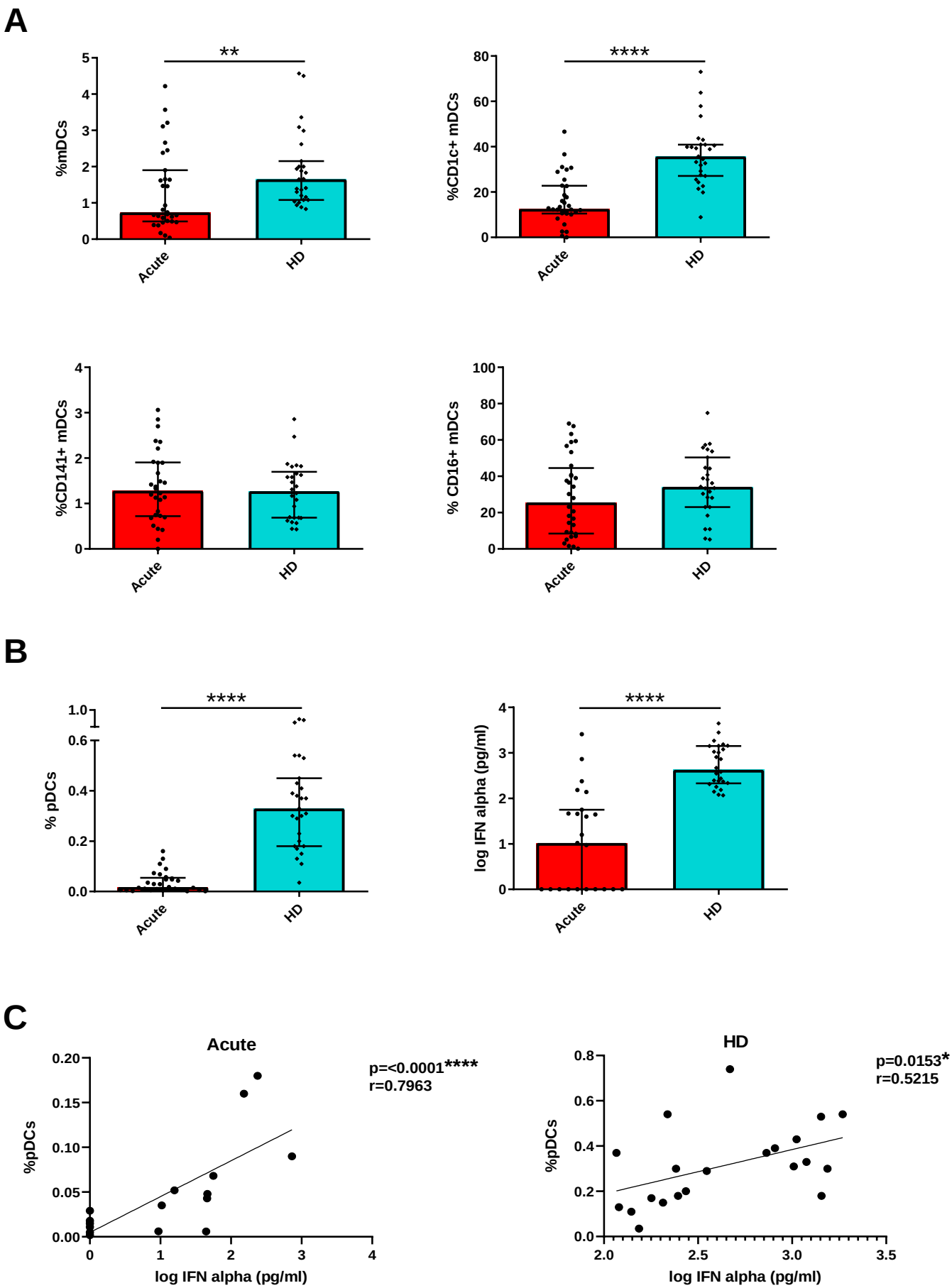

A
%mDCs
**
Acute
HD
%CD1c+ mDCs
****
%CD141+ mDCs
% CD16+ mDCs
B
% pDCs
****
log IFN alpha (pg/ml)
C
Acute
%pDCs
p=<0.0001****
r=0.7963
log IFN alpha (pg/ml)
HD
p=0.0153*
r=0.5215

