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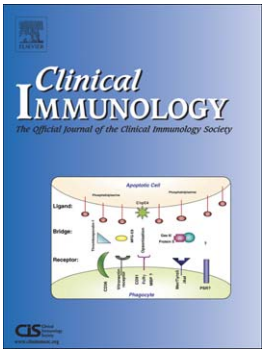

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Increased CD1c$^+$ mDC1 with mature phenotype regulated by TNFα-p38 MAPK in autoimmune ocular inflammatory disease

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Abbreviation

mDC1, myeloid dendritic cells 1; HCs, healthy controls; MoDCs, monocyte-derived dendritic cells; PBMCs, peripheral blood mononuclear cells; Th, T helper; DCs, dendritic cells; mDCs, myeloid dendritic cells; pDCs, plasmacytoid dendritic cells; Lin1, lineage 1; Alb, albumin; iNKT, invariant natural killer T.

Abstract

In this study we investigated the role of blood $CD1c^+$ myeloid dendritic cells 1 (mDC1), a key mDC subtype, in patients with autoimmune uveitis. We observed a significant increase of blood $CD1c^+$ mDC1 in uveitis patients. The increased $CD1c^+$ mDC1 exhibited high HLADR expression and less antigen uptake. $CD1c^+$ mDC1 were divided into two subpopulations. $CD1c^{hi}$ mDC1 subpopulation showed less antigen uptake and higher HLADR expression compared to $CD1c^{lo}$ mDC1 subpopulation. Importantly, the $CD1c^{hi}$ mDC1 subpopulation was increased in uveitis patients. *In vitro*, mature monocyte-derived dendritic cells (MoDCs), characterized by lower levels of antigen uptake, induced more $CD62L^-CD4^+$ T helper cell proliferation. The mature phenotype and function of $CD1c^+$ mDC1 were regulated by TNFα via a p38 MAPK-dependent pathway. These data show that alterations in the systemic immune response are involved in the pathogenesis of autoimmune uveitis and invite the therapeutic possibility of attenuating uveitis by manipulating blood $CD1c^+$ mDC1.

1. Introduction

Human autoimmune ocular inflammatory diseases, particularly non-infectious uveitis (abbreviated as uveitis), are currently responsible for 10% of the visual handicap in the United States [1]. The most commonly recognized mechanism of uveitis is $CD4^+$ T helper (Th) cell mediated immune dysregulation [2-4]. It has been suggested that the immune dysregulation is characterized by the imbalance between regulatory and pathogenic effector T cells, increased inflammatory and decreased anti-inflammatory cytokines, hyper-responsiveness to self-antigens, and dysfunctional dendritic cells (DCs), which are all suspected to contribute to the pathogenesis of autoimmune disease [5].

Dendritic cells (DCs) play a central role in immune regulation since they are involved in both innate and adaptive immune responses. An indispensable part of initiating the immune response in the eye is the permeability of the blood-ocular barrier to inflammatory cells. Infiltrating cells include monocytes/macrophages, lymphocytes, neutrophils, and myeloid DCs (mDCs). Moreover, an emerging body of literature supports a pivotal role for mDCs in uveitis in humans and mice [6-8]. However, the actual source of mDCs in eye tissue, along with the factors that trigger DC maturation, take up antigens and help T cell responses, remain unidentified.

DCs are derived from either progenitors in the bone marrow or monocytes in the peripheral blood. Blood DCs contain three subsets: $CD1c^+$ (BDCA1) myeloid dendritic cell 1 (mDC1), $CD141^+$ (BDCA3) myeloid dendritic cell 2 (mDC2), and $CD303^+$ (BDCA2) plasmacytoid DCs (pDCs) [9], which are from Lineage 1^- (Lin1, including CD3, CD14, CD16, CD19, CD20 and CD56) $HLADR^+$ cells. Myeloid DCs actively infiltrate into the eye and can be seen suspending in the aqueous humor of uveitis patients [10].

In this study, we observed an increase in blood $CD1c^+$ mDC1 with enhanced HLADR expression and lower levels of antigen uptake indicating a more mature phenotype. Moreover, this was associated with an increase in a subpopulation of $CD1c^{hi}$ mDC1, which had very low levels of antigen uptake. In an *in vitro* model we observed that MoDCs with a similar low-antigen-uptake phenotype promoted more $CD4^+$ T cell proliferation, than those taking up high levels of antigen. Our work also supports the hypothesis that in uveitis the mature phenotype of $CD1c^+$ mDC1 were regulated by TNFα via a p38 MAPK-dependent pathway.

2. Material and methods

2.1. Study population

Peripheral blood was obtained from patients with non-infectious uveitis (n=74) in the Clinical Center of the National Eye Institute (NEI), and from healthy controls (HCs, n=96) in the Blood Bank of the National Institutes of Health (NIH). All protocols were approved by the NIH Neuroscience Institutional Review Board (IRB). Informed consents were obtained from all subjects before the study commenced. Ocular disease activity, i.e. active and inactive uveitis, were recorded according to the Standardization of Uveitis Nomenclature (SUN) [11]. According to SUN criteria, less than one cell, or alternatively, no flare in the anterior chamber, constitutes a graded score of 0. SUN criteria go on to address “trace” cells in the vitreous, correlating this clinical impression with a score of 0.5. A grade of 0 is regarded as inactive, whereas, a grade >= 0.5 is considered slightly active, and a grade >= 1 or haze in vitreous is considered active. Here, we put slightly active and active uveitis into one group as “active”.

To investigate the phenotype and function of mDC1 in the peripheral blood, the effects of systemic immunosuppressive therapy on mDC1 were considered. The presence or absence of systemic immunosuppressive therapy was thus documented; this included systemic corticosteroids, secondary immunomodulatory therapy and biologic response modifiers (see the table).

