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Macrophage and dendritic cell subsets in IBD: ALDH$^+$ cells are reduced in colon tissue of patients with ulcerative colitis regardless of inflammation

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CONFLICT OF INTEREST:

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ABSTRACT

Disruption of the homeostatic balance of intestinal dendritic cells (DCs) and macrophages (MQs) may contribute to inflammatory bowel disease. We characterized DC and MQ populations, including their ability to produce retinoic acid, in clinical material encompassing Crohn's ileitis, Crohn's colitis and ulcerative colitis (UC) as well as mesenteric lymph nodes (MLNs) draining these sites. Increased $CD14^{+}DR^{int}$ MQs characterized inflamed intestinal mucosa while total $CD141^{+}$ or $CD1c^{+}$ DCs numbers were unchanged. However, $CD103^{+}$ DCs, including $CD141^{+}CD103^{+}$ and $CD1c^{+}CD103^{+}$ DCs, were reduced in inflamed intestine. In MLNs, two $CD14^{-}$ DC populations were identified: $CD11c^{int}HLADR^{hi}$ and $CD11c^{hi}HLADR^{int}$ cells. A marked increase of $CD11c^{hi}HLADR^{int}$ DC, particularly $DR^{int}CD1c^{+}$ DCs, characterized MLNs draining inflamed intestine. The fraction of DC and MQ populations expressing aldehyde dehydrogenase (ALDH) activity, reflecting retinoic acid synthesis, in UC colon, both in active disease and remission, were reduced compared to controls and inflamed Crohn's colon. In contrast, no difference in the frequency of $ALDH^{+}$ cells among blood precursors was detected between UC patients in remission and non-inflamed controls. This suggests that ALDH activity in myeloid cells in the colon of UC patients, regardless of whether the disease is active or in remission, is influenced by the intestinal environment.

INTRODUCTION

Disruption of the homeostatic balance in the intestine of genetically susceptible individuals can culminate in inflammatory bowel disease (IBD), such as Crohn's disease (CD) or ulcerative colitis (UC), where inappropriate reactivity to commensal bacteria that breach the intestinal barrier drive inflammation. Studies performed largely in mice have shown that intestinal phagocytes, such as dendritic cells (DCs) and macrophages (MQs), are central to maintaining homeostasis. In the steady state these mononuclear phagocytes are less responsive to inflammatory signals and produce anti-inflammatory mediators that promote generation of regulatory T cells (Treg) [1-4]. However, intestinal inflammation alters the differentiation of monocyte-derived cells and changes their function into cells that promote inflammation [3-6].

Compared to mice, relatively little is known about human DCs and MQs in healthy versus inflamed intestine. Intestinal MQs from healthy human intestine are hyporesponsive to inflammatory stimuli [2, 7, 8]. In the inflamed human intestine, $CD14^+$ MQs accumulate and produce proinflammatory cytokines [3, 9-12]. Accumulation of $CD14^+$ cells is also seen in mesenteric lymph nodes (MLNs) draining inflamed intestine [13].

Similar to MQs, DCs from healthy human lamina propria are hyporesponsive to some TLR ligands [14] while those from inflamed intestine exhibit proinflammatory activity [15, 16]. Consistent with this, $CD103^+$ DCs from MLN draining healthy intestine promote induction of $FoxP3^+$ T cells [17] while DCs from the MLN of active CD are proinflammatory and induce Th1 T cells [18]. Indeed, healthy colon epithelial cells seem to condition hyporesponsiveness in monocyte-derived DCs and promote $FoxP3^+$ T cells, a property that is reduced in epithelial cells from non-inflamed CD patients [17, 19]**.**

Studies of mouse intestinal DCs, using CD103 and CD11b to define populations, have identified distinct subsets with different origin and influence on intestinal homeostasis [4, 6, 20-23]. The human counterparts have been identified using CD103 combined with other markers such as Sirpα or CD141 and CD1c [21, 23-25]. Mouse $CD103^+CD11b^-$ intestinal DCs are equivalent to $CD103^+Sirp\alpha^-$ in the human intestine, which are highly similar to $CD141^+$ DCs in human blood and other tissues [24]. Mouse

$CD103^+CD11b^+$ DC, which have a role in inducing mucosal Th17 cells [21, 25, 26], are related to $CD103^+Sirp\alpha^+$ DCs in human intestine and $CD1c^+$ DCs in blood and skin. However, the role of these recently identified human DC subsets in IBD is not known.

Retinoic acid (RA) is a vitamin A metabolite that has many immunomodulatory properties depending on the context [27, 28]. It is produced from retinol in a step-wise process involving retinol dehydrogenases and aldehyde dehydrogenases (ALDH). $CD103^+$ DCs in mouse intestinal tissue produce RA, which allows these DCs to imprint intestinal homing properties to T and B cells and promotes Treg development [1, 27]. Similar to mice, human $CD103^+$ DC from MLN draining healthy intestine imprint intestinal homing properties to T cells in a RA-dependent fashion [29] and induce FoxP3 expression in T cells [17]. The ability of $CD103^+$ DCs from MLN to imprint intestinal homing on T cells is similar in MLN draining healthy or inflamed ileum [29]. Despite these findings, which subsets of mononuclear phagocytes in the human intestine have the ability to make RA and whether this is localization dependent and regulated in context of intestinal inflammation is unknown.

The pivotal role of DC and MQs in intestinal homeostasis, and the unknown contribution of recently identified human DC subsets in IBD, led us to dissect the population dynamics of MQ and DC in inflamed and non-inflamed intestinal tissue and MLN draining these sites. Extensive patient cohorts were analyzed to compare ileum *versus* colon in CD and different colon segments in CD and UC. We show that MQ and DC subsets follow a specific inflammatory pattern, both in the lamina propria and MLN, which is similar at all locations and for both diseases. We also analyzed the capacity of intestinal DC and MQ to produce RA. The data suggest that intestinal tissue of UC patients influences ALDH production by myeloid cells, regardless of whether disease is active or in remission.

RESULTS

Increased HLADR^{int} macrophages characterize inflamed intestinal mucosa

To characterize MQs infiltrating the intestinal mucosa of patients with active CD or UC, lin$^-$ HLADR$^+$CD14$^+$ cells were selected (Fig. 1a and Suppl Fig. S1a). HLADR expression of these cells differed in controls and inflamed patients so the cells were further divided into HLADR^{hi} and HLADR^{int} populations (named DR^{hi} MQs and DR^{int} MQs, respectively) (Fig. 1b). Both DR^{hi} MQs and DR^{int} MQs from inflamed colon tissue showed higher expression of CD64, CD13 and Sirpα as compared to control tissue, while CD209 expression was lower in DR^{int} MQs at inflammation (Suppl Fig. 1b). No, or only moderate, differences in total DR^{hi} MQ number (Fig. 1c) or frequency (Suppl Fig. S1c) were found at inflamed sites compared to non-inflamed tissue for both ileum and colon. The same occurred when comparing inflamed to non-inflamed tissue taken from the same patient at the same endoscopy (Fig. 1D). In contrast, there was a sharp increase in the number (Fig. 1e) and frequency (Suppl Fig. S1d) of DR^{int} MQs during inflammation, both compared to controls (Fig. 1e and Suppl Fig. S1d) and to non-inflamed sites from the same patient (Fig. 1F). Paired samples of inflamed and non-inflamed Crohn's ileum could not be obtained due to ethical considerations. The number of DR^{hi} MQs and DR^{int} MQs did not differ between controls from sigmoid and ascending colon (compare CD colon control and UC colon control in Fig. 1c and e). Likewise, the number of DR^{hi} MQs and DR^{int} MQs in controls from ileum and ascending colon were similar (compare CD ileum control and CD colon control in Fig. 1c and e).

Increased DR^{int} MQs in inflamed intestine suggests recruitment from the blood. To address this, HLADR expression on CD14$^+$ monocytes from blood (Fig. 2a) was compared to DR^{int} and DR^{hi} MQs among LPMCs from colon tissue (Fig. 1b) from the same patient. The median fluorescent intensity (MFI) of HLADR was similar between CD14$^+$ monocytes and DR^{int} MQs while DR^{hi} MQs had higher HLADR expression (Fig. 2b). In summary, in Crohn's and UC patients, recruitment of DR^{int} MQs increased in inflammation while DR^{hi} MQs remained constant.

$CD141^+CD103^+$ and $CD1c^+CD103^+$ DCs decrease in the inflamed intestinal mucosa

To further characterize antigen presenting cells in the mucosa of patients with active IBD, the $CD11c^+$ fraction of $lin^-HLADR^+CD14^-$ cells was selected and henceforth called DCs (Fig. 3a, left). Using CD141 and CD1c, two separate populations could be determined, both at steady state and during inflammation (Fig. 3a, middle and Suppl Fig. S2a). These cells were named $CD141^+$ DCs and $CD1c^+$ DCs, respectively. No double positive $CD141^+CD1c^+$ DCs were detected in either non-inflamed or inflamed tissue (Fig. 3a and data not shown). During inflammation, no change in the frequency of $CD141^+$DCs or $CD1c^+$DCs among LPMCs (Suppl Fig. S2b-c), or in the frequency among DCs, (Suppl Fig. S2d-e) was found at any location, and numbers of DCs in the ileum, ascending colon and sigmoid colon were similar (data not shown). $CD141^+$ DCs and $CD1c^+$ DCs were further analyzed for CD103 expression (Fig. 3a, right). Reduced numbers of $CD141^+$ and $CD1c^+$ DCs expressing CD103 were detected during inflammation (Fig. 3b, d). The same pattern was apparent when comparing paired samples of inflamed and non-inflamed tissue from the same patient at the same endoscopy (Fig. 3c and e). Again, no differences in numbers of $CD103^+$ DCs between intestinal locations could be identified either at inflammation or steady state (compare cell numbers in Figure 3b and d). Also, a reduction in the frequency of $CD103^+$ cells among the $CD141^-CD1c^-$ DCs was detected upon inflammation (Suppl Fig. S3). Overall, the number and frequency of $CD103^+$ DCs among DCs was decreased during inflammation (Supplementary Fig. S4). Thus, a reduction in $CD103^+$ DCs characterizes intestinal lamina propria DCs during inflammation in CD and UC.

DC composition in MLNs show distinct changes during inflammation

DCs migrate to lymph nodes to initiate T cell responses. We thus compared the composition of DCs in the MLN of patients with inflammation relative to controls. In contrast to $CD14^-CD11c^+$ lamina propria DCs, where a single population was apparent (Fig. 3a), MLNs contain two distinct populations of $CD14^-CD11c^+$ cells, one being $HLADR^{hi}$ and the other $HLADR^{int}$ (Fig. 4a). These populations were named DR^{hi} DCs and DR^{int} DCs, respectively. The ratio of these two populations

revealed differences between MLNs from inflamed patients and controls, with DR^{hi} DCs being the main population at steady state while DR^{int} DCs increased during inflammation (Fig. 4b).

Obtaining MLNs draining the colon of control patients is highly limited for ethical reasons. However, MLNs draining control ileum can be obtained in conjunction with bladder reconstruction. Thus, MLNs draining inflamed colon or ileum were compared to control MLNs draining the ileum. The DR^{hi}/DR^{int} DC ratio in MLNs draining inflamed colon from CD and UC patients was very similar to MLNs draining inflamed CD ileum (Fig. 4b). Also, no differences were detected regardless of whether the MLNs drained ascending, descending or sigmoid colon (Fig. 4b-c; colors indicate samples from the same patient). Thus, differences in the DR^{hi}/DR^{int} DC ratio in MLNs draining inflamed colon compared to ileal-draining control MLNs seemed not to depend on location *per se*, but rather that the MLNs drained inflamed intestine. The shift from predominance of DR^{hi} DCs in control MLNs to DR^{int} DCs in MLNs draining inflamed IBD intestine is shown schematically in Fig. 4d-e (compare total blue to total red).

To determine which DC subsets accounted for the shift from DR^{hi} to DR^{int} DCs during inflammation, MLN DCs were gated for CD141 and CD1c. About half of the numerically abundant DR^{hi} DCs in control MLNs expressed CD1c (Figure 4e, left). These were reduced upon inflammation while DR^{int} DCs, either expressing $CD1c^+$ DC or being $CD141^-CD1c^-$, increased in MLNs draining inflamed tissue (Fig. 4d-e). Similar trends were detected in ileal and colonic CD as well as UC. This suggests that inflammation resulted in similar relative changes in MLN DC subsets independent of the intestinal compartment or disease. Since a shift from DR^{hi} to DR^{int} DCs was apparent in MLNs, but control MLNs from the colon were not available to analyze for ethical reasons, we compared cell populations in the intestinal mucosa to MLNs. As stated above, no difference in the frequency of $CD141^+$ and $CD1c^+$ DCs in the intestinal mucosa could be detected between control and inflamed tissue (Supplementary Fig. S2). We thus examined HLADR expression on mucosal DCs from patients where inflamed and non-inflamed intestinal tissues as well as MLNs were obtained. HLADR expression on $CD141^+$, $CD1c^+$ and $CD141^-CD1c^-$ DCs was lower in inflamed compared to non-inflamed

tissue of the same patient with a similar pattern for colon and ileum (Figure 4f). To further analyze HLADR expression, blood and LPMC DCs ($CD141^+$ and $CD1c^+$) from the same patient (UC patients in remission) were analyzed and showed that DR expression increased in the tissue (Supplementary figure S5). Thus, the shift from DR^{hi} to DR^{int} DCs seen in inflamed MLNs is consistent with HLADR expression on LPMC DCs from inflamed tissue and HLADR is expressed at a higher level in tissue compare to blood.