Figure 2

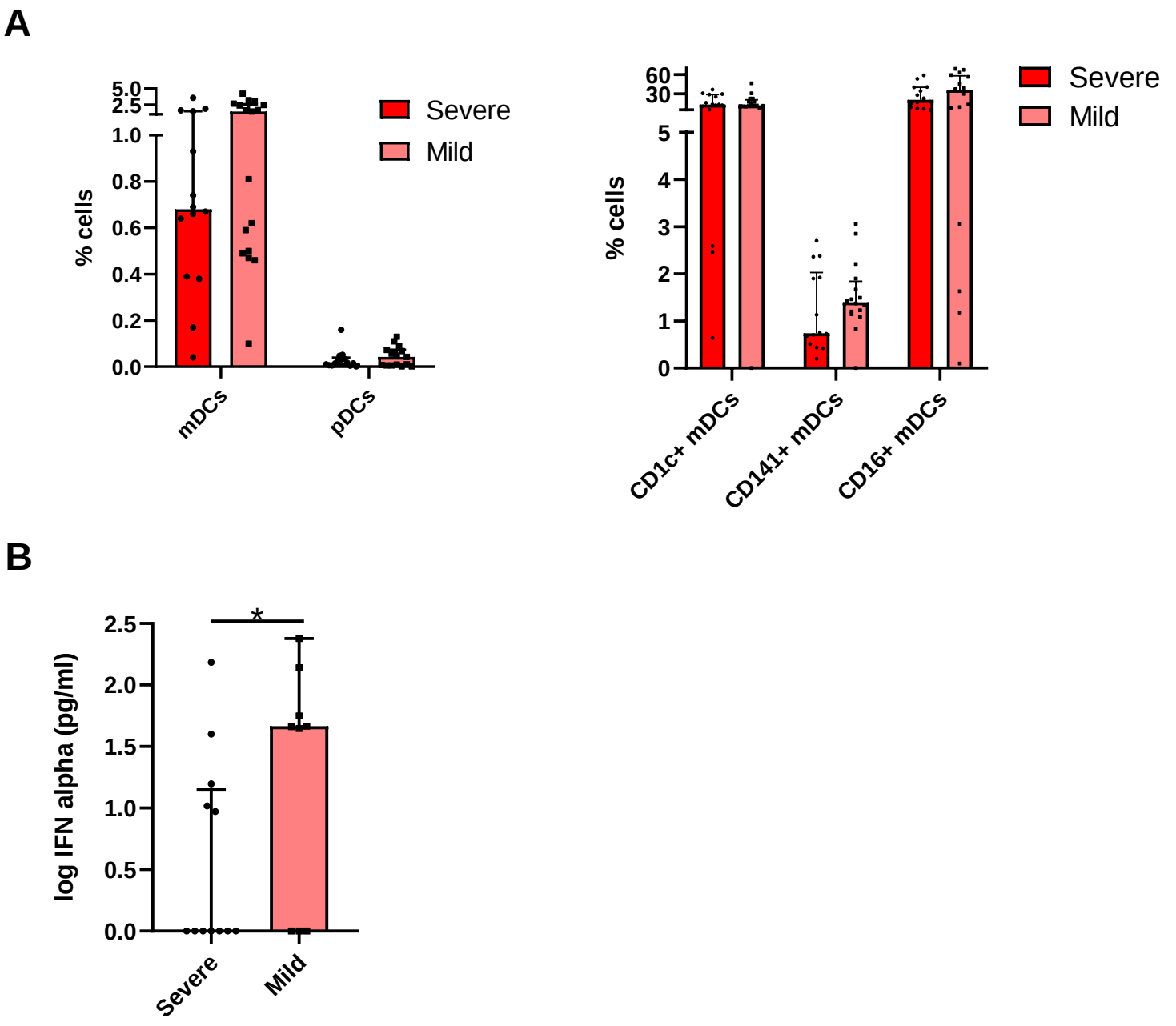