2.2. Identification of mDCs and pDCs in human peripheral blood

Blood DCs were identified by 4-color staining performed on whole fresh peripheral blood using the following monoclonal antibodies: PE-CD1c (BDCA1, Miltenyi Biotec, Auburn, CA) or PE-CD141 (BDCA3, Miltenyi Biotec), PERCP-HLADR (Miltenyi Biotec), APC-CD303 (BDCA2, Miltenyi Biotec), and FITC-labeled mAbs against lineage markers CD3, CD14, CD16, CD19, CD20 and CD56 (BD Biosciences, San Jose, CA). Cells that were not labeled with these lineage markers were designated as $Lin1^-$. Myeloid DC1 were $CD1c^+$, mDC2 were $CD141^+$, and pDCs were $CD303^+$. These DCs were gated on $Lin1^-$ $HLADR^+$ total DC population. Cells were analyzed on FACSCalibur (BD Biosciences). DC number was calculated by multiplying the percentage of DCs by the total number of white blood cells (WBC) in 1 ml blood (DC number = % of DCs $\times$ number of WBC/ml).

2.3. Generation of monocyte-derived DCs

Monocyte-derived dendritic cells (MoDCs) were generated as previously described and used as an *in vitro* model for mDCs for additional functional studies [12, 13]. Briefly, monocytes were isolated from mononuclear cell fractions of the peripheral blood of healthy controls and seeded in the presence of GM-CSF (40 ng/ml; R&D systems, Minneapolis, MN) and IL-4 (20 ng/ml; R&D Systems) at a concentration of 1×10^6

cells/well in a 6-well plate. Six to seven days after culture, monocytes were completely differentiated into dendritic cells and CD1c expression was evaluated by flow cytometry.

To investigate the effect of the TNFα-p38 MAPK signaling pathway on CD1c expression, recombinant TNFα (10 ng/ml, R&D, Minneapolis, MN) was added for 24 hours before measuring the protein expression of CD1c, and for 30 minutes before measuring phosphoryrated-p38 MAPK (BD Biosciences) expression on MoDCs by flow cytometry. For blocking experiments, the selective MAPK inhibitor SB203580 (10 μmol/l, Cell Signaling, Danvers, MA) was added at day 0 of MoDCs differentiation *in vitro.* CD1c protein expression was measured by flow cytometry on days 1, 3, and 5.

2.4. Evaluation of cytokine production

Peripheral blood were stimulated with lipopolysaccharides (LPS, 1 μg/ml, Sigma-Aldrich, St. Louis, MO) and GolgiPlug^{TM} (2 μl/ml, BD Pharmingen, San Jose, California) for 5 h at 37°C in a humidified 5% CO_2 atmosphere. Following stimulation, cells were stained with Lin1, HLADR and CD1c to identify mDC1. Then, cells were fixed and permeabilized (Fix & Perm Cell Permeabilization Kit; BD Biosciences) before staining for IL6, IL10 and IL12p40/70 (BD Biosciences). Cytokine production was analyzed by flow cytometry.

2.5. Antigen uptake assay

Quantitative analysis of DC endocytosis was performed as described previously [14], with minor modifications. To study mDC endocytosis, 1ml of fresh blood was incubated with FITC-albumin (10 μg/ml, Sigma-Aldrich, St, Louis, Mo) at either 37 or 4°C (as negative control) for 30 minutes. Freshly taken blood was stained with CD1c, HLADR

and CD19, followed by flow cytometric analysis. The $CD1c^{+}HLADR^{+}CD19^{-}$ population was classified as mDC1. Myeloid DC1 expressing FITC represented mDC1 taking up antigen (albumin). In the functional studies, 1µl sera from HC or uveitis patients was added into MoDCs in the volume of 200 µl for 24 hours before the antigen uptake assay was conducted. To investigate the effect of TNFα on antigen uptake, MoDCs were treated with recombinant TNFα at different concentrations (0.1 ng/ml, 1 ng/ml and 10 ng/ml) for 24 hours. The antigen uptake assay was subsequently conducted.

2.6. DC-stimulated T cell responses

MoDCs-stimulated T cell responses were performed as described previously [14], with minor modifications. Briefly, MoDCs with high albumin uptake (Alb^{+}) and low albumin uptake (Alb^{-}) were sorted based on FITC-albumin expression on MoDCs. Magnetic bead separation (Miltenyi Biotec) was used to isolate $CD4^{+}$ T cells in peripheral blood mononuclear cells (PBMCs) from healthy controls. Purity of isolated fractions consistently averaged to be >95% for $CD4^{+}$ T cells. Isolated DCs were co-cultured with CFSE-labeled allogenic $CD4^{+}$ T cells at a ratio of 1:10 (DC:T) for 5 days with the help of anti-CD3 stimulation (1 µg/ml). $CD4^{+}$ T cell proliferation was characterized by flow cytometric analysis (percentage of cells exhibiting CFSE fluorescence dilution) and negative expression of CD62L was characterized with activated $CD4^{+}$ T cells.

2.7. Statistical analysis

Data is presented as mean ± SD. Results shown were representative of at least three independent experiments. Statistical comparisons were performed using the Student's *t*

test for two groups, or ANOVA tests for analyzing more than two groups. P<0.05 was considered as significant. Analyses were accomplished using Prism 6 software (GraphPad Software, Inc., La Jolla, CA).

3. Results

3.1. Elevation of blood CD1c$^+$ mDC1 in uveitis patients

The percentages and absolute numbers of blood DC subsets were determined in uveitis patients and HCs. DC subsets are shown in figure 1A. Lin1$^-$HLADR$^+$ cells were defined as total DCs in which cells were subsequently identified as CD1c$^+$ mDC1, CD141$^+$ mDC2 and CD303$^+$ pDCs [9]. In uveitis patients, the frequency (p=0.0025) and number (p=0.026) of blood CD1c$^+$ mDC1 were significantly increased (Fig.1B and C), and the frequency of blood pDCs was decreased as compared to the levels observed in HCs (data not shown). No differences of mDC2 were detected between uveitis patients and HCs (data not show). Furthermore, we categorized the CD1c$^+$ mDC1 frequency based on therapy and disease status in uveitis patients. No difference was observed in untreated versus treated patients (Fig.1D). However, CD1c$^+$ mDC1 were increased in active uveitis compared to quiescent uveitis (p=0.03, Fig.1E). In summary, increased CD1c$^+$ mDC1 expression was independent of the direct effect from therapy but highly correlated with disease activity. Demographic data of the study subjects were listed in the table.

