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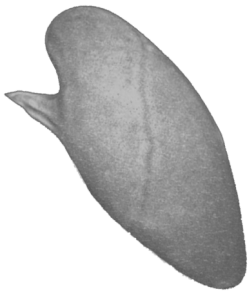
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Chapter

2



Functional impairment of human myeloid dendritic cells during *Schistosoma haematobium* infection

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Submitted

Abstract

Chronic schistosoma infection is often characterized by a state of T cell hyporesponsiveness of the host. Suppression of dendritic cell (DC) function could be one of the mechanisms underlying this phenomenon, since schistosoma antigens are potent modulators of dendritic cell function *in vitro*. Yet, it remains to be established whether DC function is modulated during chronic human schistosoma infection *in vivo*. To address this, the effect of *Schistosoma haematobium* infection on the function of human blood DCs was evaluated. We found that plasmacytoid (pDCs) and myeloid DC (mDCs) from infected subjects were present at lower frequencies in peripheral blood and that mDCs displayed lower expression levels of HLA-DR compared to those from uninfected individuals. Furthermore, mDCs from infected subjects, but not pDCs, were found to have a reduced capacity to respond to TLR ligands, as determined by MAPK signaling, cytokine production and expression of maturation markers. Moreover, the T cell activating capacity of TLR-matured mDCs from infected subjects was lower, likely as a result of reduced HLA-DR expression. Collectively these data show that *S. haematobium* infection is associated with functional impairment of human DC function *in vivo* and provide new insights into the underlying mechanisms of T cell hyporesponsiveness during chronic schistosomiasis.

Introduction

Chronic parasitic helminth infections are generally associated with a T helper 2 (Th2)-like immunological profile as well as immune hyporesponsiveness [1;2]. The latter characteristic is probably the result of immune evasion strategies that these pathogens have developed to ensure long term survival within their hosts and to allow continued transmission. Also chronic infections with blood-dwelling trematodes of the genus *Schistosoma* are well known for their capacity to suppress immune responses, which is not only reflected in negative regulation of parasite specific immune responses but also by attenuation of effector responses to third party antigens, including allergens [3-5], and other pathogens [6;7].

Dendritic cells (DCs) are the most important antigen presenting cells (APCs) that initiate immune responses and as such play a central role in the development and regulation of protective immunity against invading pathogens as well as tolerance under homeostatic conditions [8;9]. In peripheral blood, two major DC subsets can be identified: the CD11c⁺ myeloid DCs (mDCs) and CD123⁺ plasmacytoid DC (pDCs) [10]. Both are immature DCs that are in the process of migration to their target sites. It is believed that these subsets perform different functions in both innate and adaptive immune responses. Human mDCs express Toll-like receptors (TLR)-2, -4 and -8 and are thought to preferentially induce T cell responses to invading pathogens. On the other hand, human pDCs have a different but complementary TLR expression profile that includes TLR-7 and -9 and are believed to play an important role in innate anti-viral responses as well as in induction of tolerance against self-antigens [11;12].

One of the main characteristics of immune hyporesponsiveness during chronic schistosomiasis is the impairment of effector T cell responses, a process in which T cell anergy and regulatory T cells have been proposed to play an important role [13;14]. Given that DCs are central players in the priming and regulation of T cell responses, suppression of these host immune responses by schistosomes through modulation of DC function would seem plausible. Indeed, studies have demonstrated that host derived anti-inflammatory mediators, such as IL-10 [15], which is elevated during chronic schistosomiasis [5;16;16;17], as well as schistosoma derived molecules [18;19] have the potential to modulate DC function and its T cell priming capacity *in vitro*. However, it remains to be established whether human DC function is actually modulated *in vivo* during chronic schistosomiasis.

To address this question, we evaluated the function of blood DC in a cross-sectional study in Gabon, a region endemic for *Schistosoma haematobium* [5]. We find that mDCs isolated from infected subjects have an impaired capacity of to respond to TLR ligands and to prime T cell responses. In addition, our data indicate that the former observation is due to a dampened pro-inflammatory signaling rather than lower TLR

expression, and that the latter finding originates from a reduced expression of HLA-DR. Collectively, the data show a functional impairment of blood mDCs during chronic *S. haematobium* infection and shed new light on the mechanisms underlying immune hyporesponsiveness during chronic helminth infections.