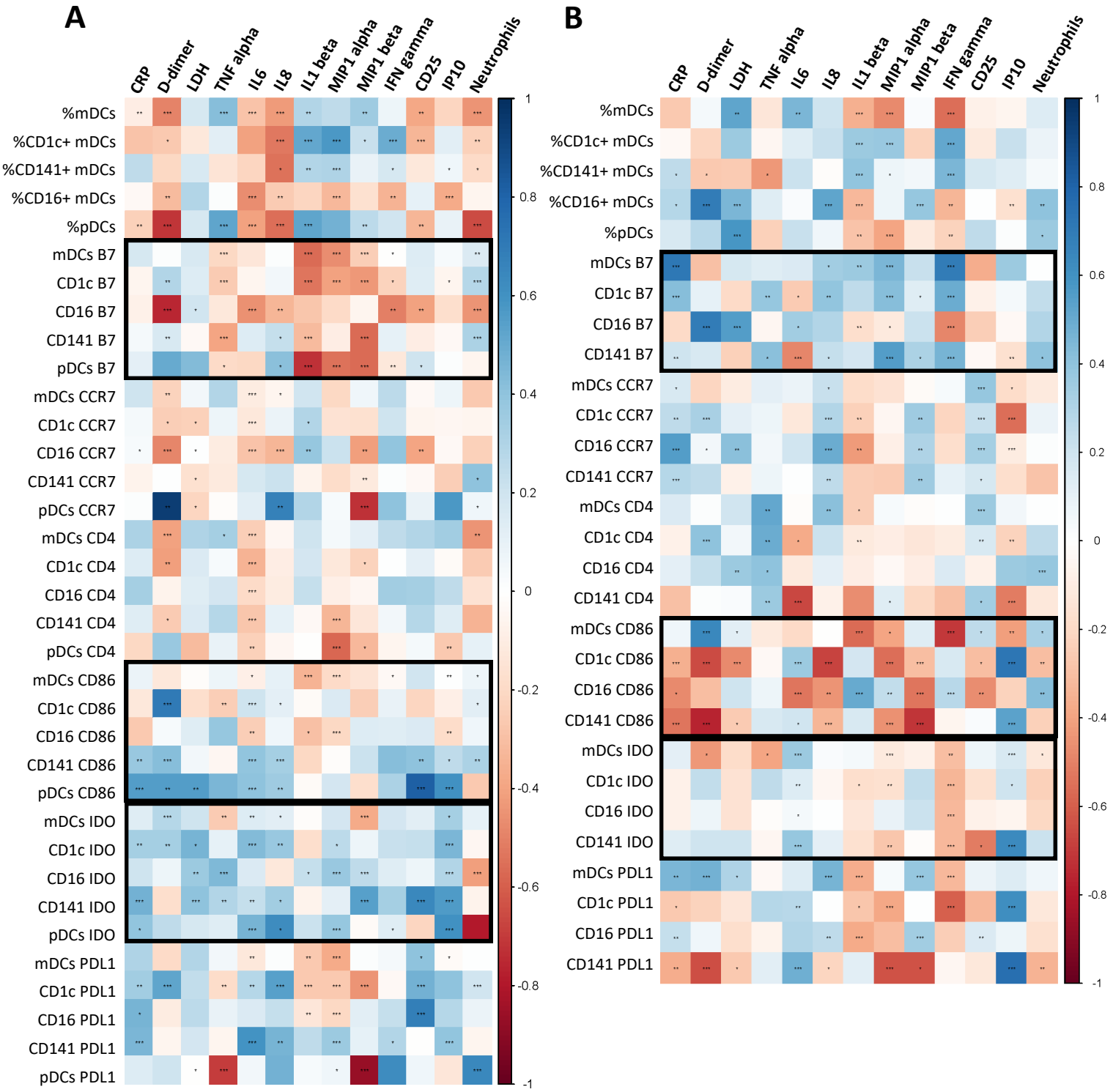