$ALDH^+$ cells are reduced in colon tissue of UC patients regardless of inflammation

The production of RA by intestinal mononuclear phagocytes has been suggested to regulate Th cell differentiation and induce intestinal homing properties [27, 28]. As generation of RA involves ALDH enzymes, we measured ALDH activity in MQs and DCs from the intestine of CD and UC patients (Fig. 5a). ALDH activity did not differ significantly between DR^{hi} and DR^{int} MQs of inflamed CD patients or between DR^{hi} and DR^{int} MQs of inflamed UC patients (compare the same patient groups in Fig. 5b-c). The same was true for $CD141^+$ and $CD1c^+$ DCs from these patient groups as well as controls (compare the same patient groups in Fig. 5d-e). However, in controls, ALDH activity was lower in DR^{int} MQs compared to DR^{hi} MQs (median % (range), 25% (21-49) *vs.* 56% (45-69), p=0.0003; compare control colon in Fig. 5b-c). In general, MQ and DC subsets from the ileum or colon of inflamed CD patients had a broad distribution in the frequency of $ALDH^+$ cells that did not differ significantly from controls (Fig. 5b-e). In contrast, ALDH activity in DCs and MQs from inflamed colon of UC patients was tightly clustered and all four subsets analyzed had a significantly reduced frequency of $ALDH^+$ cells relative to control colon (Fig. 5b-e). Similar results were found for $CD103^+$ DCs (Supplementary Fig. S6a-b). This suggests that, relative to controls, the frequency of $ALDH^+$ myeloid cells from inflamed UC colon was reduced while $ALDH^+$ myeloid cells from CD colon or ileum was similar to controls.

The data raised the possibility that inflamed UC colon reduces the frequency of $ALDH^+$ cells compared to control colon. To address this, we assessed the frequency of $ALDH^+$ cells among myeloid

cells from UC patients in remission and in non-inflamed and inflamed regions of the intestine of the same UC patient with active disease. This revealed that DCs, but not MQs, showed low Aldefluor activity at remission compared to controls (Fig. 5b-e, right panels). Also, a similar frequency of $ALDH^+$ MQs and DCs were detected in non-inflamed tissue and inflamed tissue of patients with active disease (Fig. 5f). Quantitative RT-PCR of sorted MQs and $CD141^+$ and $CD1c^+$ DCs showed similar *ALDH1A1* expression in the same cell type among the different patient groups (Fig. 5g). However, *ALDH1A2* expression was increased in all three cell types only in inflamed CD (Figure 5g). Together the data suggest that the frequency of $ALDH^+$ cells in the colon of UC patients with active disease is low regardless of whether the tissue is inflamed nor not. It also shows that ALDH activity is low among DCs and DR^{hi} MQs in UC at any disease stage while a restoration of ALDH activity in DR^{int} MQs is apparent at remission

Reduced $ALDH^+$ cell frequency is inherent to the colonic mucosa of UC patients

The ALDH data raise the possibility that the UC colon influences the frequency of ALDH-producing myeloid cells. In addition, $ALDH^+$ $CD45^-$ (stromal) cells did not compensate for reduced RA in UC patients (Supplementary Fig. S6c). We next analyzed $ALDH^+$ cells among blood precursors (Fig. 6a-b). The data revealed a high frequency of $ALDH^+$ cells among classical and intermediate monocytes, as well as among circulating $CD141^+$ and $CD1c^+$ DCs, in UC patients in remission and with active disease that was similar to healthy donors (Fig. 6c). ALDH activity was associated predominantly with *ALDH1A1* expression that was independent of disease or disease activity (Fig. 6d). Thus, the low frequency of ALDH-producing myeloid cells in the colon of UC patients in remission or with active disease is not reflected in circulating precursors. Together the data suggest that the colon environment in UC patients reduces ALDH expression of mononuclear phagocytes.

DISCUSSION

Using extensive clinical material encompassing Crohn's ileitis, Crohn's colitis and UC, as well as MLNs draining these tissues, we characterized the dynamics of MQ and DC populations, including their ability to produce RA, in health versus IBD. Our data show that CD and UC patients display the same myeloid cell imbalance in inflamed intestinal mucosa, comparisons which have not been directly made before. First, inflammation is characterized by increased $CD14^+$ MQs in the intestinal lamina propria. These cells are $CD64^{hi}$, consistent with their identity as MQs [3, 6, 30]. This increase was accounted for by $CD14^+DR^{int}$ cells, while $CD14^+DR^{hi}$ cells were unchanged in inflammation. This is also consistent with studies in Crohn's patients showing an influx of $CD14^{hi}$ cells in inflamed intestinal mucosa that have features of less mature mononuclear cells [2, 3, 10-12]. Second, we studied CD141 and CD1c DCs, which have been characterized in several healthy human tissues [14, 21, 24, 31, 32] but not in IBD. We found that the number of $CD141^+$ and $CD1c^+$ is similar in inflamed lamina propria of IBD patients and controls.

Expression of CD103, the alpha chain of the integrin $\alpha E\beta 7$ integrin, has been used to identify a population of DCs in mouse small intestine with a role in homeostasis [1, 29, 33]. CD103 is not, however, a subset-specific DC marker as it is expressed to varying degrees on both IRF8/Id2/Batf3- and IRF4-dependent DCs [20-22, 34]. Notably, we observed a marked reduction in the frequency of CD103-expressing DCs in the setting of intestinal inflammation. This was apparent among both $CD141^+$ and $CD1c^+$ DCs and the so far undefined $CD141^-CD1c^-$ DCs. It might be argued that although significant, the differences in the numbers of CD103-expressing cells during inflammation *vs.* health are small relative to the total cell count. However, the potency of DCs in controlling inflammation and tolerance is high and small numbers of DCs can have a potent influence on the ensuing response in the tissue. Inflammation-induced death of these cells, their migration from the lamina propria or negative impact of the inflammatory environment on CD103 expression may underlie reduced $CD103^+$ DCs during inflammation. The mechanisms regulating CD103 expression on DCs subsequent to their localization in the intestine is unknown. However, CD103 expression on intestinal T cells is

dependent on TGFβ [35]. This raises the possibility that the inflammatory environment of IBD negatively impacts CD103 expression by DCs.

As DCs migrate from the intestinal lamina propria to MLNs to activate naïve T cells [20, 34], we investigated DCs in MLNs draining inflamed intestine. Strikingly, we noted a marked increase in the number of DR^{int} DCs among $CD14^{-}CD64^{-}$ APCs in MLNs draining inflamed intestine, regardless of which region they drained. Similar to intestinal tissue, $CD1c^{+}$ DCs in MLNs draining inflamed or non-inflamed intestine were the major DC subset. However, in inflamed tissue, the $CD1c^{+}$ DCs were mainly DR^{int} DCs and there was also a large influx of $CD141^{-}CD1c^{-}$ DCs that were DR^{int}. The three DC subsets identified by CD103 and Sirpα in healthy human small intestine express CCR7 [24] and can thus potentially migrate to MLNs. Which MLN DC subset(s) represent lamina propria-derived migratory DCs and resident DCs in the steady state remains to be determined.

RA can potentially influence intestinal homeostasis by, for example, influencing Th differentiation and inducing intestinal homing receptors on T cells [9, 28, 29, 36]. However, little is known about RALDH activity in human intestinal MQ and DC populations including how this is influenced by IBD and whether it differs in ileal CD, colonic CD and UC. We observed ALDH activity in each of the mononuclear phagocyte populations examined from control ileum and colon. This is consistent with recent studies examining DCs and MQs from healthy ileum, jejunum and colon [24, 37] except that we observed ALDH activity in $CD141^{+}$ DCs from ileum whereas this activity was not found in $CD141^{+}$ DCs from jejunum [24].

Moreover, we found that the fraction of $ALDH^{+}$ MQs and DCs in inflamed ileum or colon of CD was similar to controls. This contrasts with a recent study showing increased ALDH activity in $CD103^{+}$ and $CD103^{-}$ DCs and $CD14^{+}$ MQs from CD patients [37]. This discrepancy could be due to how DC populations were identified in the studies. For example, Sanders et al analyzed ALDH activity in DCs gated based on the presence or absence of CD103, which does not define distinct DC populations [23-25]. In contrast, we analyzed DCs based on CD1c and CD141, which define distinct DC subsets [23-25]. Moreover, samples obtained from different intestinal sites, different patient treatment

regimens or CD complications such as strictures and fistulae could contribute to the discrepancy. Indeed, there tended to be more individual-to-individual spread in the fraction of $ALDH^+$ cells among our CD patients whereas the UC patients behaved more homogenously. Given the diversity among CD patients with, for example, respect to disease features such as structures or fistulae, it may be that a larger group of CD patients needs to be examined and/or their complications need to be formally considered, in addition to the use of identical gating strategies, to rectify the discrepancy. Interestingly, we found that mRNA levels of *ALDH1A2* were higher in MQs and DCs from inflamed colon of CD patients compared to controls. This indicates that inflamed CD patients could have higher ALDH activity and is consistent with the data of Sanders et al [37].

In contrast to inflamed CD colon, $ALDH^+$ DCs and MQs were reduced in UC colon compared to controls. This occurred even in UC patients in remission. These data, combined with the similar fraction of $ALDH^+$ blood monocytes in UC patients in remission and healthy donors, suggests that ALDH activity is influenced by UC intestinal mucosa irrespective of disease activity. Consistent with Sanders *et al*., who showed that circulating bulk $CD14^+$ monocytes express mainly *ALDH1A1* [37], we found that classical and intermediate, but not non-classical monocytes, express *ALDH1A1* but little *ALDH1A2*. Interestingly, a large fraction of circulating $CD141^+$ and $CD1c^+$ DCs expressed ALDH, both in UC and controls, despite low *ALDH1A1* expression. Enhanced efficiency of the oxidation of retinol to retinal by alcohol dehydrogenase family members and/or conversion of retinal to RA by RALDH enzymes, perhaps including *ALDH1A3* and *ALDH2,* in these circulating DCs could underlie this observation [28, 38]. Whether circulating $CD141^+$ and $CD1c^+$ DCs migrate into the intestinal mucosa is unclear but transcriptional profiling studies support this possibility [24] .

The low ALDH activity in intestinal tissue of UC patients, despite the presence of *ALDH1A1* and *ALDH1A2* mRNA, could be caused by translational control, lack of factors required to maintain enzyme activity or production of negative regulators. The microbiota composition in Crohn's disease and UC differ [39, 40], and the microbiota can influence metabolism in the host intestine [41]. Microbiota-induced alterations in local metabolites could, in turn, differentially influence ALDH enzyme stability,

translation or activity. For example, NAD^+ levels are influenced by the microbiota [42], and NAD^+ is a required co-factor for aldehyde dehydrogenases and is rate limiting, along with Mg^{2+}, in the oxidation of retinol to retinoic acid [43, 44]. Thus, disease-associated microbiota could influence ALDH activity despite the presence of *ALDH1A1* and *ALDH1A2* mRNA. For negative regulation, in a study concerning cancer, niclosamide treatment reduced Wnt/β-catenin signaling [45]. This resulted in increased *ALDH1A1* expression and highlights the link between Wnt/β-catenin signaling and ALDH expression. Interestingly, Wnt/β-catenin signaling is induced in UC but not Crohn's disease [46] and many Wnt ligands are even upregulated in the mucosa of UC patients in remission [47]. Mechanisms regulating the expression and function of enzymes involved in the oxidation of retinol in MQs and DCs in UC patients, both in the intestine and at other mucosal surfaces, warrant further investigation.

Could reduced ALDH activity in the lamina propria affect disease progression? In both human biopsies and mice, addition of RA activates the RARα receptor and inhibits inflammatory responses and increases $FoxP3^+$ T cells even without lymph node involvement [28, 36, 48]. Also, all trans RA induces suppressive functions of human nTregs [49]. Thus, local regulatory failure by a lack of RA may help drive the disease. However, studies in mice suggesting that RA can also enhance inflammation underscore the complex effects of RA [28]. Despite the complex immunomodulatory properties of RA, it is successfully being used as therapy against psoriasis and acne and may be involved in gastric homeostasis [50-52]. RA modulation is unlikely to interfere with active inflammation in UC, but may be investigated as a supplement during remission to reduce the risk of relapse.

In summary, we reveal a myeloid cell imbalance that is an inflammatory signature in the intestinal lamina propria and MLNs that is common to CD and UC. Moreover, it is similar along the colon and terminal ileum. Our data also suggest that the intestinal environment of UC influences ALDH activity of mononuclear phagocytes, irrespective of the inflammatory status of the patient *per se*. Down regulation of the retinoic acid system seems to be a feature of UC, active as well as inactive, and therapeutic normalization of this phenomenon may represent a new treatment target in UC.

MATERIALS AND METHODS

Patients and specimens

UC patients, CD patients and non-inflamed controls (Table 1) were recruited during 2010-2015 at the Endoscopy unit, Sahlgrenska University Hospital, Gothenburg, Sweden, at the Department of Surgery, Östra Hospital, Gothenburg, Sweden, at the Department of Surgery, Skåne University Hospital, SUS Malmö, Sweden and at Department of Hepatology and Gastroenterology, Aarhus University Hospital, Aarhus, Denmark. All participants gave informed written consent. The study was approved by the Regional Ethical Review Board in Gothenburg (permits 040-08 and 085-11), the Regional Ethical Board in Lund (permit 463-06) and the Local Scientific Ethical committee in Denmark (permits M-20100216 and M-20110240).

Tissue biopsies were acquired at routine colonoscopy. Inflammation was assessed macroscopically by the examining gastroenterologist and microscopically by a pathologist. Biopsies from patients without inflammation undergoing colonoscopy for other indications (polyps, weight loss) were non-inflamed control tissue (termed controls). Tissue and MLNs from surgery were acquired from UC and CD patients undergoing colectomy or ileectomy. Non-inflamed tissue and MLNs from surgery were from patients undergoing bladder reconstruction by cystectomy (also termed controls). Blood was from healthy donors, UC patients in remission and UC patients with active disease.