3.2. Elevation of HLADR expression on CD1c$^+$ mDC1 in uveitis patients

To determine whether blood CD1c$^+$ mDC1 were more mature in uveitis patients compared with HCs, HLADR and co-stimulatory molecules (CD80 and CD86) on blood

$CD1c^+$ mDC1 were analyzed by flow cytometry. HLADR expression, a marker of maturation and antigen presentation, was significantly higher on $CD1c^+$ mDC1 in uveitis patients compared to HCs (p=0.002, Fig. 2A). We sought to determine whether HLADR expression was affected by therapy or disease activity. Interestingly, HLADR expression was significantly higher in the untreated group compared to the treated group (p=0.0075) and HCs (p<0.0001, Fig. 2B), indicating that therapy suppressed HLADR expression. We further evaluated whether higher HLADR expression correlated to disease status. Unexpectedly, higher HLADR expression was observed in the quiescent uveitis patients (p=0.0009) not in the active uveitis patients, as compared to HCs (Fig. 2C). The higher level of HLADR expression in the quiescent patients was probably due to the lower rates of therapy in this group as higher HLADR expression levels were observed in quiescent patients when not on treatment (p=0.029, Fig.2D).

We hypothesized that $CD1c^+$ mDC1 from patients might be hyperresponsive to additional stimulation. To test this we stimulated $CD1c^+$ mDC1 with LPS overnight and then measured HLADR expression. The HLADR expression level was significantly higher in uveitis patients after additional LPS stimulation, as compared to that in patients without stimulation (p=0.01, Fig. 2E). The co-stimulatory markers CD80 and CD86 were similar on $CD1c^+$ mDC1 between uveitis patients and HCs (data not shown).

3.3. Impaired cytokine production of uveitic $CD1c^+$ mDC1 upon additional TLR4 stimulation

Previous studies have shown that DCs exhaust their capacity to produce cytokines after the induction of maturation [15]. Consequently, we examined whether mature $CD1c^+$ mDC1 in uveitis patients also displayed a similar exhaustion pattern in cytokine production. To test this, IL6, IL10 and IL12p40/70 produced from $CD1c^+$ mDC1 of uveitis

patients (n=12) and HCs (n=9) were detected by flow cytometry. Uveitis patients were randomly selected from the cohort along with appropriately age-matched and gender-matched healthy controls. IL6 (Fig.3A), IL10 (Fig.3B) and IL12p40/70 (Fig.3C) production were similar between uveitis patients and HCs *ex vivo*. After overnight stimulation with LPS, the production of IL6 (p=0.0002, Fig.3A), IL10 (p=0.01, Fig.3B) and IL12p40/70 (p=0.01, Fig.3C) from mDC1 was significantly increased compared with the unstimulated group from HCs. The cytokine production was not significantly increased after LPS stimulation in uveitis patients when compared with the unstimulated group from uveitis (Fig. 3). The lack of *in vitro* responses to additional LPS stimulation suggests that cytokine production abilities of mature $CD1c^+$ mDC1 from uveitis patients are exhausted.

3.4. Lower antigen uptake by $CD1c^+$ mDC1 and its subpopulation $CD1c^{hi}$ mDC1 in uveitis patients

The capacity of mDCs to take up antigens is an indicator of their level of maturation, with higher levels of antigen take-up in the immature state moving to an antigen presentation role (supported in part by increasing levels of HLADR) as the mDC matures [16]. We observed a significant decrease of antigen uptake in $CD1c^+$ mDC1 in uveitis patients compared to HCs (p=0.04, Fig. 4A, B). Flow cytometric analysis showed that $CD1c^+$ mDC1 contained two subpopulations, $CD1c^{hi}$ and $CD1c^{lo}$ mDC1 (Fig.4C). Interestingly, only 1% of $CD1c^{hi}$ mDC1 took up antigens while 43% of $CD1c^{lo}$ mDC1 took up antigens (Fig. 4D, E). Importantly, we observed that $CD1c^{hi}$ mDC1 were increased in uveitis patients compared to HCs (p=0.001, Fig. 4F), indicating that the lower antigen uptake in the whole $CD1c^+$ mDC1 population was likely due to the increased numbers of $CD1c^{hi}$ mDC1 in the uveitis group. Furthermore, high expression of CD1c was associated with

higher expression of HLADR in both HCs (p=0.0001, Fig. 4G) and uveitis patients (p=0.002, Fig. 4H).

3.5. *In vitro* model showed MoDCs with lower antigen uptake associated with higher T cell proliferation

Having shown that patients with uveitis have higher levels of $CD1c^+$ mDC1 and of the $CD1c^{hi}$ subgroup, and that these mDCs have a relatively 'mature' profile characterized by higher HLADR and lower antigen uptake, we wished to investigate the functional consequences of this. For our functional studies, we used MoDCs as a substitute for mDCs in an *in vitro* model [12, 13, 17]. Six to Seven days are usually needed for monocytes to fully differentiate into mDCs *in vitro*. This allowed us to observe the expression of CD1c on MoDCs during differentiation (Fig. 7C, D). MoDCs were loaded with FITC-albumin for 30 minutes and two subpopulations were sorted by flow cytometry based on their ability to take up antigens, which were FITC-$albumin^+$ and FITC-$albumin^-$ MoDCs (Fig. 5A). We cultured allogenic $CD4^+$ T cells with either $Albumin^+$ or $Albumin^-$ MoDCs for 5 days. $CD62L^-CD4^+$ T cell (activated T cells) proliferation was significantly increased in the group of $Albumin^-$ MoDCs, as compared to the group of $Albumin^+$ MoDCs. This was shown by the percentage of CFSE dilution on $CD62L^-CD4^+$ T dells (Fig. 5B, C), indicating that MoDCs with lower antigen uptake could induce more $CD4^+$ T cell proliferation and activation.