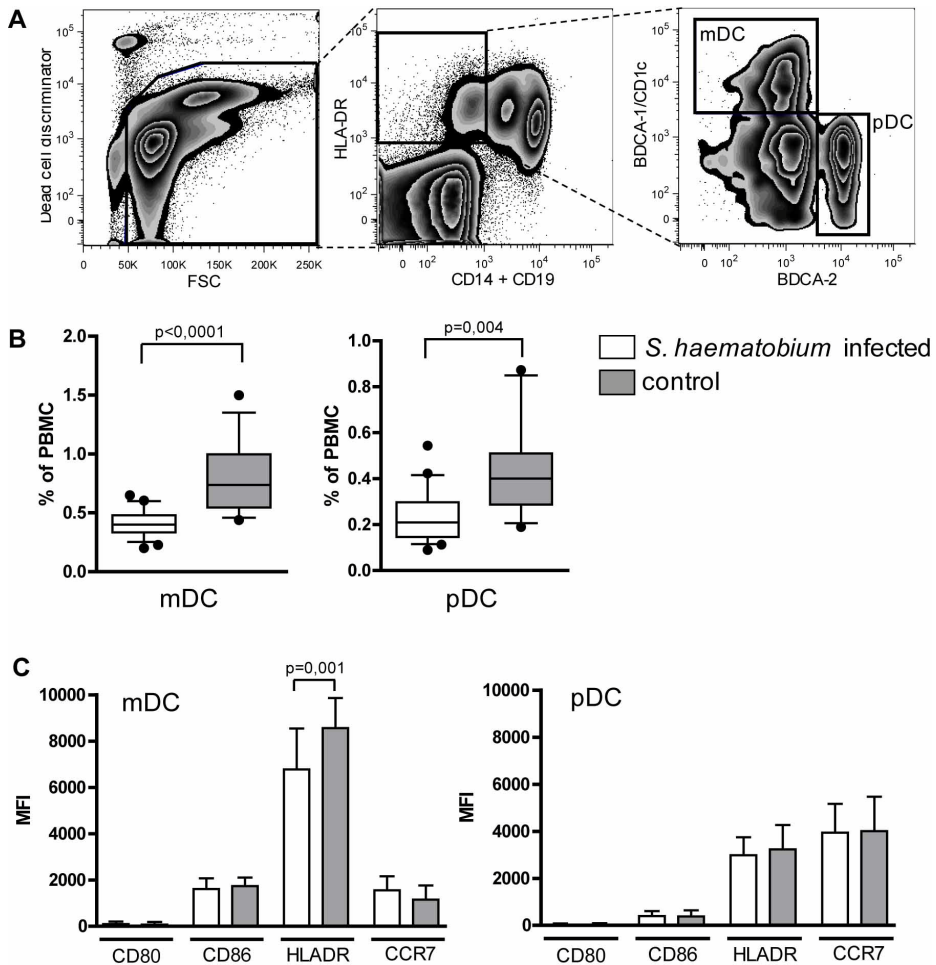


Figure 1. Reduced frequencies of mDCs and pDCs in blood from *S. haematobium*-infected subjects . (A) Blood DCs were identified in fixed PBMC as HLA-DR⁺/CD14⁻/CD19⁻ cells and subsequently subdivided into mDCs and pDCs on the basis of positive staining for BDCA-1 and BDCA-2, respectively. Data from one representative donor is shown. (B) Frequencies of blood DC subsets in total PBMCs (C) and their surface expression of HLA-DR, CD80, CD86 and CCR7 was determined by following the gating strategy shown in (A). (B) Box and whiskers with 10-90% percentile are shown. (C) Bars represent mean + SD. (B+C) Each group represents data from 20 donors.

Results

S. haematobium infected subjects have lower frequencies of mDCs and pDCs in peripheral blood

To study the effect of *S. haematobium* infection on human DC phenotype and function, we characterized the two major DC subsets present in peripheral blood, CD11c/BDC1A1⁺ mDCs and CD123⁺/BDC1A2⁺ pDCs [11], in 23 infected and 20 uninfected individuals recruited from a *S. haematobium* endemic area in Gabon (for characteristics of the study population, see Table 1). The frequencies of mDCs and pDCs were determined in peripheral blood mononuclear cells (PBMC) isolated from infected and uninfected subjects, by gating on HLA-DR⁺/BDC1A1⁺/CD14⁻/CD19⁻ and HLA-DR⁺/BDC1A2⁺/CD14⁻/CD19⁻ populations, respectively (Fig 1A). Both mDC and pDC frequencies were significantly reduced in the infected group compared to the uninfected subjects (Fig 1B). In addition, to determine whether the activation status of the DC subsets differs between infected and uninfected subjects surface expression of HLA-DR, CD80, CD86, CD40 and CCR7 was analysed. In particular HLA-DR expression on mDCs was significantly lower in the infected subjects (Fig 1C), indicating that mDCs are phenotypically different during infection. The expression of the other markers was not different between the groups.

Table 1. Study population

	<i>S. haematobium</i> infected n=23	Uninfected n=20	p value
Area	Gabon, Lambaréné & vicinity	Gabon, Lambaréné	
Male/female	17/6	17/3	0,71*
Mean age in years (range)	25,2 (17-39)	27,2 (20-37)	0,27
Mean egg counts (range) ^Δ	267 (1-5000)	0	ND
Subjects with co-infection			
Malaria*	1	0	ND
Microfilaria* [#]	11	5	0,22*

^Δ, based eggs present in 10 ml urine trapped by a 12 μm filter; *, based on Giemsa-stained whole blood smear; [#], based on presence of microfilaria in cell cultures; *, X² analysis with Fisher's exact test; ND, not determined.

mDCs, but not pDCs, from *S. haematobium* infected subjects have reduced responses to TLR ligands

Schistosomal antigens have been shown to harbour the capacity to modulate and suppress TLR-induced activation of *in vitro* generated DC [18-20]. To address whether TLR-mediated activation of human blood DCs during schistosoma infection are also affected, mDCs and pDCs were isolated from peripheral blood of infected and uninfected individuals. Subsequently mDCs were stimulated by LPS (TLR4 ligand) or