Blood and intestinal samples

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by density-gradient centrifugation on Ficoll-Paque (GE Healthcare, Sweden). Biopsies and surgical samples were collected in PBS and put on ice. Surgical samples were stripped of muscle and fat and cut into small pieces. Epithelial cells were removed by incubating for 15 min at 37°C with HBSS-EDTA (HBSS containing 2% FCS, 1.5 mM Hepes and 2 mM EDTA) three times followed by a wash in RPMI

1640 containing 10% FCS and 1.5 mM Hepes. Lamina propria mononuclear cells (LPMCs) were prepared by a 45-60 minute incubation at 37°C with 25 CDU/ml collagenase type VIII (Sigma-Aldrich) or 40 CDU/ml collagenase D (Roche Diagnostics) and 60 Kunitz units/ml DNase I (Sigma-Aldrich) diluted in RPMI 1640 with 10% FCS, 1.5 mM Hepes and 2 mM $CaCl_2$. Following digestion, surgical samples were further dissociated using a GentleMACS (Miltenyi Biotec, Germany). Supernatants were collected by filtration through a nylon mesh and analyzed by flow cytometry. Cell suspensions from MLNs were obtained by cutting it into pieces and performing collagenase digestion as above but for 30 min.

Flow cytometry

Cells were washed twice in HBSS containing 2% FCS, 5 mM EDTA and 20 mM HEPES and stained in the same buffer for 30 min at 4°C in the dark. 7-Aminoactinomycin D (7AAD, Sigma-Aldrich) or Live/Dead Fixable Aqua Dead Cell Stain Kit (Gibco Life Technologies) were used to exclude non-viable cells. Antibodies used were: anti-HLADR-Alexa700/APC-Cy7, anti-CD3-PE-CF594/FITC, anti-CD19-PE-CF594/FITC, anti-CD56-PE-CF594, anti-CD11c-Pacific Blue/PeCy7, anti-CD14-PeCy7/FITC, anti-CD103-PE/APC, anti-CD141-PE, anti-CD1c-PerCP-Cy5.5, anti-CD45-APCH7, anti-CD16-APC, anti-CD64/APC, anti-CD13/APC, anti-CD33/APC, anti-CD209/APC and anti-Sirpα/PeCy7. Antibodies were from BD Biosciences (San Jose, CA) except anti-CD103 (eBioscience), anti-HLA-DR (BioLegend), anti-CD1c (BioLegend) and anti-CD141 (Miltenyi). Samples were collected with a LSRII- or FACSCAnto (BD Biosciences) using DIVA software (BD Biosciences) and analyzed using FlowJo software (Tree Star). Lineage cocktail was anti-(CD3/CD19/CD56). Isotype controls were used for anti-CD14-PeCy7/FITC, anti-CD103-PE/APC, anti-CD141-PE, anti-CD1c-PerCP-Cy5.5, anti-CD64/APC, anti-CD13/APC, anti-CD33/APC, anti-CD209/APC and anti-Sirpα/PeCy7.

Classical ($Lin^-CD14^+CD16^-$), intermediate ($Lin^-CD14^+CD16^+$) and non-classical ($Lin^-CD14^{low}CD16^+$) monocytes as well as $CD141^+$ ($Lin^-CD14^-CD16^-HLADR^+CD141^+$) and $CD1c^+$ ($Lin^-CD14^-CD16^-HLADR^+CD1c^+$) cells were sorted from peripheral blood. For Fig. 5g, total $CD14^+$ MQs were

analyzed (called DR^{hi}/DR^{int} MQs in Fig. 5g) since DR^{int} MQs were too few from control tissue and DR^{hi} MQs were too few from inflamed tissue. Total $CD14^+$ MQs, $CD141^+$ DCs and $CD1c^+$ DCs were sorted from intestinal tissue from the indicated patient groups. Samples were sorted using a FACS Aria III (BD Biosciences). Purity was >98%.

ALDH activity

ALDH activity was evaluated using the Aldefluor assay (Stemcell Technologies, France) according to the manufacturer's protocol with some modifications. Briefly, $7x10^6$ cells were resuspended in 0.5 ml assay buffer containing 5 μl Aldefluor reagent without or with 10 μl of the ALDH inhibitor DEAB (15 μM). Samples were incubated at 37°C for 45 min in the dark. $ALDH^+$ cells were identified as cells with brighter fluorescent intensity than a DEAB-inhibited sample run in parallel.

RNA isolation and qPCR

RNA was extracted using NucleoSpin RNA kit (Macherey-Nagel GmbH, Belgium) and cDNA was produced using Quantitect Reverse Transcription Kit (Qiagen). Quantitative real-time PCR (qPCR) was performed using TaqMan Universal PCR Master Mix (Applied Biosystems). Expression of *ALDH1A1* (Hs00946916_m1), *ALDH1A2* (Hs00180254_m1), *GAPDH* (Hs03929097_g1) and *HPRT* (Hs01003267_m1) (all from Applied Biosystems) were quantitated using the 7500 Real Time PCR system (Applied Biosystems). Standard conditions for relative gene expression analysis as recommended by the manufacturer were applied. Expression data was normalized to GAPDH and HPRT using the $2^{-\Delta Ct}$ method.

Statistics

Statistical evaluations were performed using GraphPad Prism 6.0 (GraphPad Software, USA). Wilcoxon signed rank test was used to evaluate differences between paired samples, Mann Whitney

U test was performed to evaluate differences between two groups and Kruskal-Wallis test followed by Dunn's multiple comparison was used for comparison between three or more groups.

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FIGURE LEGENDS

Figure 1. DR^{int} MQs infiltrate inflamed intestinal mucosa in CD and UC. (a) Gating strategy used to select live, Lin$^-$HLADR$^+$CD14$^+$ cells. (b) Lin$^-$HLADR$^+$CD14$^+$ were further divided into DR^{hi} and DR^{int} populations. (c) The number of DR^{hi} MQs and (e) DR^{int} MQs among 10^5 LPMCs from the indicated tissue is shown. Control tissue for CD and UC were from ascending and sigmoid colon, respectively. Horizontal lines indicate the median. (d) The number of DR^{hi} MQs and (f) DR^{int} MQs among 10^5 LPMCs from non-inflamed and inflamed areas of the same patient is shown. ns = non-significant.

Figure 2. HLADR expression on intestinal DR^{int} MQs resembles CD14$^+$ blood monocytes. (a) Gating strategy used to select live, Lin$^-$CD14$^+$ blood cells (monocytes). (b) Median fluorescence intensity (MFI) of HLADR on CD14$^+$ monocytes and intestinal DR^{int} and DR^{hi} MQs. Paired blood and tissue samples were obtained from five UC patients in remission. Horizontal lines indicate the median.

Figure 3. Lamina propria CD141$^+$CD103$^+$and CD1c$^+$CD103$^+$ DCs decrease during inflammation. (a) Gating strategy for DCs. The CD14$^-$ population in Figure 1a was further gated as CD11c$^+$ (left). These cells were then divided into CD141$^+$ and CD1c$^+$ DC populations (middle) and further gated into CD141$^+$CD103$^+$ and CD1c$^+$CD103$^+$ DCs (right). Isotype control for CD103 (total DC population) and CD103 control staining of DR^{hi} MQs are shown. The number of CD141$^+$CD103$^+$DCs (b) and CD1c$^+$CD103$^+$DCs (d) among 10^5 live LPMCs from the indicated tissue is shown. Control samples are as in Fig. 1. Horizontal lines indicate the median. The frequency of CD103$^+$ cells among CD141$^+$ DCs (c) and CD1c$^+$ DCs (e) from non-inflamed and inflamed areas of the same patient is shown.

Figure 4. Increased DR^{int} DCs characterize MLNs draining inflamed ileum and colon. (a) Gating strategy to identify MLN DCs. Live, lin$^-$CD45$^+$HLADR$^+$CD14$^-$ cells were gated for CD11c and further divided into DR^{hi} and DR^{int} DCs. (b) The DR^{hi}/ DR^{int} DC ratio in the MLN draining the indicated tissue is shown. Horizontal lines indicate the median. (c) Schematic showing where the colon-draining MLNs

used to generate the DR^{hi}/ DR^{int} DC ratio in (b) were taken. Each color in (c) correlates to the DR^{hi}/ DR^{int} DC ratio of the same color (individual) in (b). (d) DR^{hi} DCs (blue) and DR^{int} DCs (red) were further gated into $CD141^+$, $CD1c^+$ and $CD141^-CD1c^-$ DCs and displayed as pie charts in (e). (f) MFI of HLADR expression on lamina propria $CD141^+$, $CD1c^+$ and $CD141^-CD1c^-$ DCs from non-inflamed and inflamed colon tissue of patients from whom MLNs were also obtained (a subset of the patients shown in 4a-e; CD ileum n=2, CD colon n=2 and UC colon n=2). Open triangles are ileal CD; open circles are colonic CD and closed circles are UC.

Figure 5. Low frequency of $ALDH^+$ DCs and MQs characterizes inflamed UC lamina propria . (a) Histograms of $ALDH^+$ cells among the indicated populations from the colon of an inflamed CD colitis patient. Cells were incubated with ALDEFLUOR in the absence (filled) or presence (dotted line) of DEAB inhibitor. The percent $Aldeflour^+$ cells is shown above the horizontal line indicating the positive gate. The frequency of $ALDH^+$ cells among DR^{hi} MQs (b), DR^{int} MQs (c), $CD141^+$ DCs (d) and $CD1c^+$ DCs (e) in inflamed tissue (CD and UC) and in remission (UC) compared to controls is shown. Horizontal lines indicate the median. (F) The frequency of $ALDH^+$ cells among DR^{hi} MQs, DR^{int} MQs, $CD141^+$ DCs and $CD1c^+$ DCs from non-inflamed and inflamed areas of the same patient is shown. (g) RT-PCR analysis of *ALDH1A1* and *ALDH1A2* expression relative to *HPRT* by total $CD14^+$ MQs (called DR^{hi}/DR^{int} MQs in the figure), $CD141^+$ DCs and $CD1c^+$ DCs purified from intestinal tissue by sorting using the gates in Figure 1a and 3a is shown (control n=4, UC n=4 and CD n=4). Bars show the median and range. Statistical significance is indicated with the *p*-value and all other comparisons were non-significant.

Figure 6. Similar frequency of circulating $ALDH^+$ cells in UC patients and healthy donors. (a-b) Gating strategy showing PBMCs of a healthy donor. Live, $Lin^-HLADR^+CD45^+$ cells were further gated into (a) non-classical ($CD14^{low}CD16^+$), intermediate ($CD14^+CD16^+$) and classical ($CD14^+CD16^-$) monocytes and $CD14^-CD16^-$ cells. (b) The double negative cells were further gated into $CD141^+$ and $CD1c^+$ cells. $ALDH^+$

cells were identified as in Fig. 5. The frequency of $ALDH^+$ cells among (c) classical, intermediate and non-classical monocytes as well as $CD141^+$ and $CD1c^+$ cells from the blood of healthy donors, UC patients with active disease and UC patients in remission is shown. Horizontal lines indicate the median. (d) RT-PCR analysis of *ALDH1A1* and *ALDH1A2* expression relative to *HPRT* by the indicated cell populations purified from the blood of healthy donors (n=5), UC patients with active disease (n=4) and UC patients in remission (n=3) by sorting using the gates in (a-b). nd = not detected. Bars show median and range. All comparisons were non-significant.

Supplementary Figure S1. Surface marker expression and frequency of DR^{hi} and DR^{int} MQs. (a) LPMCs from a control patient were pregated as in Figure 1a (left) and gating for $CD14^+$ and $CD14^-$ cells compared to isotype control is shown.. (b) Histograms showing surface expression of CD64, CD13, CD33, CD209 and Sirpα on DR^{hi} and DR^{int} MQs from control colon (open histogram) and inflamed Crohn's colon (filled histogram) is shown. Isotype control is indicated with a dotted line. (c-d) The frequency of DR^{hi} MQs (c) and DR^{int} MQs (d) among LPMCs from the indicated tissue is shown. Control tissue for CD and UC were from ascending and sigmoid colon, respectively. Horizontal lines indicate the median. ns = non-significant.

Supplementary Figure S2. The frequency of $CD141^+$ and $CD1c^+$ DCs among total LPMCs or lamina propria DCs remains constant during inflammation. (a) LPMCs from the colon of a control patient were pregated as in Figure 3a (left) and further gated for CD141 or CD1c as shown relative to isotype control. The frequency of $CD141^+$ DCs (b) and $CD1c^+$ DCs (c) among total live LPMCs and the frequency of $CD141^+$ DCs (d) and $CD1c^+$ DCs (e) among lamina propria DCs are shown. Horizontal lines indicate the median. ns = non-significant.

Supplementary Figure S3. The frequency of $CD103^+$ cells among $CD141^-CD1c^-$ DCs is reduced during colitis. (a) Gating strategy for $CD103^+$ DCs among $CD141^-CD1c^-$ DCs. The $CD14^-$ population in Figure 1a

was further gated as $CD11c^+$ (left) and then into $CD141^-CD1c^-$ DCs (middle) for analysis of CD103 (right). Isotype control for CD103 (total DC population) and CD103 control staining of DR^{hi} MQs are shown. (b) The frequencies of $CD103^+$ cells among $CD141^-CD1c^-$ DCs is shown for the indicated tissues. Horizontal lines indicate the median. ns = non-significant.