3.6. Uveitic sera and exogenous TNFα reduced antigen uptake in MoDCs

Having confirmed in our *in vitro* model that the MoDCs with lower antigen uptake (modeling the low-antigen-uptake mDC1 identified earlier) induced higher levels of T cell proliferation with a higher proportion of activated $CD62L^-$ T cells, we wished to investigate this further, specifically whether there was a factor in uveitis serum that might be inducing this more mature DC phenotype. In order to investigate this, we performed overnight incubation with MoDCs treated with the addition of sera from patients with uveitis or HCs. We found that MoDCs had less antigen uptake after they were treated with sera from uveitis patients compared with MoDCs treated with sera from HCs (Fig. 6A, B and C).

We hypothesized that this might be related to higher levels of inflammatory cytokines such as TNFα and IL-17a in patient sera. TNFα is known to contribute to DC maturation, plays a key role in the pathogenesis of uveitis, is present at high concentration levels both in the aqueous humor and in the serum [18-22], and is a successful target in the treatment of uveitis [23]. Although less well-established IL17a has also shown to be increased in patients with uveitis, and an anti-IL17 agent, secukinumab, has been trialed for treatment of uveitis [24, 25]. We found that TNFα, but not IL17a (data not shown), significantly reduced MoDCs' antigen uptake in a dose-dependent manner (Fig.6D, E and F). Furthermore we found that treatment with TNFα increased CD1c expression compared to controls (Fig.7A).

One of the key pathways that TNFα exerts its effect is via phosphorylation of p38 MAPK [26]. We hypothesized that TNF-induced upregulation of CD1c expression was mediated via this pathway. Treatment of MoDCs with TNFα caused an increased phosphorylation of p38 MAPK (Fig.7B). Conversely, blockage of this pathway by addition of SB203580, an inhibitor of p38 MAPK (Fig.7C, D), inhibited CD1c expression during MoDC differentiation. Although monocytes don't express CD1c, CD1c expression was

induced in MoDCs on day 1 and increased dramatically every subsequent day of differentiation. The inhibition of CD1c expression by SB203580 was observed on day 1 and reached statistical significance on days 3 and 5 (Fig. 7C, D). Taken together, this data suggests that the known increased TNFα levels in patient sera induces a more mature DC phenotype characterized by high CD1c expression and less antigen uptake, and this is mediated (at least in part) by the p38 MAPK pathway.

4. Discussion

In this study, we have shown that blood CD1c^{+} mDC1 is increased in autoimmune non-infectious uveitis patients compared with HCs. Blood CD1c^{+} mDC1 from uveitis patients show a mature phenotype, characterized by less antigen uptake and high HLADR expression. CD1c^{hi} mDC1 is identified as the mDC1 subpopulation with lower antigen uptake, and associated with higher HLADR expression and a capability to induce higher levels of CD4^{+} T cell activation and proliferation. Additionally, we present data suggesting these effects are mediated by the increased level of TNFα in uveitis patient sera, which appears to promote CD1c^{+} mDC1 maturation through the p38 MAPK pathway. Our data provide support for the role of blood CD1c^{+} mDC1, especially CD1c^{hi} mDC1, in the pathogenesis of non-infectious uveitis in humans.

Previous studies have provided strong evidence that non-infectious uveitis is an autoimmune disease [27]. A key step in autoimmune disease centers on mDC taking up self-antigens thereby initiating antigen-specific T cell immune responses. Among blood mDCs, CD1c^{+} mDC1 represent a major mDC population in the peripheral blood, and have been shown to be increased in other autoimmune disease such as rheumatoid arthritis [28]. Our study shows that CD1c^{+} mDC1 is increased in uveitis patients

compared with HCs. It should be noted from our data that the increased $CD1c^+$ mDC1 appear to be independent of therapy but positively correlated with disease activity [29].

The maturation state of DCs affects their ability to induce adaptive immune responses [30]. We noted that $CD1c^+$ mDC1 from uveitis patients were more mature characterized by an increased expression of HLADR, although interestingly we observed no differences in the expression of co-stimulatory molecules (CD80 and CD86). Interestingly, in another study of mDCs from patients with uveitis, Kim et al did observe an increase in CD86. The differences may lie in the broader spectrum of DCs considered in their study since their isolation process selects out mDCs with subsequent co-staining of CD11c. This will lead to both mDC1 and mDC2 populations being included whereas our study was restricted to changes in the mDC1 population. Interestingly even in their study the changes on CD86 were relatively minor compared to the significant elevation in HLADR seen in both studies [8]. It remains of interest to understand why $CD1c^+$ mDC1 only exhibits high HLADR but no other co-stimulatory molecules, particularly as alterations in relative contribution of signal 1 and signal 2 may alter function and differentiation of T cells [31].

In addition to changes in their surface phenotype, maturation is associated with a number of other important alterations in mDC function. Cytokine production is affected, with DCs displaying a different cytokine repertoire as they mature [32], and activated DCs becoming refractory to further stimulation due to exhaustion of their cytokine-producing capacity [15]. In keeping with these finding, our data show that mature $CD1c^+$ mDC1 from uveitis patients fail to increase their cytokine production in response to additional LPS stimulation *in vitro*.

It is also well established that normal DC maturation is associated with their transition from a phenotype with high levels of antigen uptake, to one, which primarily presents antigen (and has low levels of uptake). We have confirmed that this behavior is