R848 (TLR7/8 ligand), while pDCs were stimulated by CpG (TLR9 ligand) or R848. To characterize the responses of the DC subsets to the different TLR ligands, the surface expression of maturation markers was determined. As expected, expression of all markers (CD40, CD80, HLA-DR and CCR7) on mDCs was increased in response to LPS, except for CD86 (Fig 2A: fold increase compared to medium cultured mDCs). Interestingly, the fold increase in CD80 and CCR7 expression was significantly lower in the mDC derived from the schistosoma infected group compared to the uninfected group (fig 2A). The mean fluorescence intensity (MFI) of CD80 and HLADR tended to be lower on mDCs derived from *S. haematobium* infected subjects after LPS stimulation (Fig 2B). CpG induced an increase in surface markers on pDCs, but with no significant differences between the two groups (Fig 2A), although in absolute expression levels, there was a tendency towards a lower CD80 expression in the infected group (Fig 2B). For both mDCs and pDCs similar results were obtained when they were stimulated with R848 (data not shown).

To further characterize the TLR responsiveness of blood DCs, several cytokines were analysed in supernatants (Fig 2C). LPS-stimulated mDCs produced only very low levels of IL-10 and IL-6, whereas R848 induced much more and higher cytokine responses in mDCs. Interestingly, mDC from infected subjects had a significantly impaired IL-12 and IL-6 production in response to R848 relative to the mDCs isolated from uninfected individuals. In contrast, comparison of CpG or R848-induced cytokine production by pDCs from the two groups revealed no differences, including the classical pDC-derived cytokines IFN- α and CXCL-10. Taken together, these data show that mDCs but not pDCs from *S. haematobium* infected individuals are functionally impaired in their responses to TLR ligands in terms of upregulation of maturation markers and cytokine expression.

Reduced TLR responses of mDCs coincides with reduced pro-inflammatory MAPK signaling, but not with altered TLR expression

To explore the mechanisms underlying the reduced TLR-responsiveness in mDCs from infected subjects, TLR4 expression on mDCs was analysed. However, no differences were seen in surface expression of TLR4 on mDCs between both groups (fig 3A), suggesting that other mechanisms underlie the observed differences in TLR-responsiveness, at least for TLR4. TLR-mediated DC activation is known to be mediated by signaling molecules that include the mitogen activated protein kinases (MAPK) p38 and ERK. Activation of p38 downstream of TLR-triggering in DCs has been shown to be important for expression of maturation markers and cytokines production, whereas