Supplementary Figure S4. Lamina propria $CD103^+$ DCs decrease during inflammation. (a) Gating strategy for $CD103^+$ DCs. The $CD14^-$ population (top left) was further gated as $CD11c^+$ cells (left) that were divided into $CD103^+$ and $CD103^-$ populations (right). (b) The number of $CD103^+$ DCs among 10^5 live LPMCs and (c) the frequency of $CD103^+$ DCs among total APCs from the indicated tissue is shown. Control samples are as in Fig. 1. Horizontal lines indicate the median. (d) The frequency of $CD103^+$DCs from non-inflamed and inflamed areas of the same patient is shown.

Supplementary Figure S5. HLADR expression is higher on $CD141^+$ and $CD1c^+$ cells from the intestine compared to the blood. (a) Gating strategy used to select live, Lin^-HLADR^+ blood cells (left) further gated into $CD141^+$ (top) and $CD1c^+$ (bottom) cells. (b) MFI of HLADR on $CD141^+$ and $CD1c^+$ cells from the blood and intestinal tissue of the same patient. Paired blood and tissue samples were obtained from UC patients in remission.

Supplementary Figure S6. ALDH activity in intestinal $CD103^+$ DCs and lamina propria $CD45^+$ and $CD45^-$ cells. (a) Histogram of ALDH expression by $CD103^+$ DCs from the colon of an inflamed CD colitis patient is shown. Cells were gated as in Supplementary Figure S4a. Cells were incubated with ALDEFLUOR in the absence (filled) or presence (dotted line) of DEAB inhibitor. The percent of $Aldeflour^+$ cells is shown above the horizontal line indicating the positive gate. (b) The frequency of $ALDH^+$ cells among $CD103^+$ DCs in inflamed CD or UC tissue and in UC patients in remission compared to controls is shown. (c) The frequency of $ALDH^+$ cells among and $CD45^+$ and $CD45^-$ LPMCs for UC patients in remission and controls is shown. Horizontal lines indicate the median.

Figure1

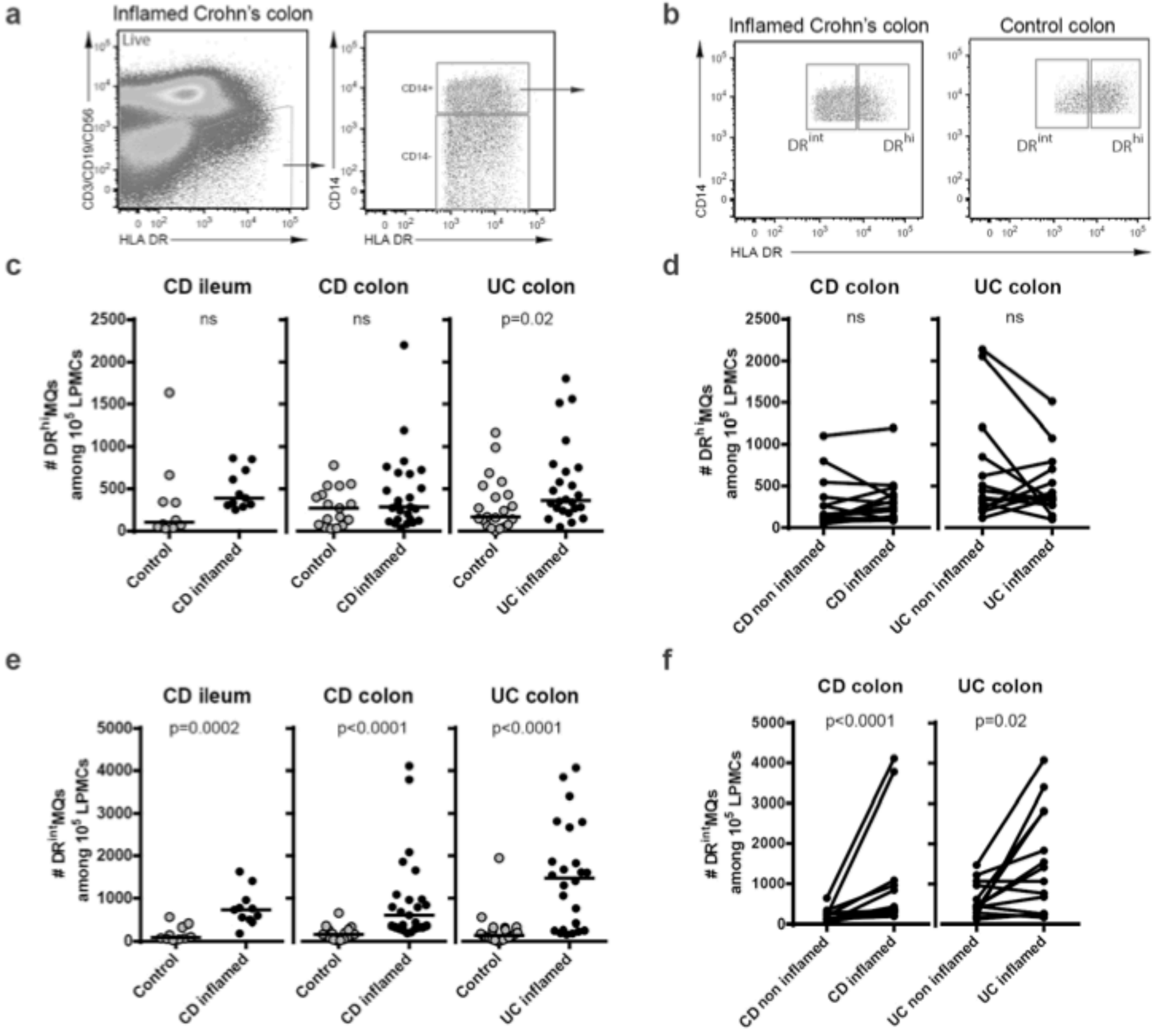

a
Inflamed Crohn's colon
Live
CD3/CD19/CD56
HLA DR
CD14
CD14+
CD14-
b
Inflamed Crohn's colon
Control colon
CD14
HLA DR
DR^int
DR^hi
c
CD ileum
CD colon
UC colon
ns
ns
p=0.02
# DR^hiMQs among 10^5 LPMCs
Control
CD inflamed
Control
CD inflamed
Control
UC inflamed
d
CD colon
UC colon
ns
ns
DR^hiMQs among 10^5 LPMCs
CD non inflamed
CD inflamed
UC non inflamed
UC inflamed
e
CD ileum
CD colon
UC colon
p=0.0002
p<0.0001
p<0.0001
DR^intMQs among 10^5 LPMCs
Control
CD inflamed
Control
CD inflamed
Control
UC inflamed
f
CD colon
UC colon
p<0.0001
p=0.02
# DR^intMQs among 10^5 LPMCs
CD non inflamed
CD inflamed
UC non inflamed
UC inflamed

Figure 2

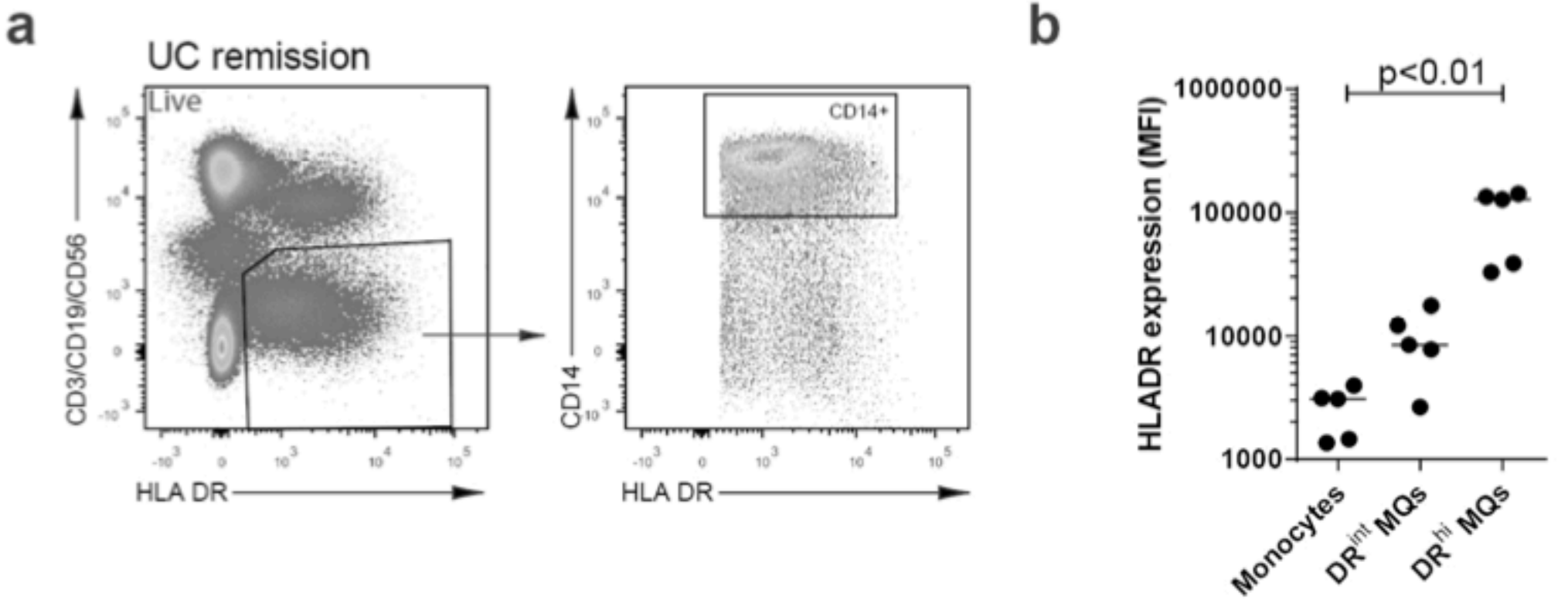
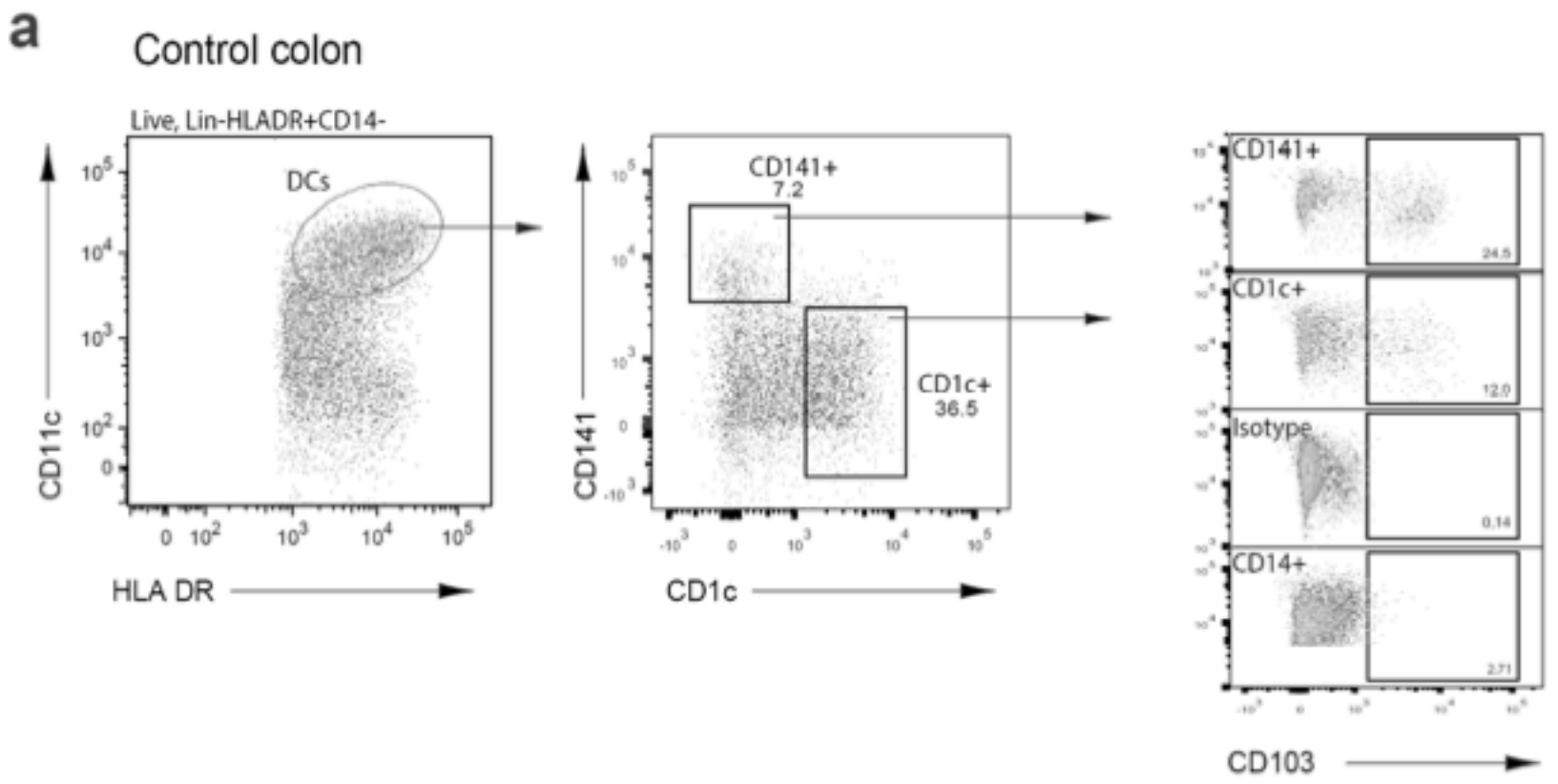
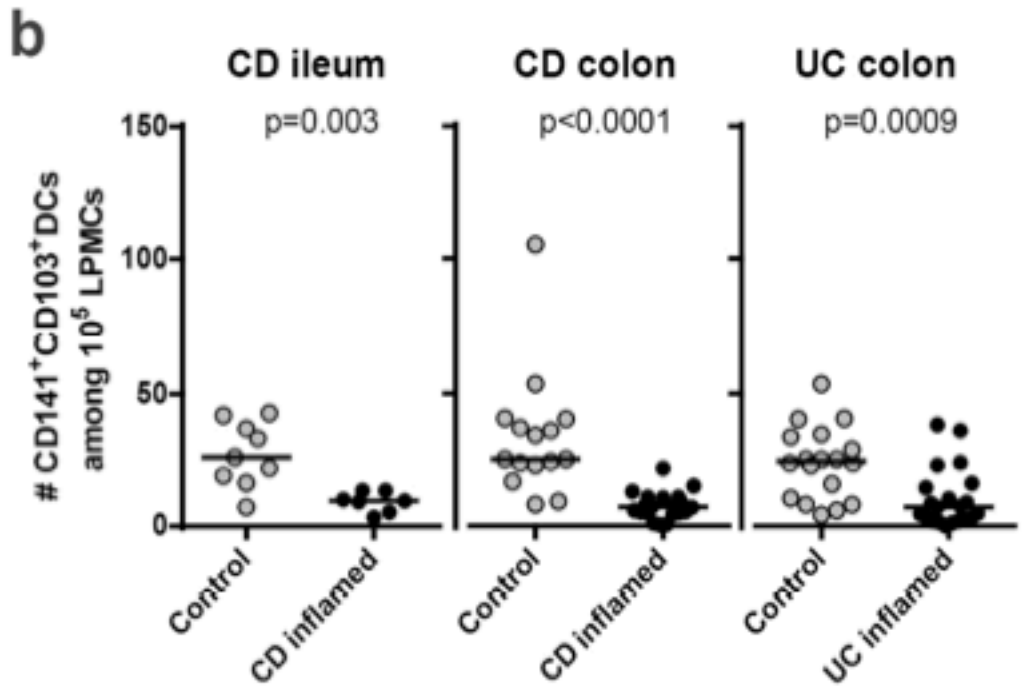
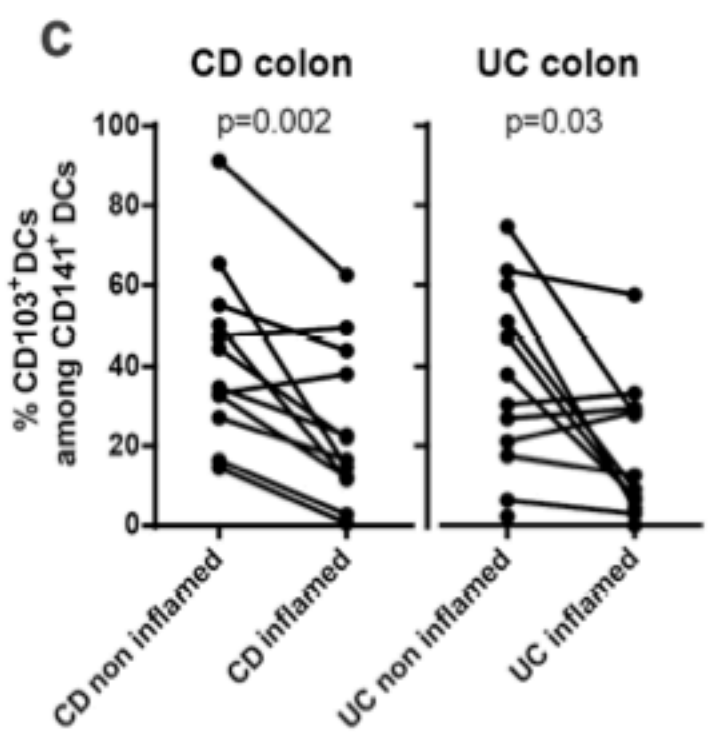
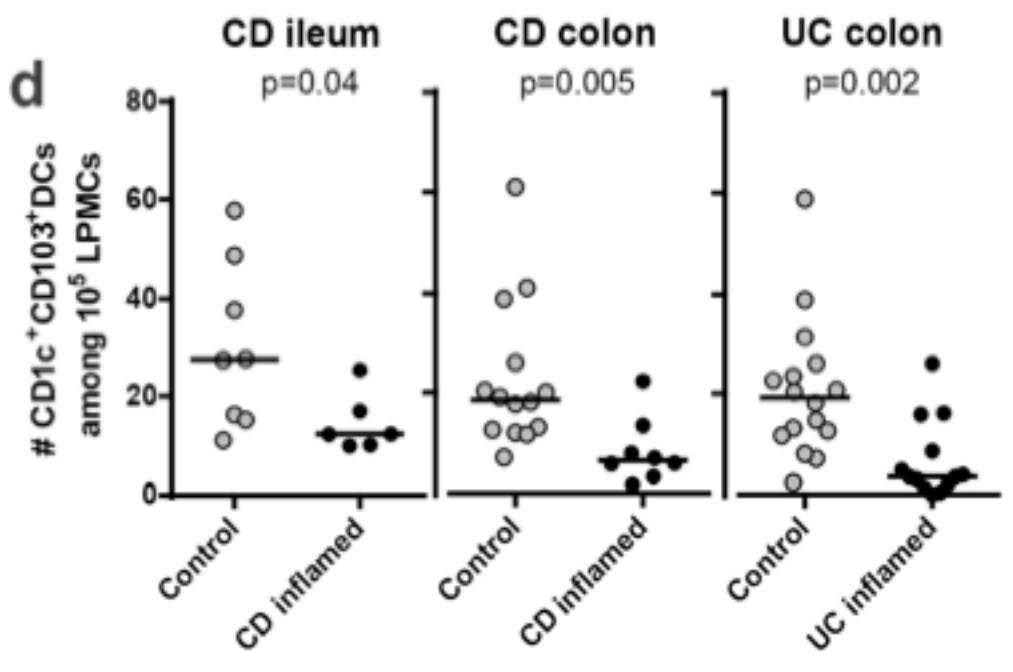