A
% cells
5.0
2.5
1.0
0.8
0.6
0.4
0.2
0.0
Severe
Mild
mDCs
pDCs
% cells
60
30
5
4
3
2
1
0
Severe
Mild
CD1c+ mDCs
CD141+ mDCs
CD16+ mDCs
B
log IFN alpha (pg/ml)
2.5
2.0
1.5
1.0
0.5
0.0
*
Severe
Mild

Figure 3


A
B
CRP
D-dimer
LDH
TNF alpha
IL6
IL8
IL1 beta
MIP1 alpha
MIP1 beta
IFN gamma
CD25
IP10
Neutrophils
%mDCs
%CD1c+ mDCs
%CD141+ mDCs
%CD16+ mDCs
%pDCs
mDCs B7
CD1c B7
CD16 B7
CD141 B7
pDCs B7
mDCs CCR7
CD1c CCR7
CD16 CCR7
CD141 CCR7
pDCs CCR7
mDCs CD4
CD1c CD4
CD16 CD4
CD141 CD4
pDCs CD4
mDCs CD86
CD1c CD86
CD16 CD86
CD141 CD86
pDCs CD86
mDCs IDO
CD1c IDO
CD16 IDO
CD141 IDO
pDCs IDO
mDCs PDL1
CD1c PDL1
CD16 PDL1
CD141 PDL1
pDCs PDL1
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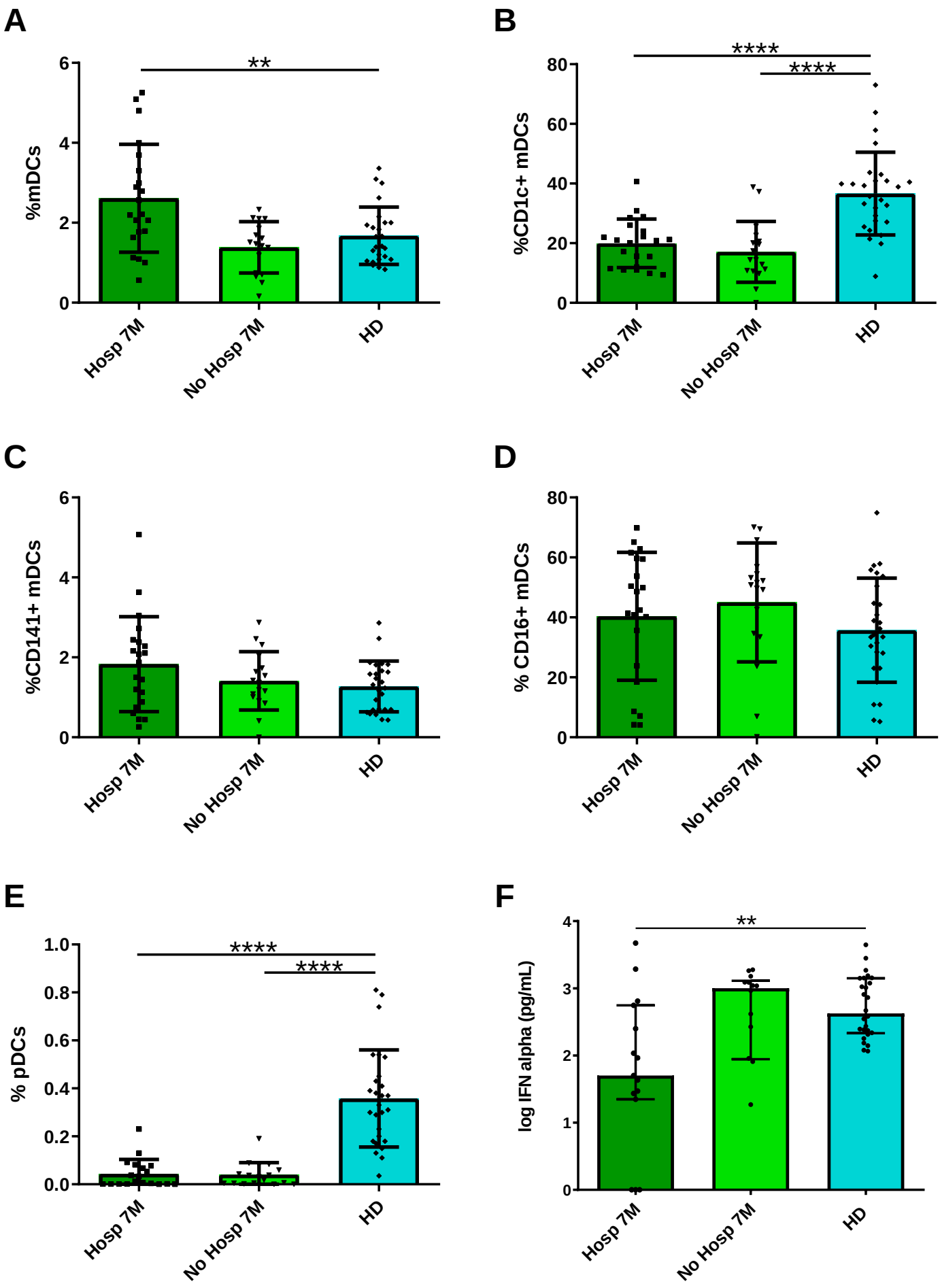
0.2
0
-0.2
-0.4
-0.6
-0.8
-1