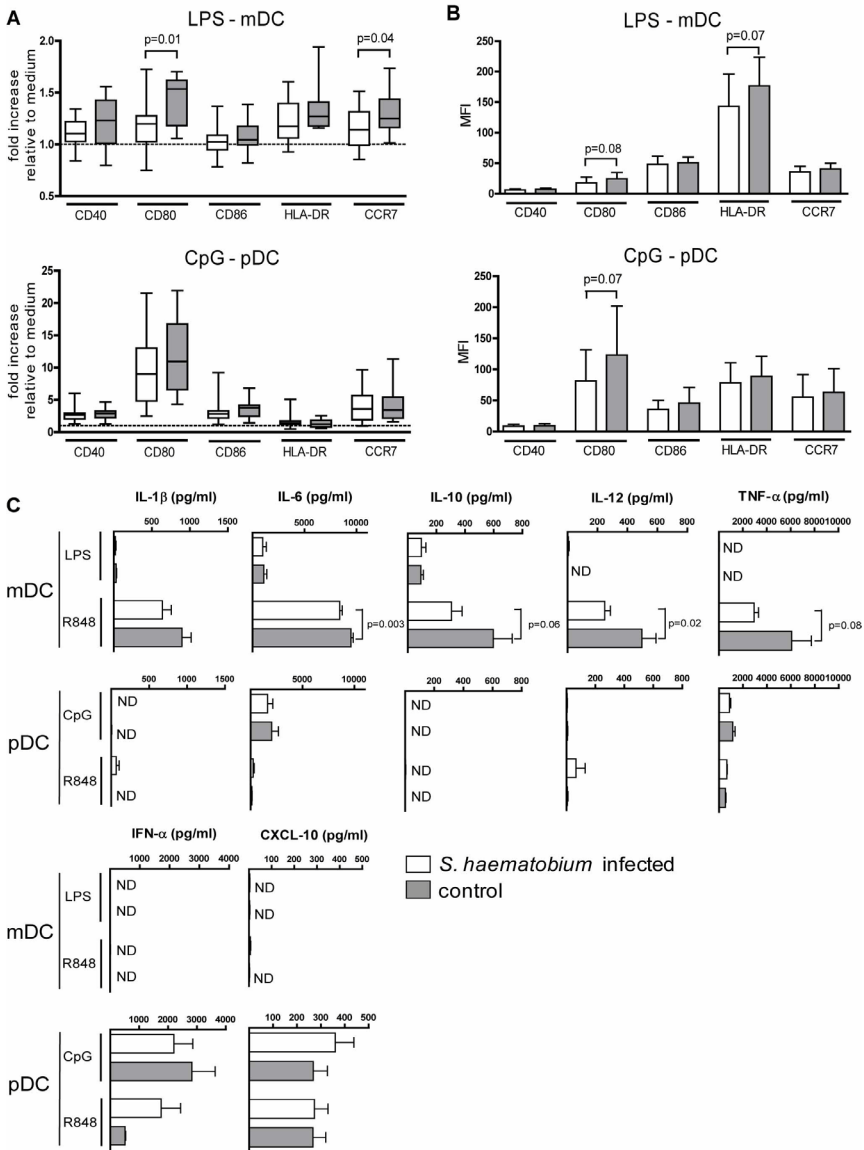


Figure 2. mDCs, but not pDCs, from *S. haematobium* infected subjects have impaired TLR responses. Isolated blood DCs were stimulated with 100ng/ml LPS (mDC), 1 μ g/ml R848 (mDCs & pDCs) or 1 μ g/ml CpG (pDCs) for 40 h. (A+B) LPS-matured mDCs and CpG-stimulated pDCs were analysed for maturation marker expression by FACS. Expression of surface marker is either shown (A) as fold increase relative to medium or (B) as absolute mean fluorescence intensity. (C) Cytokine levels in 40 h culture supernatants from TLR-stimulated DCs were determined by multiplex Luminex. Values represent cytokine concentration from which medium control cytokine levels have been subtracted. (A) box plots represent 25-75 percentile range with error bars showing minimum to maximum. (B+C) Bars represent mean + SD. Each group represents data from 20 donors. ND: not detectable.

ERK phosphorylation is linked to dampening of these responses [21]. Given that schistosomal antigen preparations have been shown to modify TLR responses through modulation of these MAPK [19;20;22] we asked whether altered TLR-signaling via p38 and ERK could provide an explanation for the reduced TLR-responsiveness in the infected group. Phosphorylation of MAPK p38 and ERK was determined at 20 and 60 minutes following TLR stimulation. We found that stimulation of mDCs by LPS resulted in a higher phosphorylated ERK/p38 ratio in the schistosome infected group compared to the controls, which was primarily due to lower p38 phosphorylation (Fig 3B and data not shown). These data show that mDCs from infected individuals display a reduced induction of pro-inflammatory MAPK activation in response to LPS. These results indicate that a change in downstream signaling, rather than TLR expression itself, may be responsible for the impaired responsiveness of mDCs towards TLR ligands from *S. haematobium* infected individuals.

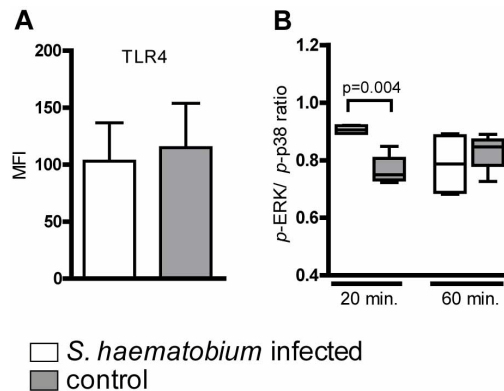


Figure 3. mDCs from *S. haematobium* infected subjects have an altered MAPK signaling, but not TLR4 expression profile. (A) TLR4 expression was analysed on mDCs present in PBMCs following the gating strategy as described in legend of Fig 1. (B) 20 and 60 minutes after stimulation with LPS, mDCs were intracellularly stained for phosphophorylated p38 and ERK and the ratio between *p*-ERK and *p*-p38 was determined by dividing the respective mean fluorescence intensities per sample. Each group represents data from 5 donors. (A) Bars represent mean + SD. (B) box plots represent 25-75 percentile range with error bars showing minimum to maximum.

mDCs from *S. haematobium* infected subjects have impaired T cell activation capacity, due to reduced HLA-DR expression

DCs are instrumental in the priming and regulation of T cell responses. In this process antigen presentation, costimulation and cytokine production by DCs play a crucial role. Since these processes were affected in mDC from *S. haematobium* infected, we assessed the T cell priming capacities of these DC. To address this, allogeneic naïve T helper cells were cocultured together with LPS-stimulated mDCs. After 6 days T cell

expansion and T cell cytokines were analysed. Interestingly, T cells primed by mDCs from schistosoma infected subjects had expanded significantly less than the T cells in the presence of mDCs from uninfected subjects (Fig 4A). Schistosomes are known to condition DCs to promote Th2 and regulatory type T cell responses [2;9], characterized by an increased production of IL-4, IL-5, IL-13 and IL-10, respectively. Yet, total cytokine production in day 6 culture supernatants (data not shown) or intracellular cytokines following polyclonal restimulation (Fig 4B) when primed by mDCs from infected subjects compared to uninfected individuals. In contrast, the proportion of IL-10- or TNF- α -secreting T cells was unchanged (Fig 4B). In addition, these T cells expressed lower levels of the activation markers HLA-DR and CD25 revealed no differences in T cell polarization by mDCs from infected or uninfected individuals. However, the frequency of IL-4- or IFN- γ -producing T cells was significantly lower [23] (Fig 4C). This suggests, that mDCs from *S. haematobium* infected subjects do not drive distinct polarized effector T helper cell responses, but seem to have a general impairment to induce T cell activation and effector T cell expansion.