a
UC remission
Live
CD3/CD19/CD56
HLA DR
CD14+
CD14
HLA DR
b
p<0.01
HLADR expression (MFI)
1000000
100000
10000
1000
Monocytes
DR^int MQs
DR^hi MQs

Figure 3


a
Control colon
Live, Lin-HLADR+CD14-
DCs
CD11c
HLA DR
CD141
CD1c
CD141+
7.2
CD1c+
36.5
CD141+
24.5
CD1c+
12.0
Isotype
0.14
CD14+
2.71
CD103


b
CD ileum
CD colon
UC colon
p=0.003
p<0.0001
p=0.0009
# CD141+CD103+DCs among 10^5 LPMCs
Control
CD inflamed
UC inflamed


c
CD colon
UC colon
p=0.002
p=0.03
% CD103+DCs among CD141+ DCs
CD non inflamed
CD inflamed
UC non inflamed
UC inflamed


d
CD ileum
CD colon
UC colon
p=0.04
p=0.005
p=0.002
# CD1c+CD103+DCs among 10^5 LPMCs
Control
CD inflamed
UC inflamed

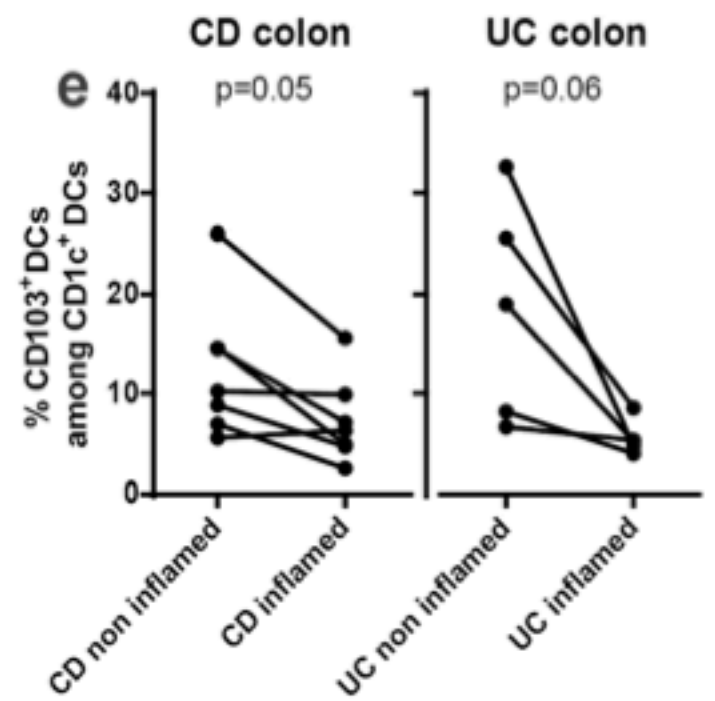

e
CD colon
UC colon
p=0.05
p=0.06
% CD103+DCs among CD1c+ DCs
CD non inflamed
CD inflamed
UC non inflamed
UC inflamed

Figure 4

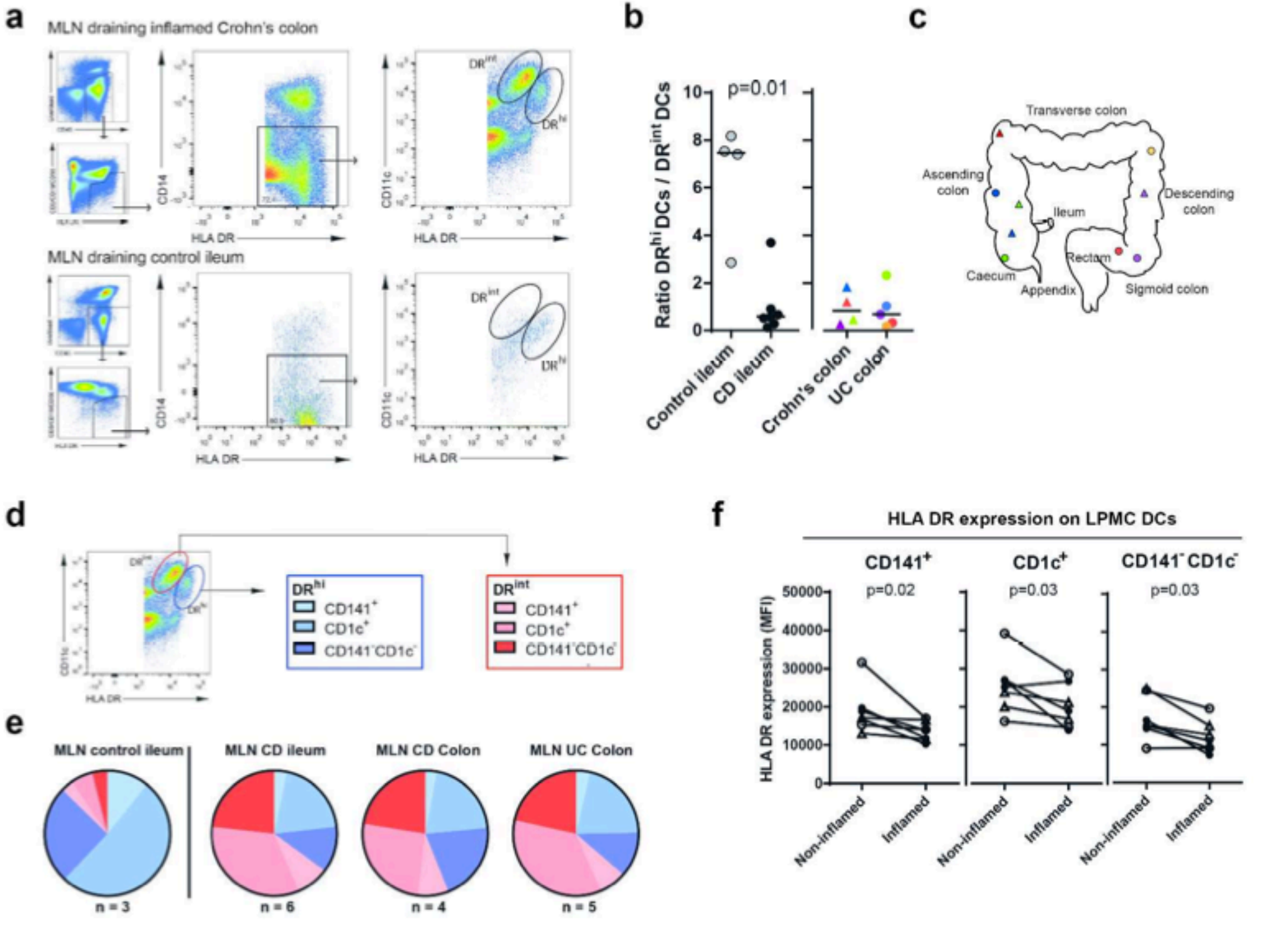

a
MLN draining inflamed Crohn's colon
CD14
HLA DR
CD11c
DR^int
DR^hi
MLN draining control ileum
b
Ratio DR^hi DCs / DR^int DCs
p=0.01
Control ileum
CD ileum
Crohn's colon
UC colon
c
Transverse colon
Ascending colon
Descending colon
Ileum
Rectum
Caecum
Appendix
Sigmoid colon
d
DR^hi
CD141+
CD1c+
CD141-CD1c-
DR^int
CD141+
CD1c+
CD141-CD1c-
e
MLN control ileum
MLN CD ileum
MLN CD Colon
MLN UC Colon
n = 3
n = 6
n = 4
n = 5
f
HLA DR expression on LPMC DCs
CD141+
CD1c+
CD141-CD1c-
p=0.02
p=0.03
p=0.03
HLA DR expression (MFI)
50000
40000
30000
20000
10000
0
Non-inflamed
Inflamed
Non-inflamed
Inflamed
Non-inflamed
Inflamed