Figure 4


A
%mDCs
**
Hosp 7M
No Hosp 7M
HD
B
%CD1c+ mDCs
****
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C
%CD141+ mDCs
D
% CD16+ mDCs
E
% pDCs
****
****
F
log IFN alpha (pg/mL)
**

Figure 5

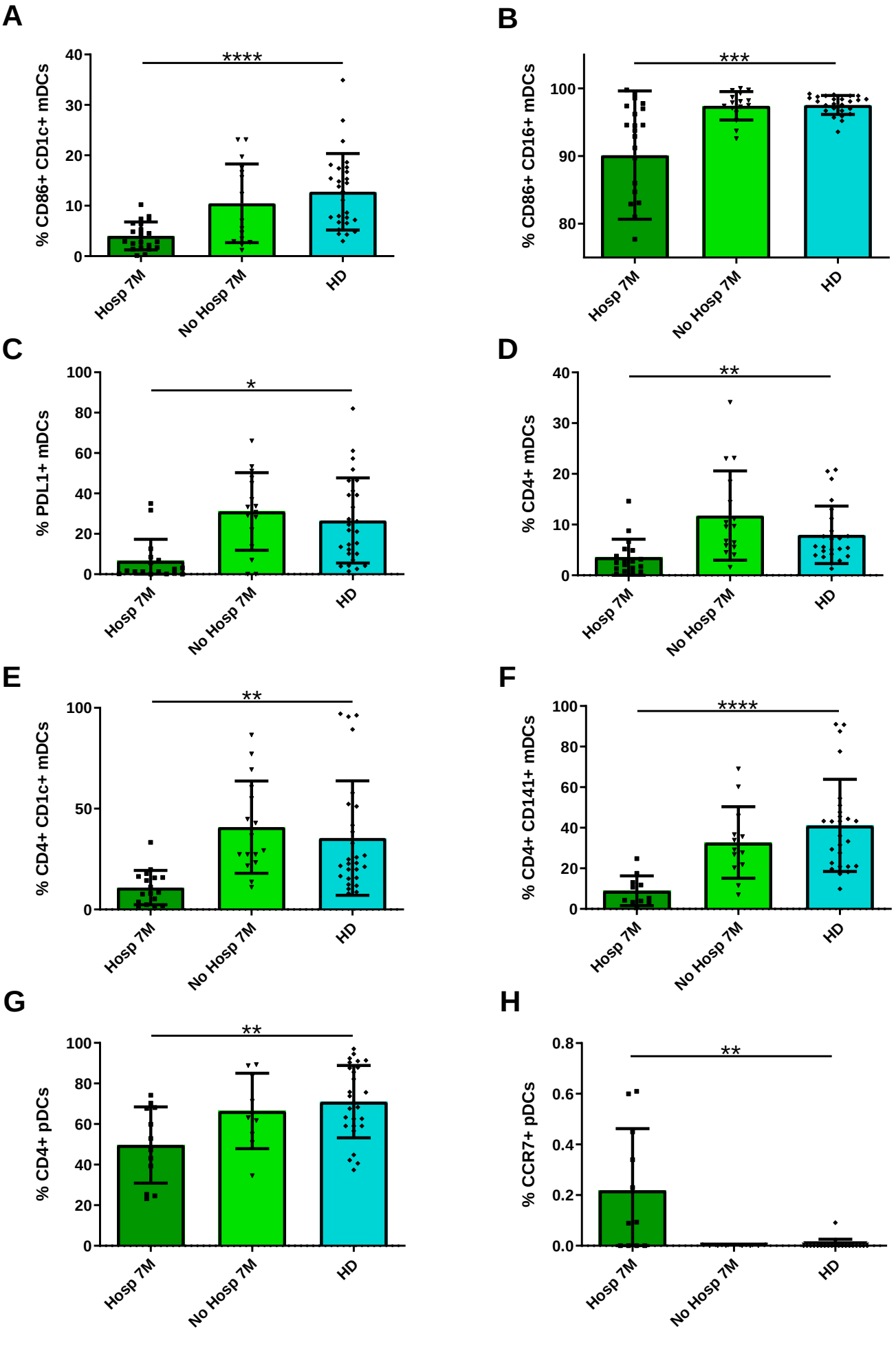
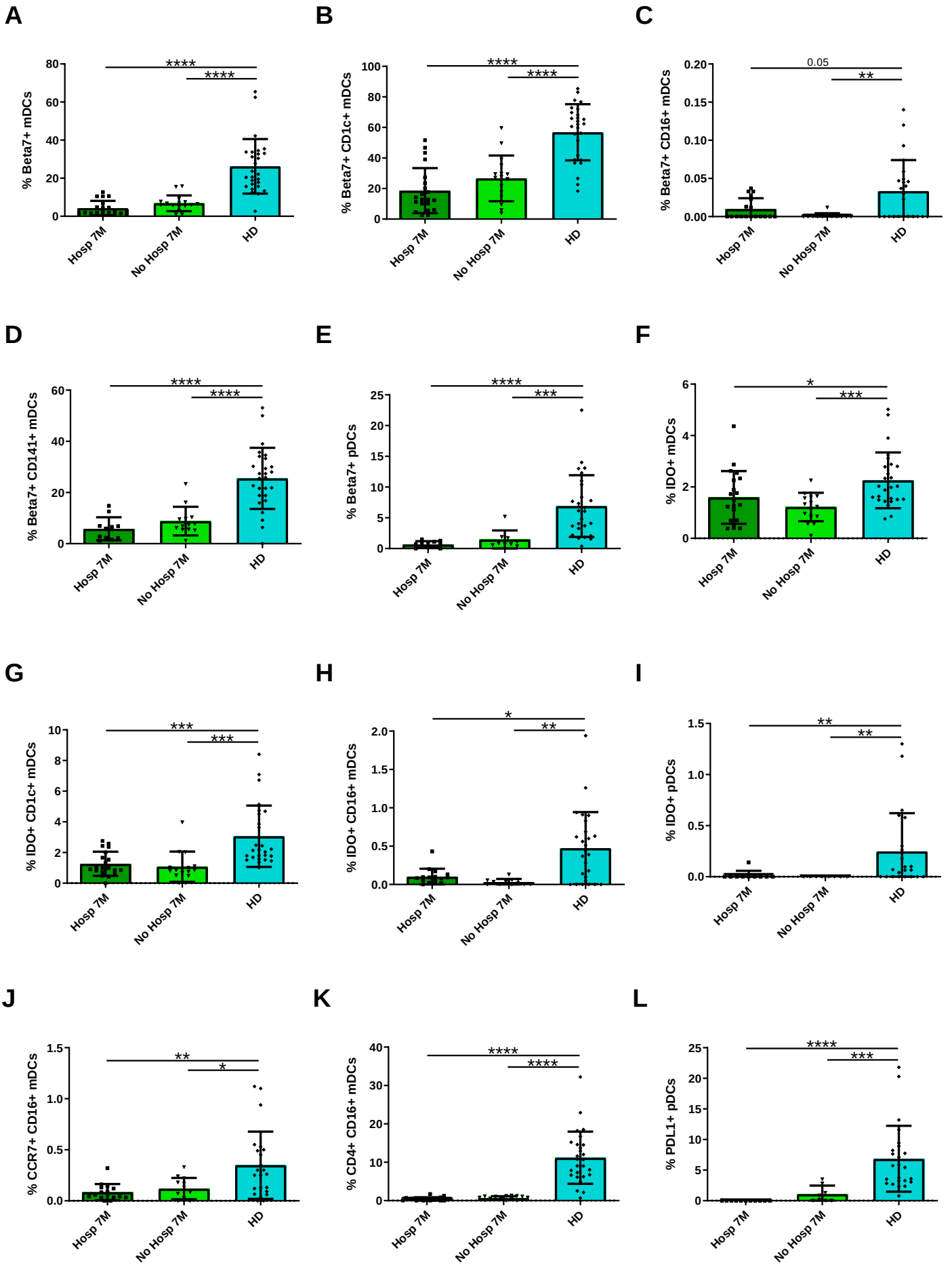

A
% CD86+ CD1c+ mDCs
****
Hosp 7M
No Hosp 7M
HD
B
% CD86+ CD16+ mDCs
***
C
% PDL1+ mDCs
*
D
% CD4+ mDCs
**
E
% CD4+ CD1c+ mDCs
**
F
% CD4+ CD141+ mDCs
****
G
% CD4+ pDCs
**
H
% CCR7+ pDCs
**

Figure 6


A
% Beta7+ mDCs
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J
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K
% CD4+ CD16+ mDCs
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% PDL1+ pDCs
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Hosp 7M
No Hosp 7M
HD