For proper T helper cell activation and expansion, TCR-triggering (signal 1) and costimulation (signal 2) are provided by DCs through peptides/MHC class II complexes and costimulatory molecules CD80 and CD86, respectively [24;25]. Since HLA-DR and CD80 surface expression on mDCs from infected individuals following TLR stimulation tended to be lower compared to levels found on mDC from uninfected subjects (Fig 2B), we assessed whether blocking of HLA-DR and/or CD80 on control mDCs could mimic their functional impairment to activate T cells. Indeed, neutralization of HLA-DR during the DC-T cell cocultures strongly suppressed the T cell activating capacity of mDCs as determined by T cell proliferation (Fig 4D) and cytokine production (data not shown). In contrast, complete blocking of CD80 only minimally inhibited T cell expansion (Fig 4D). This observation, together with the fact that only minimal concentrations of HLA-DR blocking antibody could already interfere with mDC-induced T cell activation (Fig 4E), point in the direction that the observed differences in HLA-DR expression, although small, play a major role in the different capacity of the mDCs from infected and uninfected subjects to drive T cell activation.

Discussion

There is a wealth of evidence from both *in vitro* and murine models that helminth parasites or their secreted products are able to modulate and suppress DC function [26;27]. However, apart from a recent report that has documented a suppressed function of *in vitro* generated monocyte-derived DCs isolated from hookworm infected subjects [28], there is currently little known about the actual consequences of chronic helminth infections on human DC function *in vivo*. In the study presented here, we

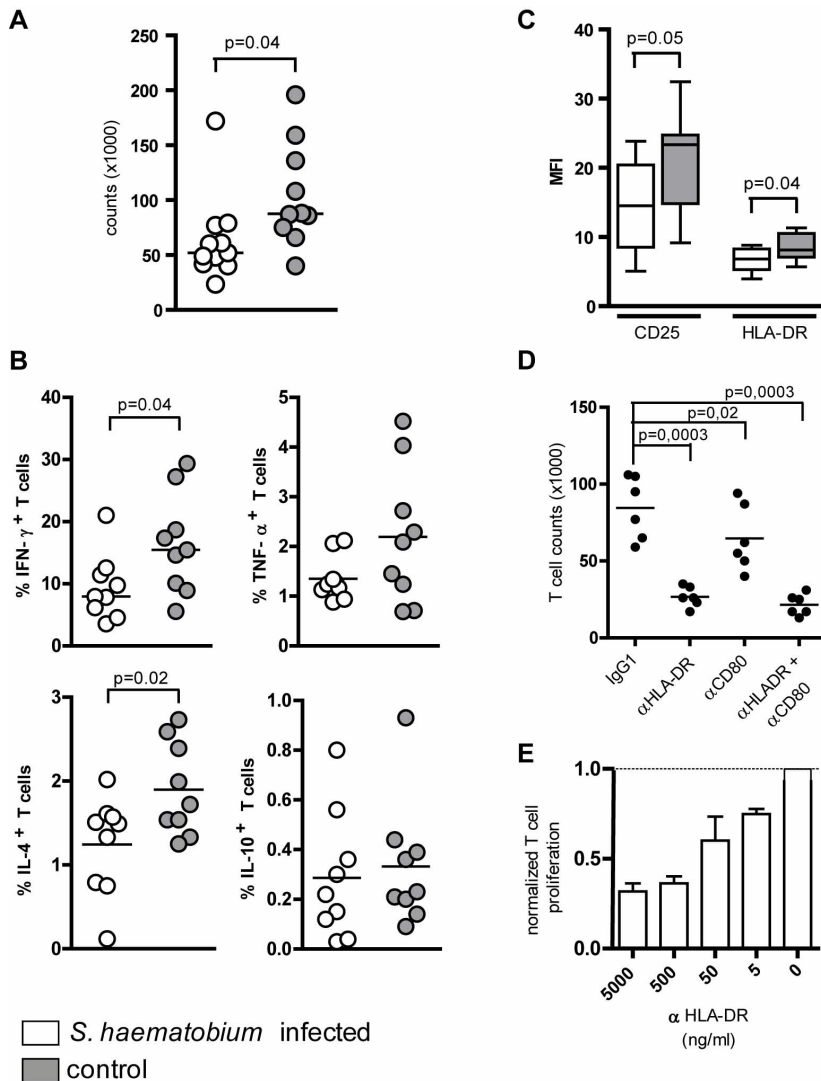


Figure 4. mDCs from *S. haematobium* infected subjects have a reduced T cell activating capacity due to lower HLA-DR expression. LPS-matured mDCs were cocultured with allogeneic naïve T helper cells for 6 d after which (A) T cell expansion was determined with a counting chamber and (B) Intracellular cytokine production or (C) CD25 and HLA-DR expression was assayed by FACS, 6 h after restimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin in the presence of brefeldin A for the last 2 h. (D+E) LPS-matured mDCs from European controls were cocultured with allogeneic naïve T helper cells for 6 d in the presence of depicted neutralizing antibodies and T cells were counted as in (A). T cell counts are shown as (D) absolute values or (E) relative to the control condition. (A,B,D) Horizontal bars represent mean based on data from 9 donors in each group. (C) Box plots represent 25-75 percentile range with error bars showing minimum to maximum based data of 9 donors in each group. (E) Bars represent mean + SD of 3 independent experiments.

now provide support for the notion that human blood DC are functionally impaired during chronic helminth infection *in vivo*, by showing that circulating mDCs isolated from schistosoma-infected subjects have an reduced capacity to respond to TLR ligands and to initiate T helper cell responses.