Figure 5

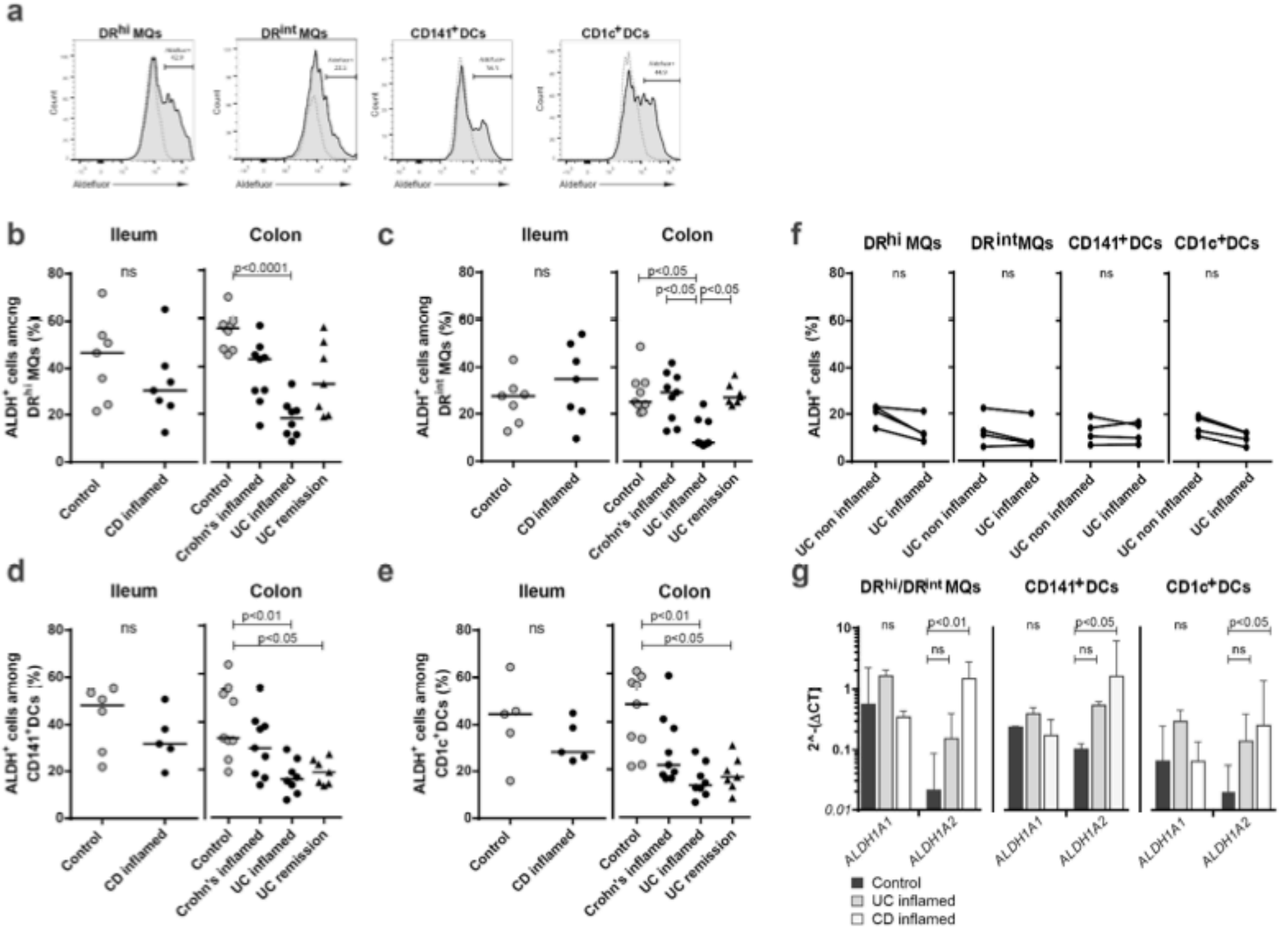
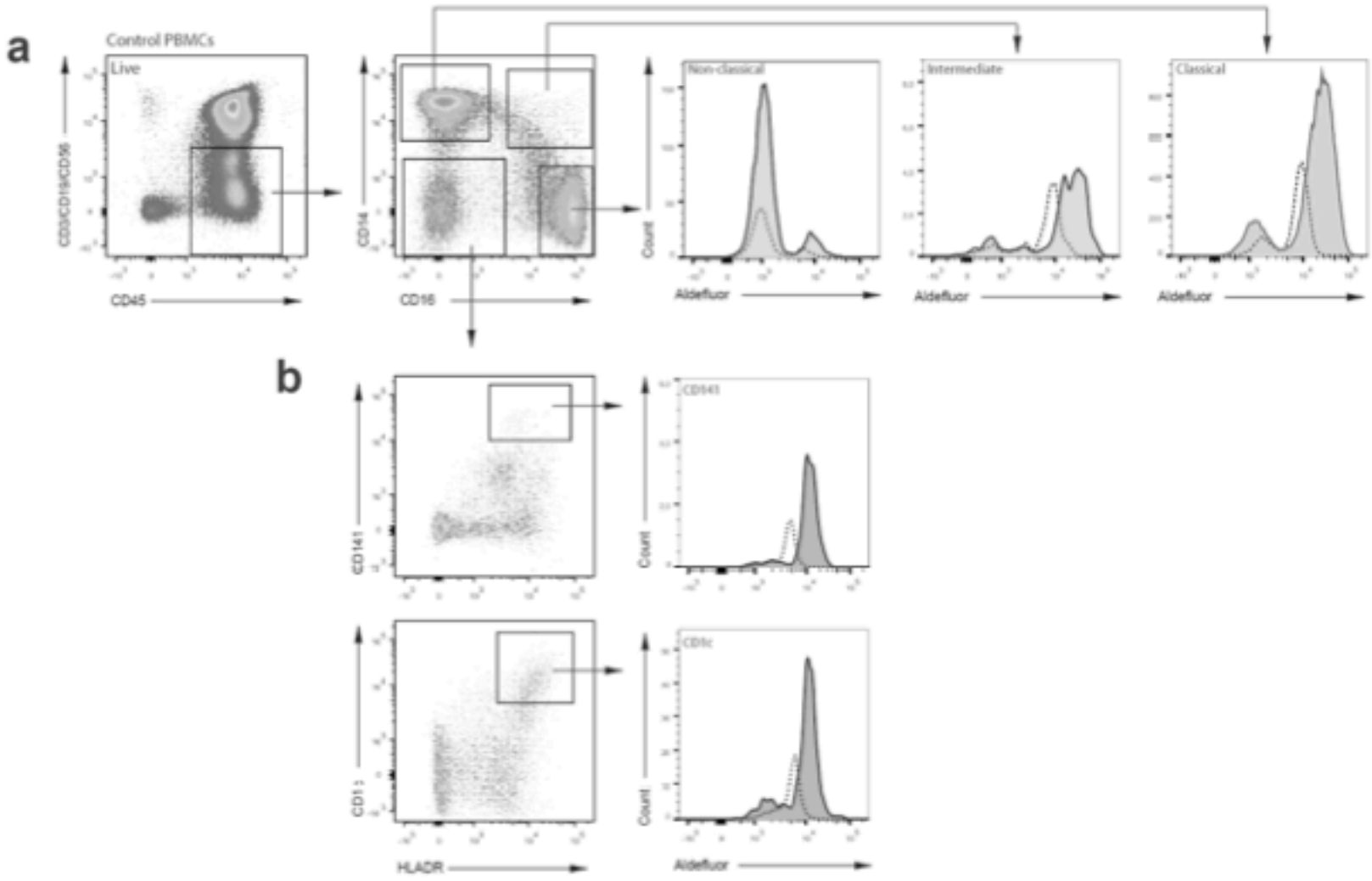

a
DRhi MQs
DRint MQs
CD141+DCs
CD1c+DCs
Count
Aldefluor
b
Ileum
Colon
ns
p<0.0001
ALDH+ cells among DRhi MQs (%)
Control
CD inflamed
Crohn's inflamed
UC inflamed
UC remission
c
ns
p<0.05
p<0.05
p<0.05
ALDH+ cells among DRint MQs (%)
d
ns
p<0.01
p<0.05
ALDH+ cells among CD141+DCs (%)
e
ns
p<0.01
p<0.05
ALDH+ cells among CD1c+DCs (%)
f
DRhi MQs
DRintMQs
CD141+DCs
CD1c+DCs
ALDH+ cells (%)
UC non inflamed
UC inflamed
g
DRhi/DRint MQs
CD141+DCs
CD1c+DCs
2^-(ΔCT)
ALDH1A1
ALDH1A2
Control
UC inflamed
CD inflamed

Figure 6


a
Control PBMCs
Live
CD3/CD19/CD56
CD45
CD14
CD16
Count
Aldefluor
Non-classical
Intermediate
Classical
b
CD141
CD1c
HLADR

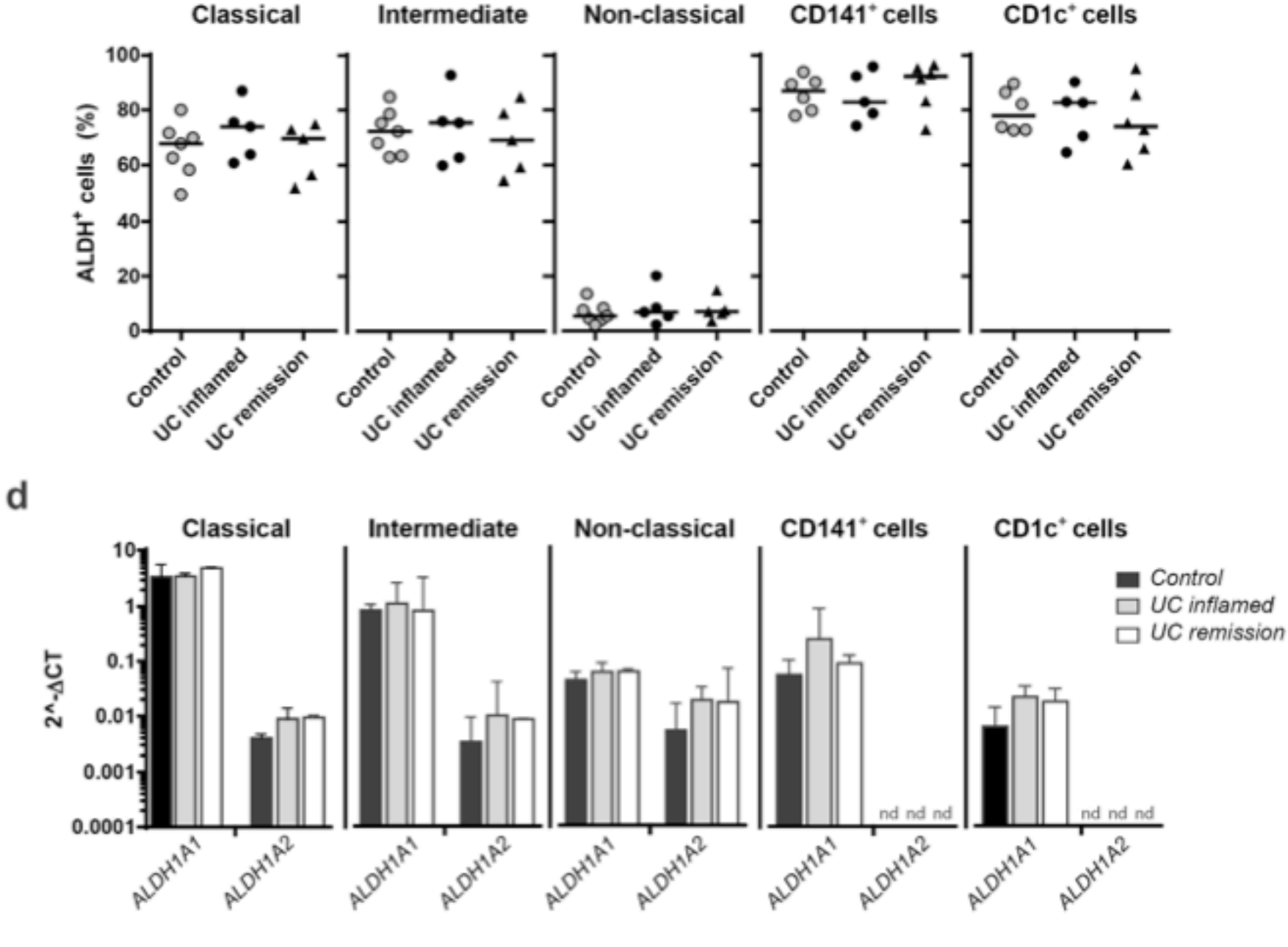

c
Classical
Intermediate
Non-classical
CD141+ cells
CD1c+ cells
ALDH+ cells (%)
Control
UC inflamed
UC remission
d
2^-ΔCT
ALDH1A1
ALDH1A2
nd nd nd
Control
UC inflamed
UC remission

Table 1. Patient demographics.