also seen in the context of uveitis, with $CD1c^+$ mDC1 in uveitis patients showing lower levels of antigen uptake than healthy controls, a characteristic which could also be induced by co-culture of MoDC with sera from uveitis patients [33]. Interestingly, we observed significant numbers of a $CD1c^{hi}$ subpopulation in the uveitis patients, associated with a more mature phenotype, as indicated by higher HLADR expression and lower levels of antigen uptake, which is also supported by the study from mouse DCs [34]. The function of CD1 molecules is to present lipid, both endogenous and pathogen-derived [35]. Similar to class I MHC they associate with β2-microglobulin on the surface of various types of cells, particularly antigen-presenting cells (APCs), but differ in that (1) they are not polymorphic, and (2) have hydrophobic binding pockets that allow them to bind the hydrocarbon chains of lipids [36-38]. CD1c (along with CD1a and CD1b) is recognized by CD1-restricted T cells whereas CD1d is recognized by invariant natural killer T (iNKT) cells. CD1c may present a wide range of antigens and indeed its ability to present antigen from important human pathogens such as mycobacteria, leading to its proposal as a candidate for vaccine development [36]. $CD1c^+$ mDC1 can be found not only in the peripheral blood but also in various tissues including the intestinal lamina propria [39] and in the synovial fluid in patients with rheumatoid arthritis [28]. In both these studies the $CD1c^+$ mDC1 identified in the tissues express higher levels of activation markers than those in the peripheral blood. $CD1c^+$ mDC1 levels can however be affected by treatment, as demonstrated in the study by Jolivel, et al., where $CD1c^+$ mDC1 levels were reduced in laquinimod-treated multiple sclerosis patients in comparison to untreated patients and healthy donors [40]. However, our study supports that level of CD1c is not due to the treatment but is associated with disease activity.

A critical role of mature mDCs is to promote T cell proliferation. In our study we showed, as expected, that not only did uveitis patients have a higher proportion of mDC1 with a more mature phenotype, but that (1) these mDC1 induced higher levels of T cell

proliferation and (2) a higher proportion of these were CD62L$^-$. CD62L is expressed on naïve T cells and central memory T cells, which help T cells to localize in secondary lymphoid organs. Effector memory T cells do not express CD62L. They circulate in the periphery and have immediate effector functions upon encountering antigens [41]. It is likely that this lies at the center of the generation of autoimmunity in non-infectious uveitis. It is known that IRBP and S-antigen can initiate innate and adaptive immune responses by attracting immature DCs [42]. In this context our finding of increased levels of CD1c$^+$ mDC1 cells in uveitis patients, is of interest, particularly in its implications for the generation of auto-reactive uveitic T cell immune responses, which may underlie the pathogenesis of noninfectious uveitis.

Several studies have demonstrated that TNFα is increased in sera of uveitis patients [18-22]. We have found that TNFα-treated MoDCs show lower antigen uptake and increase CD1c expression. TNFα can promote DC maturation [43] and activate p38 MAPK [26]. Studies have shown that SB203580 (p38 inhibitor) inhibits the up-regulation of CD1a, CD40, CD80, CD86, HLA-DR, and DC maturation marker CD83, which is induced by LPS and TNFα [26]. In addition, SB203580 inhibits the enhancement of the allostimulatory capacity and partially prevents the down-regulation of FITC-dextran uptake induced by LPS and TNFα [26]. Consistently, we have found that TNFα decreases FITC-albumin antigen uptake and increases CD1c expression through regulating p38 MAPK. These data suggest that phosphorylation of p38 MAPK may be a key pathway in the maturation of DCs in uveitis.

In conclusion, a significant increased blood CD1c$^+$ mDC1 with mature phenotype is identified in uveitis patients. A CD1c^{hi} mDC1 subpopulation is identified which is increased in patients and is characterized by a relatively mature phenotype including lower antigen uptake and increased induction of T cell responses. We also provide

evidence that these changes may be caused by the higher levels of TNFα present in the serum of patients with uveitis, and which is p38 MAPK-dependent. Further understanding of the role of blood $CD1c^+$ mDC1 in non-infectious uveitis may answer key questions regarding the initial events in the pathogenesis of uveitis and provide novel therapeutic opportunities for treating patients with this potentially blinding autoimmune disease.

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Conflict of Interest Disclosure

The authors have no financial conflicts of interest.

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Figure legends

Figure 1.

Increased frequency and number of blood $CD1c^+$ mDC1 in uveitis patients (n=32) as compared to HCs (n=64). **(A)** Representative scattergrams from flow cytometry analysis of dendritic cell subtypes $CD1c^+$ mDC1, $CD141^+$ mDC2 and $CD303^+$ pDCs gated on $Lin1^-HLADR^+$ total DCs. Percentages **(B)** and absolute numbers **(C)** of mDC1 were analyzed between uveitis patients and HCs. Comparison of $CD1c^+$ mDC1 percentage in treated and untreated groups **(D)**, as well as in quiescent and active uveitis groups **(E)**. Student's t test was used in **(B**) and **(C)**. One-way ANOVA analysis and post ANOVA analysis (Tukey's multiple comparisons test) were used. ANOVA $p < 0.0001$ in **(D)** and **(E).**

Figure 2.

Higher HLADR expression on $CD1c^+$ mDC1 in uveitis patients compared to HCs. **(A)** Mean fluorescence intensity (MFI) of HLADR was measured by flow cytometry between uveitis patients (n=37) and HCs (n=39). **(B)** HLADR expression on mDC1 was compared among untreated, treated and HC groups. **(C)** HLADR expression on mDC1 was compared in quiescent, active and HC groups. **(D)** HLADR expression was compared in untreated and treated groups of quiescent uveitis patients, and HCs. **(E)** HLADR expression was measured on mDC1 with or without LPS stimulation overnight *in vitro* in both HCs (unstimulated group; LPS group) and uveitis patients (unstimulated group; LPS group). Student's t test was used in **(A**). One-way ANOVA analysis and post ANOVA analysis (Tukey's multiple comparisons test) were used in **(B)** through **(E)**. The ANOVA p values are 0.03 in **(B),** 0.002 in **(C),** 0.0003 in **(D)** and less than 0.0001 in **(E).**

Figure 3.

Cytokine production of mDC1 was exhausted upon additional TLR4 stimulation in uveitis patients. Myeloid DC1 producing-IL6 **(A),** IL10 **(B)** and IL12P40/70 **(C)** were measured in HCs (n=9) and uveitis patients (n=12) with or without LPS stimulation *in vitro*. The cytokine production was detected by intracellular staining and analyzed by flow cytometry. One-way ANOVA analysis and post ANOVA analysis (Sidak's multiple comparisons test) were used. ANOVA overall p values are 0.049 **(A)**, 0.008 **(B)**, and 0.039 **(C)**.

Figure 4.