Quantification of circulating mDCs and pDCs in blood from infected subjects revealed that these cells are present in reduced frequencies compared to controls. This appears to be a phenomenon that is associated with more chronic infections in general, since similar observations have been made in patients with chronic Hepatitis B [29], HIV [30], tuberculosis [31] and malaria infection [32]. The reason for lower frequencies remains unclear, but similar to what has been documented for microfilaria of the species *Brugia malayi* [33;34], schistosoma parasites may promote direct apoptosis in DC. An increase in migration out of the circulation would also be a possible explanation, since chronic schistosoma infection may favor migration of DCs into inflamed tissues or lymphoid organs. Finally, the output of DC from the bone marrow could be altered, since we also observed lower monocyte, but not T cell frequencies in the circulation of the infected individuals (unpublished data), a cell type closely related to mDCs and originating from same bone marrow precursors [35;36].

In order to exert their function as innate immune cells, DCs have to be able to sense and respond rapidly to external stimuli, including pathogen-derived components. This is reflected in the release of cytokines and expression of a set of membrane bound molecules crucial for the initiation of the adaptive arm of the immune response [25]. We found that mDCs from infected subjects displayed reduced responses towards TLR ligands LPS and R848. Although, R848 generally induced stronger responses than LPS, the selective impairment to upregulate HLA-DR and CD80 by mDC from infected subjects was found after stimulation with both LPS and R848. This suggests that TLR responses in general may be impaired instead of being restricted to specific TLRs. Impaired TLR responsiveness of blood DCs has been reported during other chronic infections as well, where both reduced TLR expression [37;38] as well as altered signaling [38;39] has been implicated as underlying mechanisms. We could not observe any difference in TLR4 expression between the two groups; nevertheless we did find lower p38 activation in mDCs from the infected group when compared to the uninfected group in response to LPS. Since p38 activation is known to be important for mediating pro-inflammatory signaling in DCs following triggering of multiple TLRs [40], support the notion that a deficit of pro-inflammatory signaling, rather than TLR expression itself, underlies the impaired responsiveness of mDCs towards TLR ligands from *S. haematobium* infected individuals.

In addition to defects in innate responses of the DCs, we observed an impaired capacity of these cells to prime T cell responses. Given that chronic

schistosoma infection is generally associated with a Th2-like as well as anti-inflammatory immunological profile [1;2] and several *in vitro* studies have shown that schistosomal antigens can drive these responses via DCs [18;18;20;41-43], one could hypothesize that blood DCs from infected individuals would favor the differentiation of naïve T cell towards these subsets. However, we observed a general failure to prime and activate a T helper cell response by mDCs, reflected by reduced T cell expansion and a general impairment in production of both Th1- and Th2-associated cytokines after restimulation. An explanation for this discrepancy may be provided by the current view that only after migration into tissues blood DCs receive the right differentiation signals to acquire their full T cell polarizing potential [44]. Alternatively, the level of potentially polarizing factors in blood may be too low to affect the polarizing capacity of these DCs.

With regard to the mechanisms that may underlie the impaired capacity of these DCs to prime T cell responses, we found a tendency towards lower HLA-DR as well as CD80 expression on LPS-matured mDCs derived from infected individuals. Both TCR-triggering and costimulation are instrumental for DC-mediated T cell activation [25]. However, the fact that even partial neutralization of HLA-DR in mDC – T cell cocultures had a similar impact on T cell activation than total blockade of CD80, makes it more likely that primarily the reduced HLA-DR expression, though small, accounts for the observed differences in T cell activating capacity. Nonetheless, involvement of other membrane bound or soluble factors in the observed differences in T cell priming cannot be excluded. A recent *in vivo* murine study has shown that schistosoma infection can lead to T cell anergy through induction of PD-L1 on macrophages [13]. However, we did not find any differences in expression of PD-L1 between the mDCs of the two groups (data not shown), making a role for PD-L1 in the observed differences unlikely. Thus, we conclude that probably not an increase in negative costimulatory signals, but most likely a lack of antigen presentation underlies the reduced capacity of blood DCs from infected individuals to prime a T cell response.