	UC	CD	Controls
Total number of patients	45	51	39
Male/female	34/11	27/24	21/18
Age	43 (20-76)[1]	38 (22-80)	60 (18-90)
Smoking habit (active/ex smoker/never)	4/6/35	7/7/35[2]	8/8/23
Disease duration, years	9 (1-48)	12 (1-55)	NA[3]
Disease status (active inflammation/remission)	36/9	51/0	NA
Examination (endoscopy/surgery/blood only)	27/9/9	34/17/0	21/10/8
Samples obtained (colon/ileum/MLN/blood)	36/0/5/15	31/20/11/0	25/12/4/8
Treatments:			
Corticosteroids, thiopurines, anti-TNF	2	3	0
Corticosteroids, 5-ASA, anti-TNF	3	0	0
Corticosteroids, 5-ASA, thiopurines	2	0	0
Corticosteroids, thiopurines	0	1	0
Corticosteroids, 5-ASA	4	1	0
Corticosteroids, anti-TNF	0	1	0
Thiopurines , anti-TNF	0	1	0
Thiopurines , 5-ASA	2	4	0
Anti-TNF	0	2	0
Corticosteroids	1	4	1
Thiopurines	1	15	0
5-ASA	22	7	0
Neoadjuvant chemotherapy	0	0	2

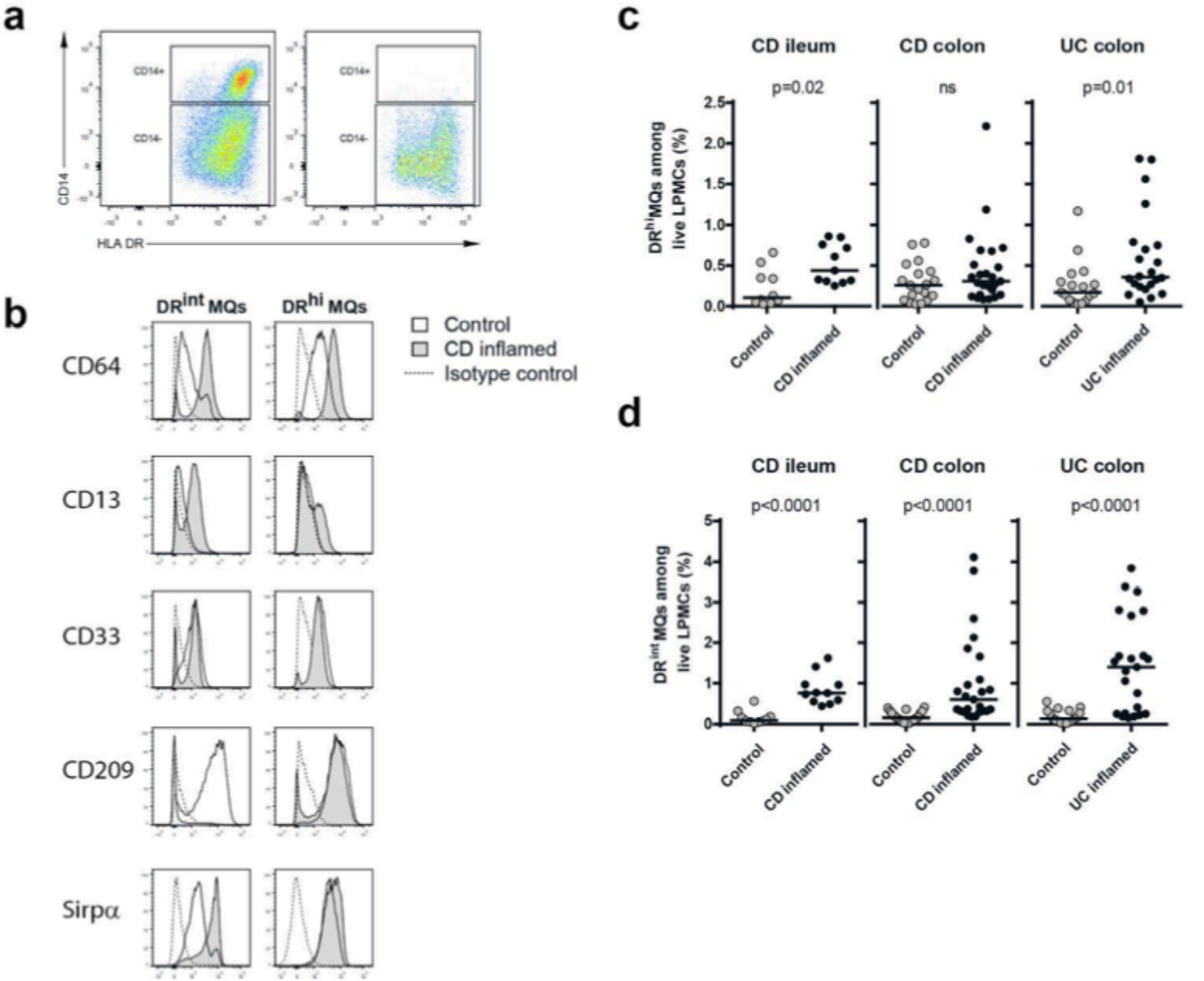
No treatment	8	12	36

[1] Data are shown as median (range).

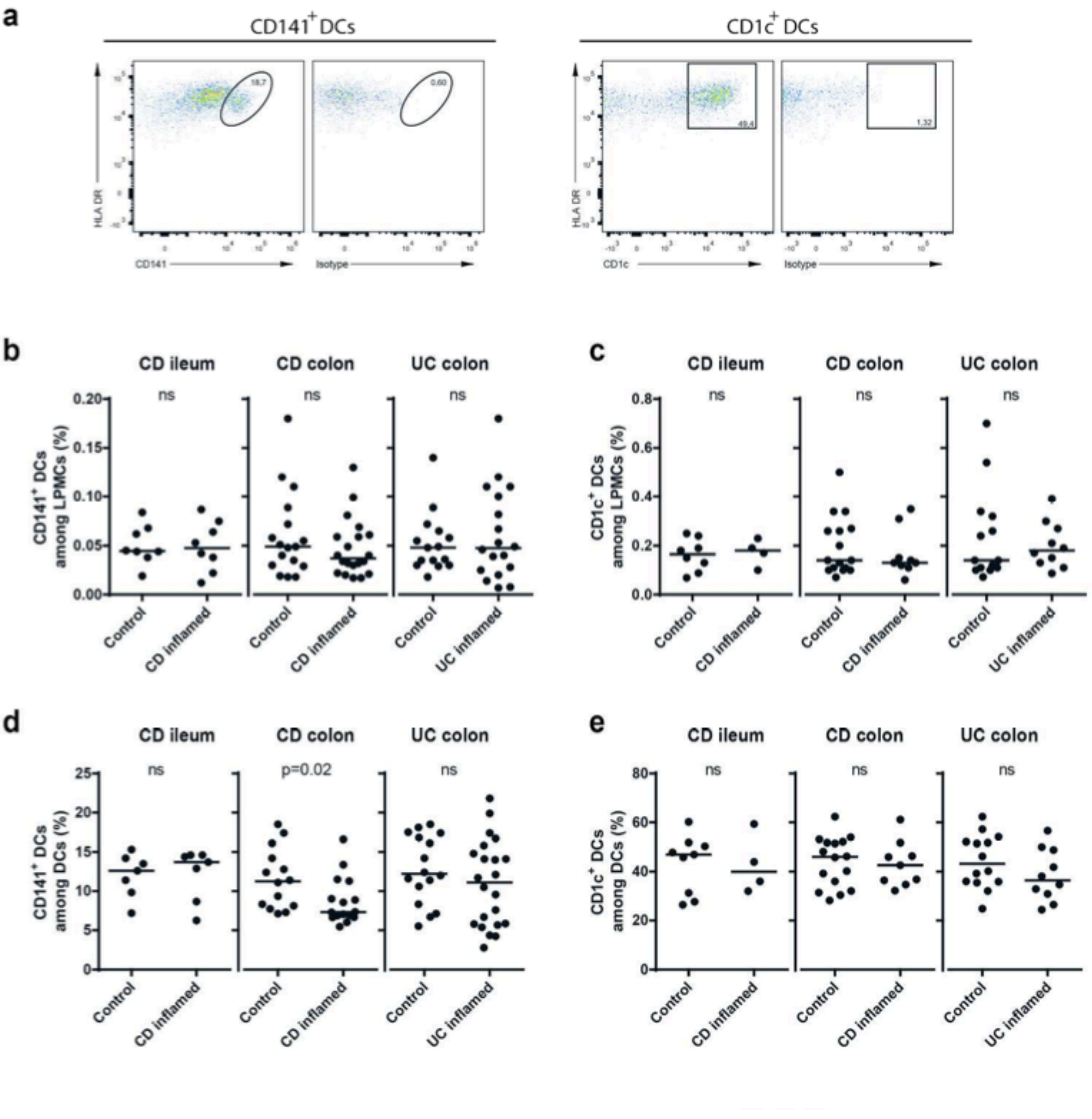
[2] Data from 2 patients are missing.

[3] NA, not applicable.