Low antigen uptake in $CD1c^+$ mDC1 in uveitis patients. **(A)** Fresh blood from either HCs (n=23) or uveitis patients (n=30) was loaded with FITC-albumin (antigen) for 30 minutes. Representative antigen uptake of mDC1 measured by flow cytometry is shown. The negative control was MoDCs cultured in 4°C. **(B)** Significant differences in antigen uptake of mDC1 from uveitis patients and HCs. **(C)** $CD1c^+$ mDC1 were separated as $CD1c^{hi}$ and $CD1c^{lo}$ mDC1 by flow cytometry. $CD1c^{hi}$ mDC1 **(D)** had less antigen uptake when compared with $CD1c^{lo}$ mDC1 **(E)**. **(F)** $CD1c^{hi}$ mDC1 were increased in uveitis patients (n=20) as compared with HCs (n=23). High CD1c expression on mDC1 correlated with high HLADR expression in both HCs **(G)** and uveitis patients **(H)**.

Figure 5.

MoDCs with lower antigen uptake enhanced $CD62L^-CD4^+$ T cell proliferation. **(A)** To address the function of mDC1 with decreased antigen uptake, monocyte-derived DC (MoDCs) were used *in vitro*. MoDCs were loaded with FITC-albumin for 30 minutes and sorted by flow cytometry based on high and low expression of FITC-albumin ($Albumin^+$ and $Albumin^-$). **(B, C)** $Albumin^+$ or $Albumin^-$ MoDCs co-cultured with allogenic CFSE

labeled $CD4^+$ T cells for 5 days. T cell proliferation and activation were measured by diluted expression of CFSE and low expression of CD62L. The figures are representative of four independent experiments. One-way ANOVA analysis was used; p=0.04 in **(C)**.

Figure 6.

Treatment with uveitis sera or TNFα reduced the antigen uptake of MoDCs. **(A)** Antigen uptake assay was done in two groups: MoDCs were treated with sera from either HCs (HC group, n=6) or patients with uveitis (Uveitis group, n=6). The percentage of FITC-albumin$^+$ **(B)** and MFI of FITC-albumin$^+$ (**C**) on MoDCs were calculated. The experiment was repeated six times. **(D)** An antigen uptake assay was done in five groups: MoDCs were cultured at 4°C (negative control); MoDCs were cultured at 37°C without TNFα stimulation (untreated group), or with TNFα stimulation overnight with different concentrations (0.1 ng/ml, 1 ng/ml and 10 ng/ml). The percentage of FITC-albumin$^+$ **(E)** and MFI of FITC-albumin$^+$ **(F)** on MoDCs were calculated. The dose-dependent experiment was repeated two times. One-way ANOVA analysis was used and the total p=0.0008 in **(E)** and 0.04 in (**F**).

Figure 7.

TNFα increased CD1c expression by p38 MAPK signaling. **(A)** CD1c expression was measured by flow cytometry on MoDCs with (TNFα group) or without (control group) TNFα stimulation *in vitro* for 24 h. The experiment was repeated two times. Dotted line is control group; solid line is TNFα group. **(B)** Phosphorylated p38 was measured on MoDCs treated with (TNFα group) or without (control group) TNFα for 30 minutes. The experiment was repeated four times. Dotted line is isotype control; Dash line is control

group; solid line is TNFα group. **(C)** MoDCs were induced from monocytes by GM-CSF and IL-4. P38 inhibitor SB203580 was added at a concentration of 10 μmol/L into the culture at day 0. During MoDC differentiation, CD1c expression was measured by flow cytometry on days 1, 3 and 5. The filled grey area is the p38 inhibitor group; and the solid line is the untreated group. The experiment was repeated two separate times. Unpaired student's *t* test was used in **(A)** and **(B)**. One-way ANOVA analysis was used and ANOVA $p<0.0001$ in **(D)**.