It remains to be established what the mechanisms are that underlie the impaired DC function in schistosoma infected individuals. Modulation of DC function by direct interactions with schistosoma derived antigens would provide a plausible explanation. Several studies have documented potent modulatory effects of schistosomal antigens on the function of *in vitro* generated DCs, that include suppression of TLR induced cytokine production, maturation marker expression and MAPK signaling [19;20;45] and manipulation of DC driven T cell responses [13;18]. Adult *S. haematobium* worms are found in the venous plexuses around the urinary bladder, thereby providing a physiological situation in which circulating DCs could constantly be exposed to potentially modulatory antigens released by these parasites

in the blood. In this respect, an interesting finding was that mDCs from infected subjects, but not pDCs, were less responsive to TLR ligands. Schistosomal antigens can be recognized and have been shown to exert their modulatory effects on DC through engagement of certain TLRs, such as TLR2 [18;20] and c-type lectins like DC-SIGN [45-47], which are preferentially expressed by myeloid, and not the plasmacytoid DC lineage [48;49]. Apart from this direct mechanism, chronic schistosome infection may also lead to attenuation of DC function indirectly by induction of regulatory immune responses. For instance, important regulatory cytokines produced by the host, such as IL-10, have the potential to suppress DC function [15] and to be elevated in *S. haematobium* infected subjects [17]. Lastly, chronic schistosomiasis has also been shown in several studies to negatively affect the nutritional status of the host [50;51]. Although there is currently little known about the consequences of malnutrition on the functionality of DCs during schistosomiasis, experimental models show that undernutrition as such can lead to an impaired function of APCs resulting in a diminished induction of adaptive immune responses [52;53].

Despite the fact the infected and healthy individuals enrolled in this study were sex and age matched (Table 1) and living in the same endemic area, we cannot formally exclude that other environmental factors, such as co-infections, could contribute to observed differences in DC phenotype and function. In this respect, since filarial and malarial infections are also highly endemic in Gabon and have been documented to modulate APC function [38;54;55], the participants in this study were screened for infection by these parasites. While only a single individual was tested positive for malaria infection, several cases of filariasis were found in both groups (Table 1). However, stratification based on filarial infection revealed that these parasitic infections could not account for the observed differences in DC function (data not shown). Furthermore, other endemic chronic infections such as HIV, hepatitis and tuberculosis, are not likely to underlie the selective dysfunction of mDCs either, since in contrast to our findings, these types of infections have been found to be associated with functional impairment of pDCs [31;37;56;57].

In summary, the data presented in this study provide support for the notion that chronic schistosomiasis results in suppression of human DC function *in vivo*. This sheds new light on the mechanisms that could underlie the immune hyporesponsiveness observed during chronic helminth infections [2;14]. Importantly, a general impairment in TLR responses of mDCs and subsequent priming of T cell responses, may not only lead to impediment of immune responses against the worms, but may also have consequences for proper induction of protective immunity against concurrent infections, especially from bacterial or viral origin, which draws heavily on TLR-driven APC activation [58]. Finally, suppressed function of DCs, as we documented

here during a chronic helminth infection, has also been documented in chronic infections caused by other pathogens such as bacteria [31] or viruses [29;56;57]. This suggests that an impaired DC function during persistent infections is a widely distributed phenomenon and points in the direction that targeting of DCs is a common strategy evolved by pathogens to subvert immune responses and establish chronic infections.

Materials and Methods

Study population

Venous blood was obtained from 43 young adults (mean age $25,3 \pm 5,8$ years) living in or in the vicinity of Lambaréné, Gabon, a semi-urban municipality and an area in which *Schistosoma haematobium* infection is endemic [5]. Infection with *S. haematobium* was determined by passing 10 ml urine through 12 μm diameter filter. From every subject two independent urine screenings were performed to increase accuracy. The subjects were grouped into an infected group and non-infected group, which were sex and age matched. Furthermore, subjects were screened for other parasitic blood infections, including malaria and filariasis, as determined by microscopic analysis of Giemsa-stained blood smears (Table 1). For HLA-DR and/or CD80 neutralization experiments in the DC-T cell cocultures, mDC were isolated from venous blood from healthy European subjects. The study was approved by the Comité d’Ethique Régional Independent de Lambaréné (CERIL). Written informed consent was obtained from all subjects participating in the study.

Blood DC isolation

PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation from 40 ml of heparinized venous blood. Myeloid DCs (mDCs; BDCA-1+) were isolated by CD19-negative selection, followed by CD1c-positive selection using MACS and BDCA-1 Dendritic Cell Isolation Kit (Miltenyi Biotec). Subsequently, to maximize cell yield plasmacytoid DCs (pDCs; BDCA4⁺/CD123⁺) were isolated from the flowthrough of the mDC isolation by using and BDCA-4 Dendritic Cell Isolation Kit (Miltenyi Biotec). Purity based on BDCA-1/CD11c and BDCA-4/CD123 expression for myeloid and plasmacytoid DCs, respectively, was routinely more than 90%.

Characterization of DC populations in peripheral blood

For immunophenotyping and determination of frequencies of mDCs and pDCs present in peripheral blood, part of freshly isolated PBMCs were fixed using a ‘fixation and dead cell discrimination kit’ (Miltenyi Biotec), according to the manufacturers recommendations. Subsequently, cells were washed in 0,5%BSA/PBS and stained for

30 minutes at 4°C with anti-CD19-Pacific blue (Biolegend), anti-CD14-Pacific blue (Biolegend), anti-HLA-DR-APC/cy7 (Biolegend), anti-BDCA-1-APC (Miltenyi Biotec) and anti-BDCA-2-biotin/streptavidin-Qdot526 (both eBioscience) in combination with either anti-CD80-PE/Cy5 (BD) and anti-TLR4-FITC (BD) or anti-CD86-FITC (BD) and anti-CCR7-PE/Cy7 (BD). As gating strategy, blood DCs were selected as the CD14⁺/CD19⁻/HLA-DR⁺ population, followed by gating on BDCA-1 and BDCA-2 positive cells as selective markers for mDCs and pDCs, respectively. Frequencies as well as expression levels of TLR4, CD80, CD86, HLA-DR, CCR7 on these subpopulations were determined.