Supplementary Figure S1

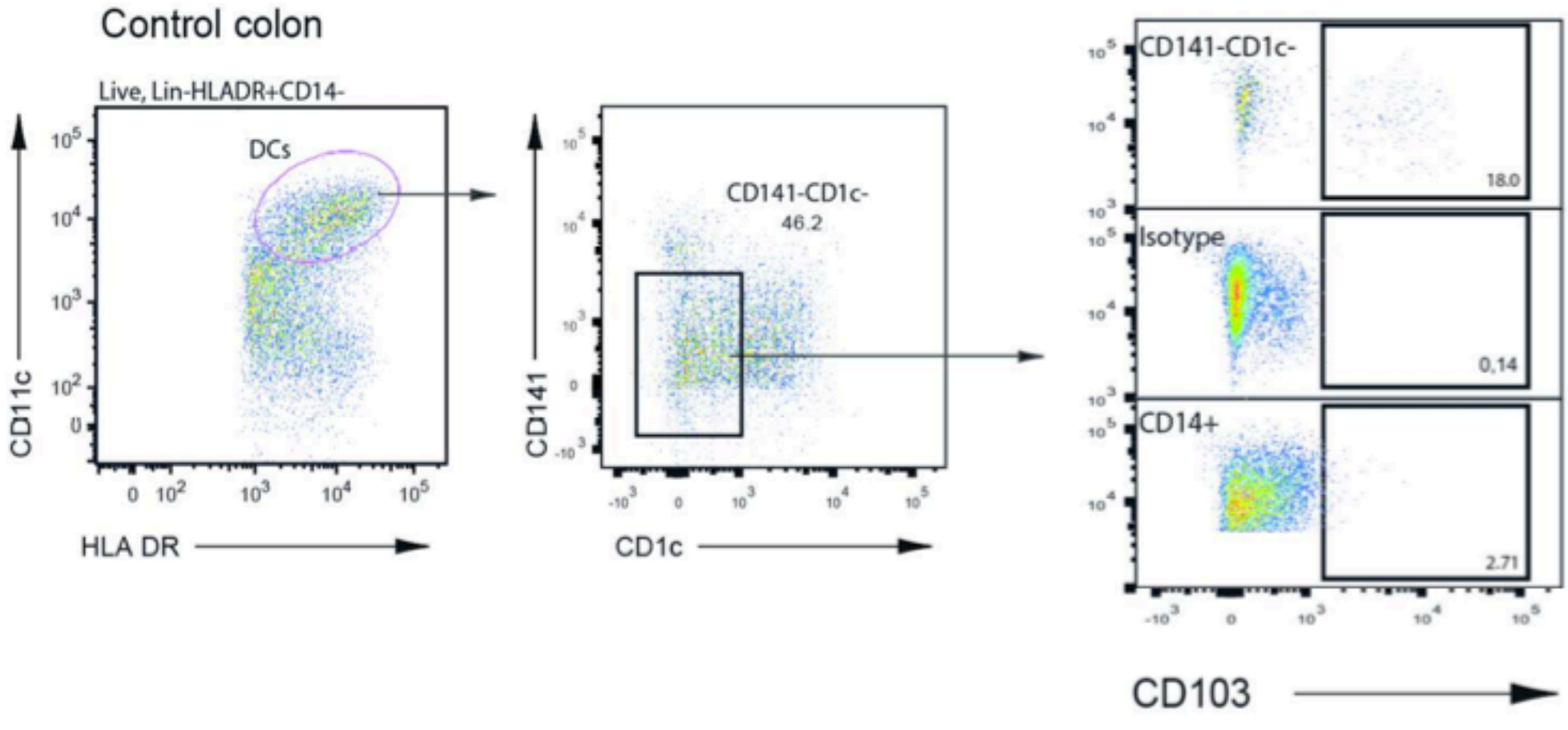


Supplementary Figure S2

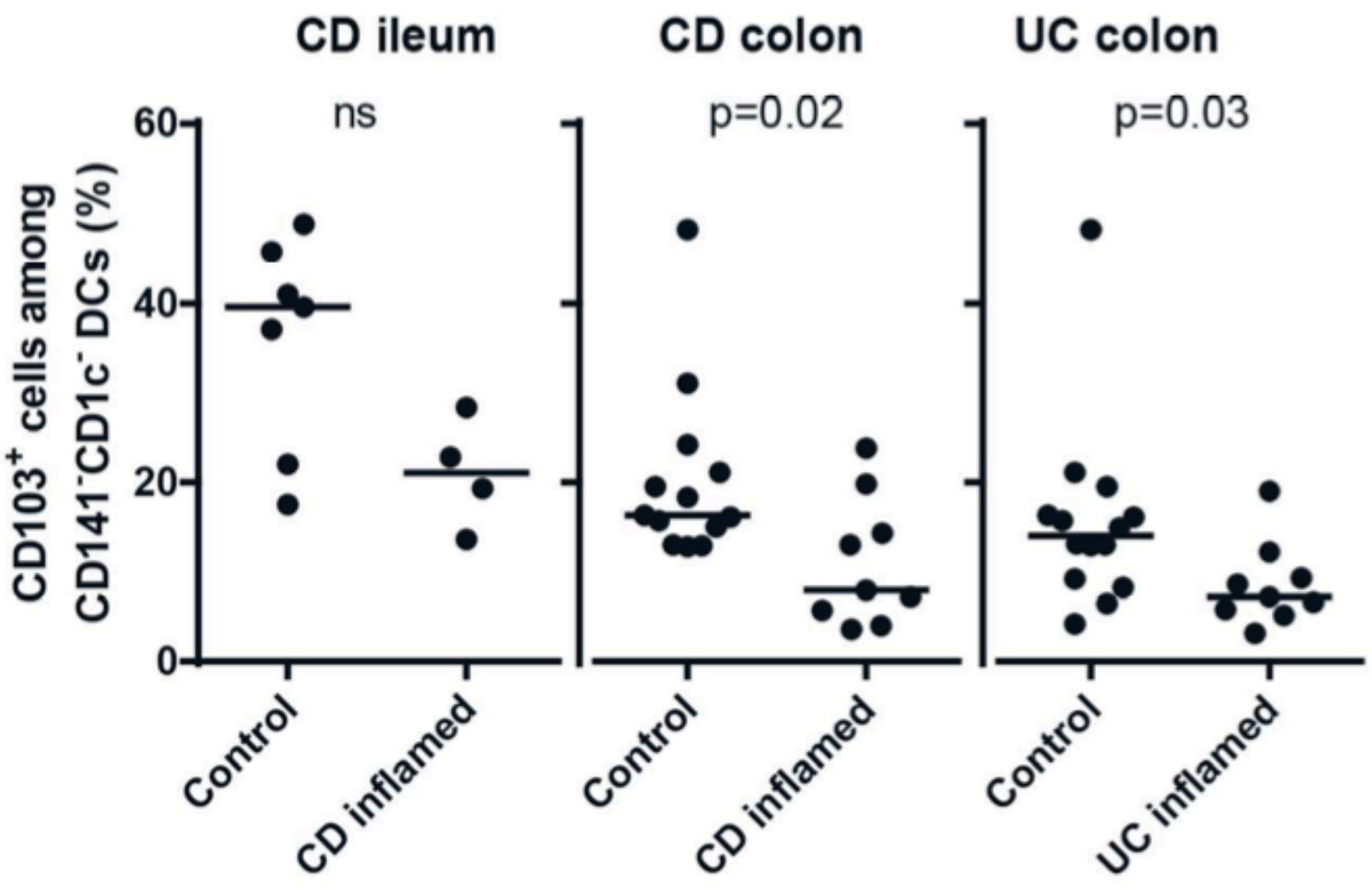


Supplementary figure S3

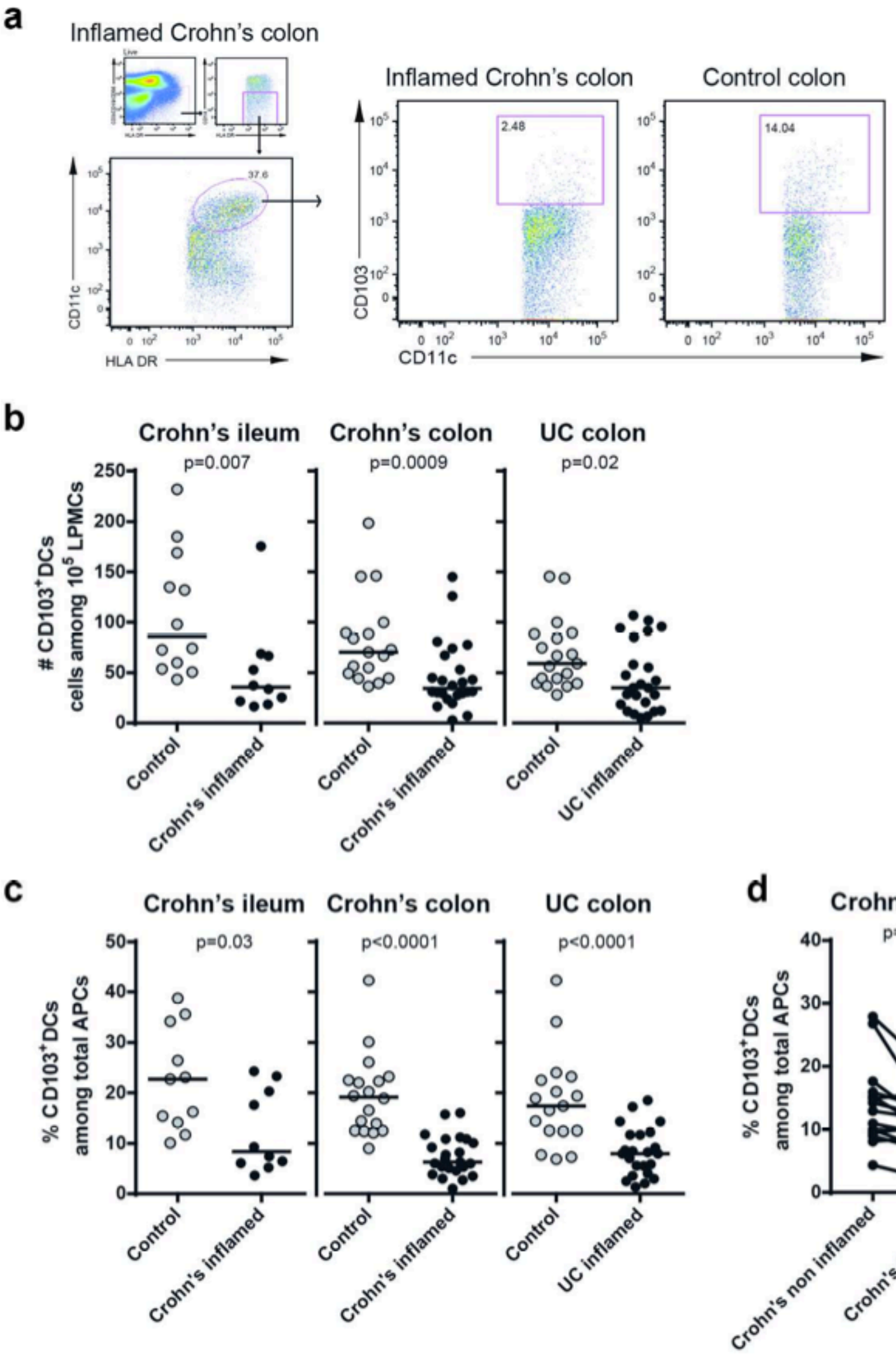
a



b

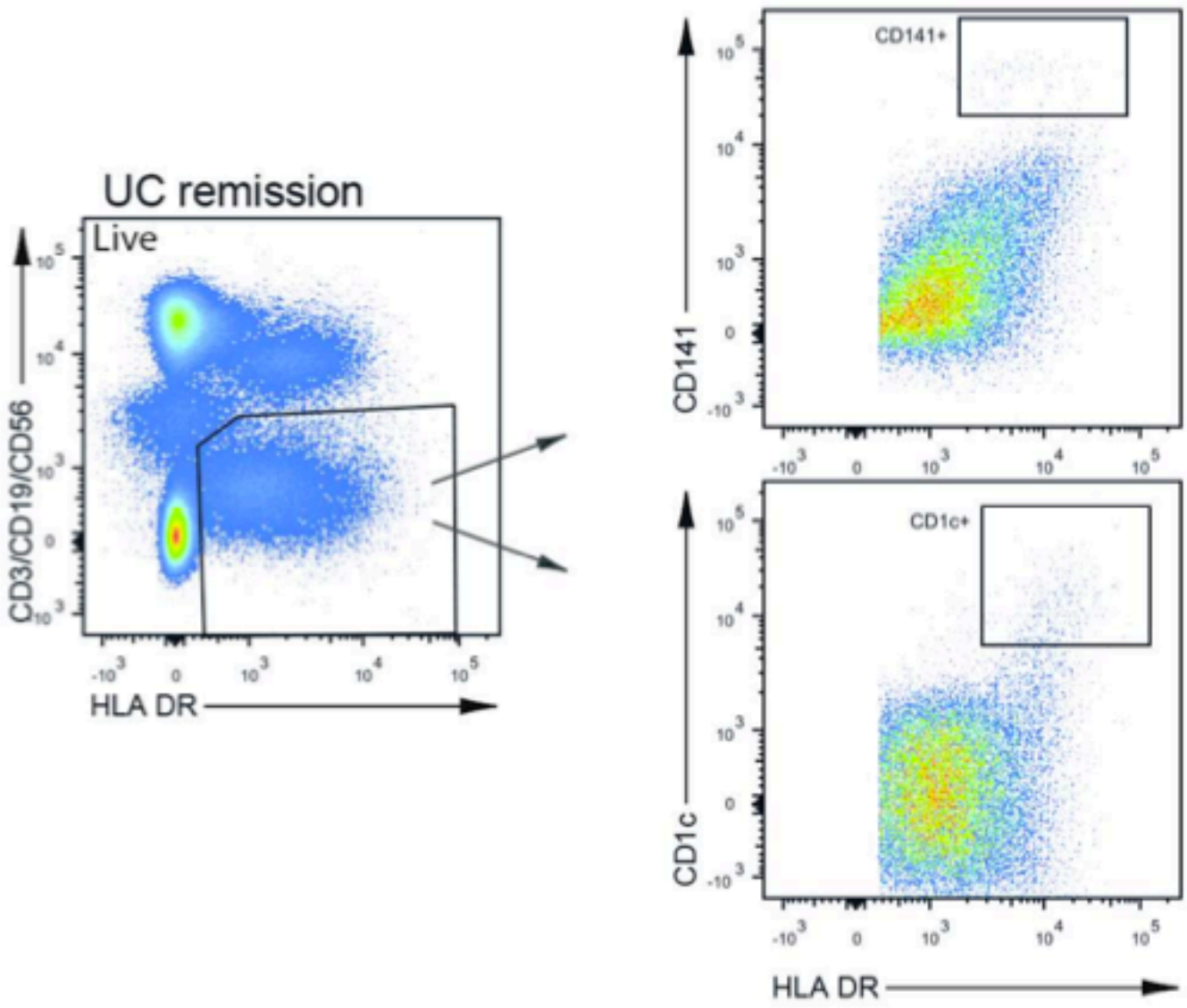


Supplementary figure S4

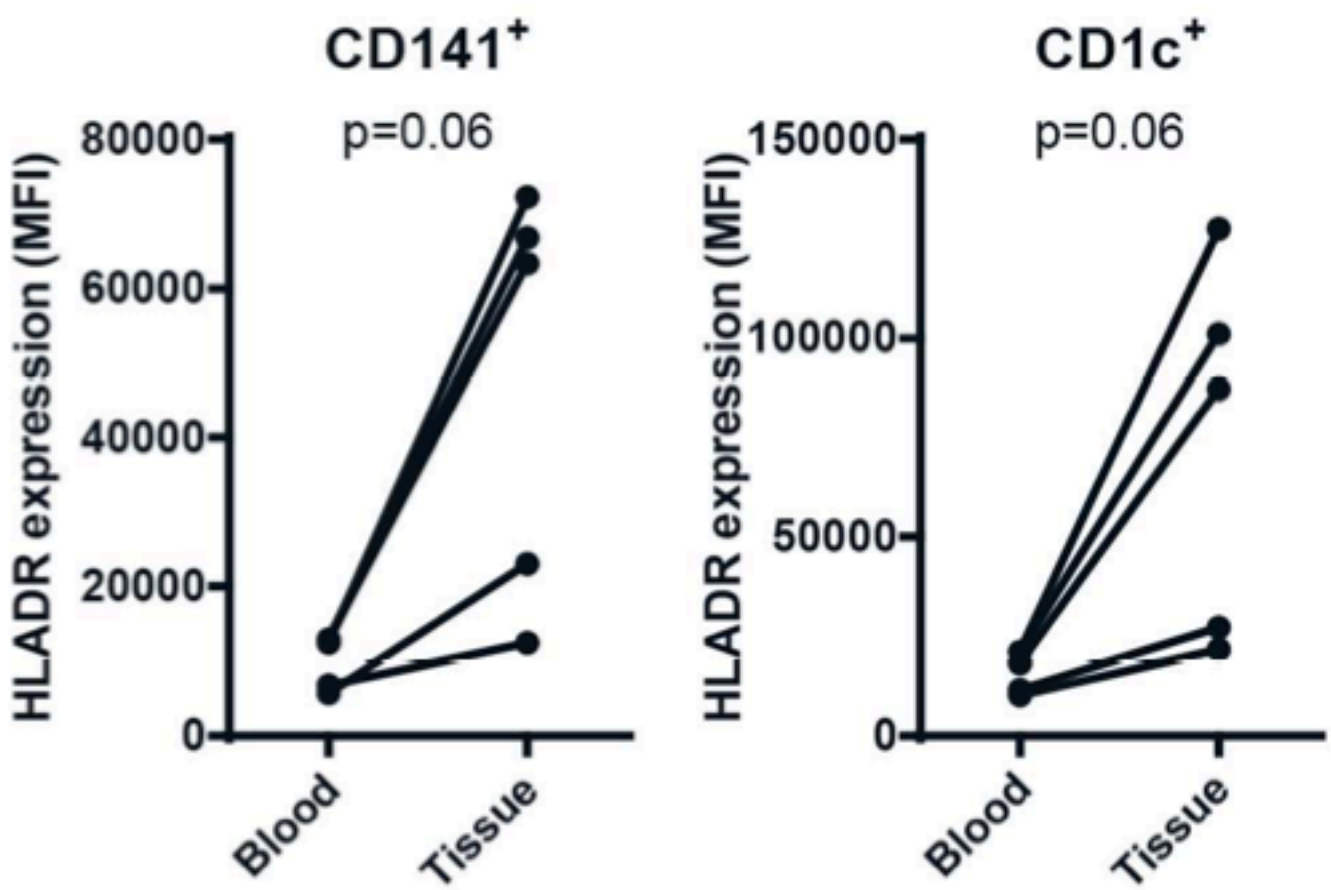


Supplementary figure S5

a



b



Supplementary figure S6