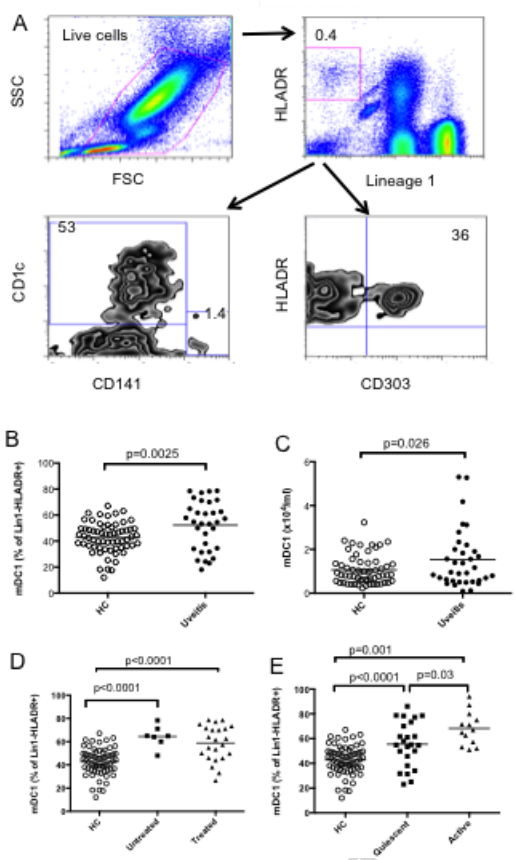

Fig. 1

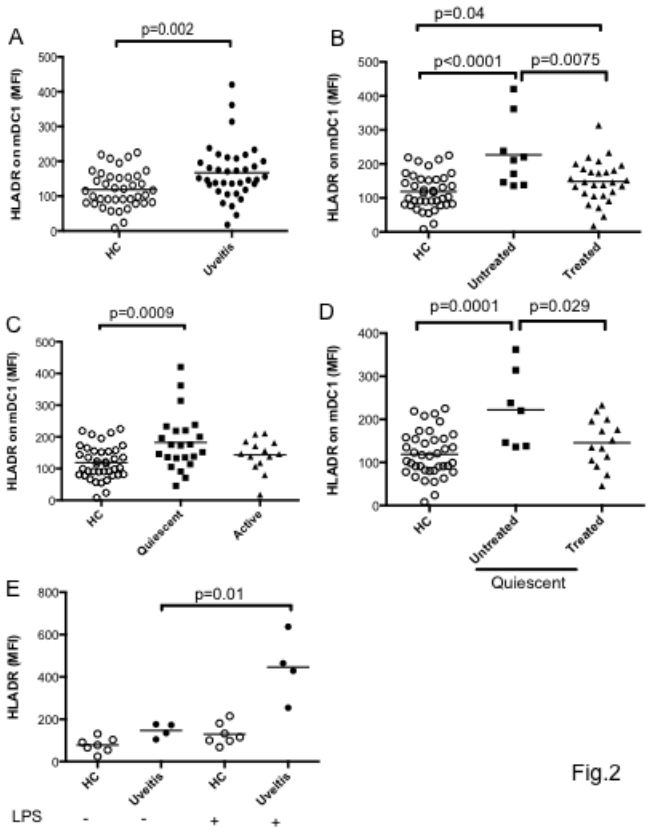

Fig.2

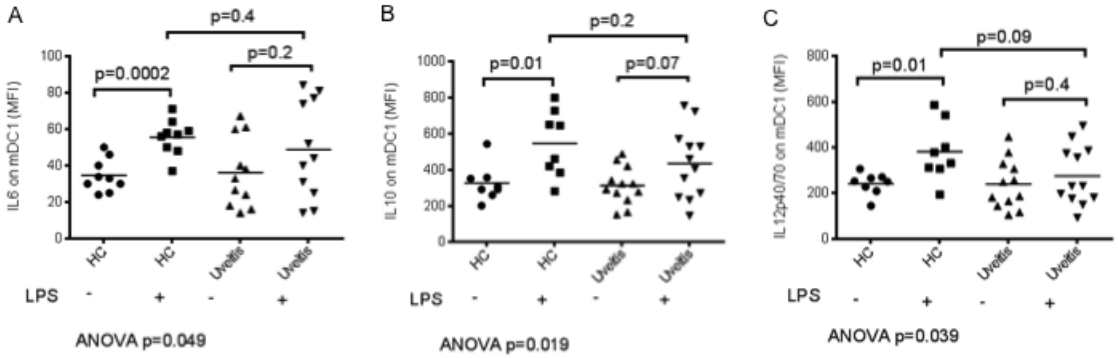

Fig.3

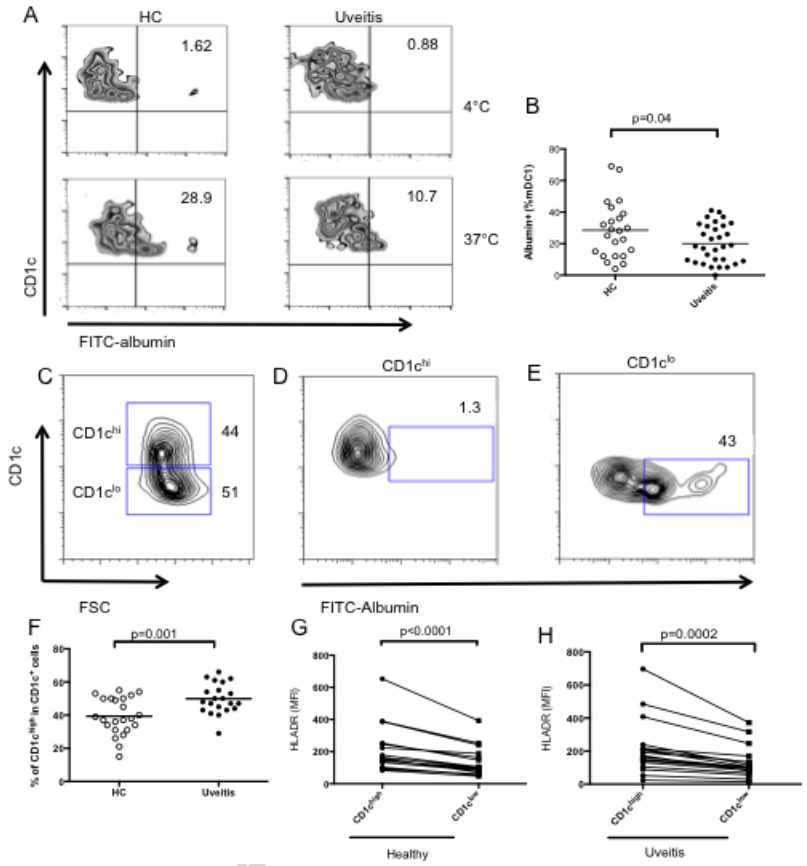

Fig.4

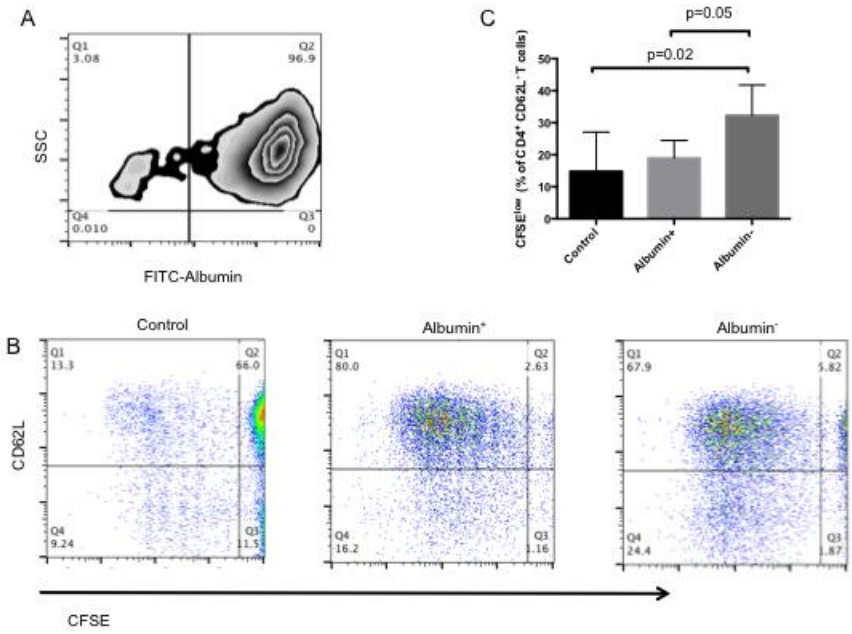

Fig.5

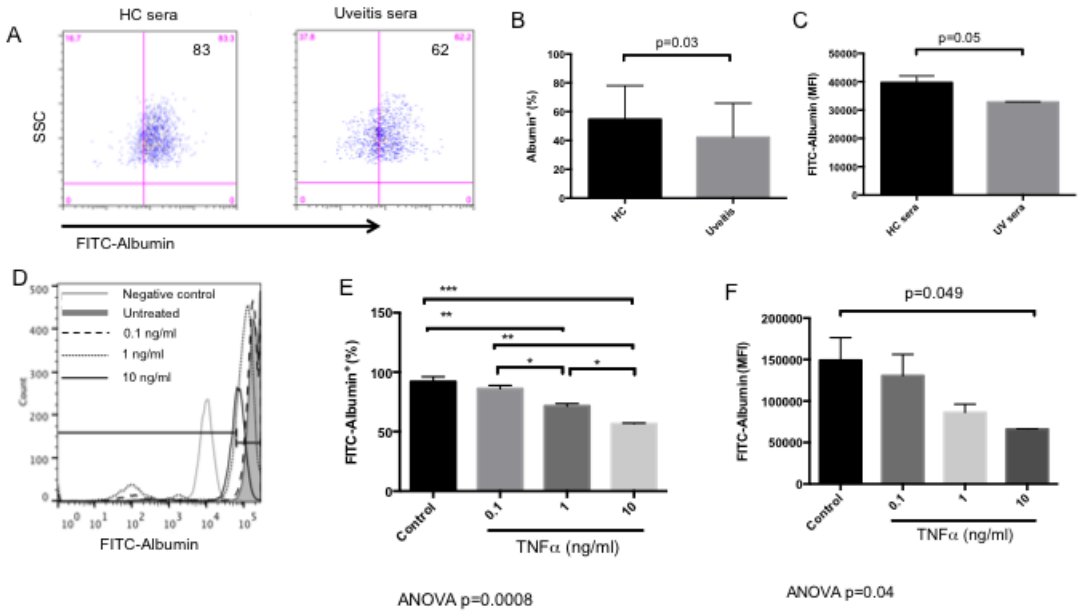

Fig.6

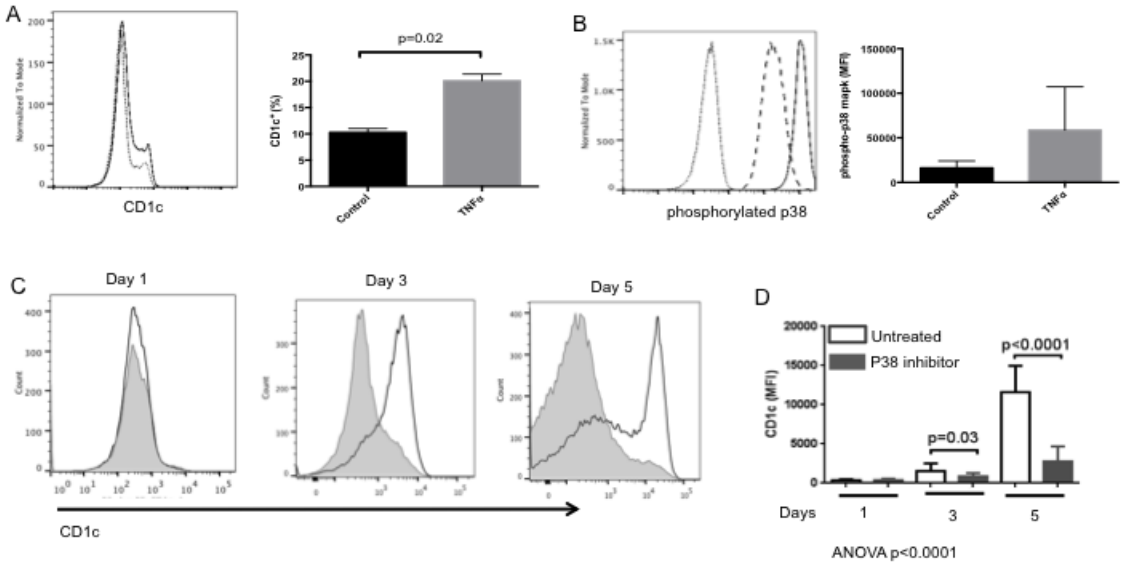

Fig.7

Table. Characteristics of Uveitis Patients and HCs

Parameter	Uveitis (n=74)	HCs (n=96)
Age (y), median (range)	44 (17-74)	46 (19-76)
Gender [n (%)]		
Male	44 (59)	61 (64)
Female	30 (41)	35 (36)
Race [n (%)]		
Caucasian	28 (38)	80 (83)
African American	28 (38)	15 (16)
Others	18 (24)	1 (1)
Anatomic type of uveitis [n (%)]		N/A
Anterior	10 (14)	
Intermediate	7 (9)	
Posterior	17 (23)	
Panuveitis	40 (54)	
Disease association [n (%)]		N/A
Sarcoidosis	17 (23)	
Idiopathic	32 (43)	
Birdshot	8 (11)	
VKH	5 (7)	
Behcet's disease	8 (11)	
Serpiginous choroidopathy	4 (5)	
Systemic Immunosuppressive Therapy [n (%)]	57 (77)	N/A
Active uveitis [n (%)]	18 (24)	N/A

Highlights

- $CD1c^+$ mDC1 were increased in uveitis patients.
- $CD1c^+$ mDC1 exhibited less antigen uptake in uveitis patients.
- $CD1c^{hi}$ mDC1 subpopulation with less antigen uptake was increased in patients.
- MoDCs with less antigen uptake induced more $CD4^+CD62L^-$ T cells proliferation.
- TNFα contribute to less antigen uptake in MoDCs through p38 MAPK pathway.