Blood DC culture

Freshly isolated DCs (2×10^4 cells/well in 200 μ l) were cultured in complete RPMI 1640 medium containing 10% FCS, 100 U/ml penicillin, and 100 μ g/ml streptomycin and supplemented with 500 U/ml GM-CSF or 10ng/ml IL-3 (both Strathmann, Germany) for mDCs and pDCs respectively. DCs were stimulated with 100ng/ml LPS (ultrapure, *E. coli* O111 B4 strain), 1 μ g/ml R848, or 1 μ g/ml CpG-B 2006 (all Invivogen). After 40h supernatants and cells were harvested. For analysis of surface marker expression of TLR-stimulated DCs, the cells were fixed in 2% formaldehyde (Sigma-Aldrich) for 15 minutes and, after two washing steps in 0,5% BSA/PBS, stained for 30 minutes at 4°C with a combination of either anti-HLA-DR-PerCP, anti-CD80-PE, anti-CD86-FITC and anti-CD40-APC or anti-PD-L1-APC, anti-ICOS-L-PE and anti-CCR7-FITC (all BD).

MAPK assay

Freshly isolated DCs (2×10^4 cells/well in 200 μ l) were seeded to rest for 24h in 96-well round bottom plates before stimulation with LPS. After 20 or 60 minutes cells were fixed for 10 minutes with 4% ultrapure formaldehyde (Polysciences) directly in the plate. Cells were harvested and washed twice in PBS/0.5% BSA. Subsequently, the DCs were permeabilized in 700 μ l ice-cold 90% methanol in PBS and stored at -80°C until analysis. For intracellular staining of phosphorylated MAPK, fixed permeabilized DC were washed twice in PBS/0.5%BSA and subsequently stained with anti-phospho-p44/42 MAPK AF-488 (T202/Y204) and anti-phospho-p38 MAPK AF-647 (T180/Y182), (both Cell Signalling Technology) for 2 hours at room temperature in the dark.

mDC coculture with naive T helper cells

5×10^3 LPS-matured mDCs were cocultured with 2×10^4 allogeneic naive T helper cells in 96-well flat-bottom plates in 200 μ l (Corning). Naive T helper cells were purified from a single European donor using the human CD4⁺/CD45RO- column kit (R&D, Minneapolis, MN). In some experiments cocultures were performed in the presence of 10 μ g/ml IgG1, blocking mAbs against HLA-DR (varying concentrations, clone L243, Biolegend) or

CD80 (5 µg/ml, clone 37711) (both R&D systems). After 6 days supernatants were harvested for determination of cytokine levels. In addition, T cells were counted with a counting chamber. For intracellular cytokine analysis T cells were restimulated with 200ng/ml PMA and 2 µg/ml ionomycin in the presence of 10 µg/ml brefeldin A (all Sigma-Aldrich) during 6 h, followed by fixation in 4% formaldehyde (Sigma-Aldrich) for 15 minutes at RT. After washing in 0.5% saponin/PBS, T cells were stained for 30 minutes in 0.5% saponin/PBS with anti-IL4-PE, anti-IFN γ -FITC, anti-TNF- α -biotin, anti-IL-10-APC (all BD Biosciences) followed by a second incubation with streptavidin-PerCP (eBioscience) at RT. In addition, T cells were stained for activation markers CD25-APC and HLA-DR-FITC (both BD).

Cytokine analysis

Cytokine levels in supernatants from 40 h stimulated DCs and 6d T cells cultures were determined using Luminex-100 cytometer (Luminex Corporation, TX, USA). DC supernatants were analysed simultaneously for IL-1 β , IL-6, IL-10, IL-12, TNF- α and IFN- α , while in T cell supernatants levels of IL-10, TNF- α , IL-4, IL-5, IL-13 and IFN- γ were determined. Buffer reagent and Luminex cytokine kits (Biosource, CA, USA) for cytokine analysis were used according to the manufacturers' recommendations. Samples with concentrations below the detection limit, as determined by the provided standards, were given half the value of this threshold.

Flow cytometric analysis

FACS experiments were performed on a Becton Dickinson FACSCalibur flowcytometer (BD) with CellQuest software (BD), except for the direct characterization of blood DCs in PBMCs, which was performed on a FACSCanto II (BD) using FACSDiva software. For the latter analysis, Fluorescence-minus-one (FMOs) was taken along as controls. FACS data were analysed using FlowJO software (Treestar, USA).

Statistical analysis

Data were analysed using SPSS (v14.0) and GraphPad Prism (v5). For multiple comparisons, the Kruskal-Wallis H nonparametric test was applied. Statistical difference between two groups was determined by applying the Mann-Whitney *U* nonparametric test. A paired *t* test was used to show the effect of treatment with specific blocking or stimulating Abs on T cell function. Differences were considered significant when P-values were below 0.05.

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