Multifaceted Defects in Monocytes in Different Phases of Chronic HBV Infection: Persistence After Antiviral Therapy

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Abstract

Background

Monocytes play an important role in the control of microbial infection but monocyte biology during chronic HBV infection (CHI) remains inadequately studied. We investigated the frequency/phenotype/functions of monocytes in different phases of CHI namely, Immune-tolerant (IT), HBeAg-positive/HBeAg-negative (EP/EN) chronic hepatitis B (CHB) and Inactive carriers (IC), identified factors responsible for their functional alterations and determined the impact of antiviral-therapy on these cells.

Methods

Multicolor flow cytometry/cell-sorting/co-culture experiments/confocal microscopy were performed.

Results

HLA-DR⁺CD14⁺⁺CD16⁻ classical-monocytes were found to be significantly reduced while HLA-DR⁺CD14⁺⁺CD16⁺ intermediate- and HLA-DR⁺CD14⁺CD16⁺⁺ non-classical-monocyte-subsets were expanded in IT and EP-/EN-CHB than IC and healthy controls (HC). In comparison to IC/HC, monocytes (including all subsets) in IT/CHB exhibited diminished expression of TLR-2/TLR-4/TLR-9 and cytokines IL-12/TNF- α /IL-6 but produced higher levels of IL-10/TGF- β . Further, monocytes in CHB/IT showed impaired phagocytosis and oxidative response relative to IC/HC. *In vitro* assays indicated that high titres of hepatitis B surface antigen (HBsAg) present in IT/CHB and IL-4 in CHB triggered the functional defects in monocytes via induction of β -catenin. Additionally, monocyte-derived M1-macrophages of CHB/IT produced less pro-inflammatory and more anti-inflammatory cytokines than those of IC/HC whereas, monocytes in CHB/IT skewed the differentiation of CD4⁺-T-cells towards regulatory T-cell and Th2-dominated phenotype. Moreover, in CHB/IT, monocytes overexpressed chemokine-receptor CCR2, which coincided with increased intrahepatic accumulation of β -catenin⁺CD14⁺-cells. One-year of Tenofovir-therapy failed to normalize monocyte functions or reduce serum HBsAg/IL-4 levels.

Conclusions

Monocytes are functionally perturbed mostly in IT and EP-/EN-CHB phases. Targeting intramonocytic β -catenin or reducing HBsAg/IL-4 levels might restore monocyte function and facilitate viral clearance.

Introduction

The outcome of chronic HBV infection [CHI] and the pathogenesis of liver disease are largely determined by immune-mediated host-virus interactions [1]. Inability to achieve sustained viral control in CHI has been correlated with the incapacity of the host to evoke an effective immune response against the virus [1]. Monocytes represent a critical component of innate immunity that play a fundamental role in the control of microbial infection and also contribute to the pathogenesis of inflammatory diseases [2]. They recognise pathogen-associated molecular patterns (PAMPs) by a set of Toll-like receptors (TLRs), and trigger intracellular signalling cascades leading to the expression of pro-inflammatory cytokines, enhanced phagocytic activity and generation of reactive oxygen and nitrogen intermediates [2–3]. These events orchestrate the early host response to infection that promote the clearance of pathogen. Based on the expression of CD14 and CD16, human monocytes can be divided into three subsets, CD14⁺⁺/CD16⁻ [classical], CD14⁺⁺/CD16⁺ [intermediate] and CD14⁺/CD16⁺⁺ [non-classical], whose relative percentages, functional properties and disease association had been reported to vary when studied *in vitro* and *in vivo* [4]. Monocytes traffic to the site of infection/inflammation and depending on microenvironmental stimuli, they differentiate into either M1-macrophages with pronounced pro-inflammatory phenotype, or M2-macrophages with anti-inflammatory attributes [2]. Monocytes can also drive the CD4⁺ T-cell differentiation into distinct effector cells that could impact the elimination of microbes and disease pathogenesis [5]. Relatively little is known about the effects of chronic viral infection on monocytes. Monocytes/macrophages serve as an important reservoir of HIV and the disease progression is closely linked to the expansion of CD16⁺-monocytes [6]. Altered TLR-signalling and cytokine production by monocytes had been described in HCV-infected patients [7]. In chronically HBV-infected patients, changes were observed in the frequencies of monocyte-subsets and regulation of TLR-expression by HBV precore and surface protein had been reported [8–9]. However, there is still a lack of comprehensive understanding of monocyte biology during CHI, whose natural history includes four dynamic phases namely, immune-tolerant (IT), hepatitis B e antigen (HBeAg)-positive chronic hepatitis B (EP-CHB), inactive carrier (IC) and HBeAg-negative chronic hepatitis B (EN-CHB) [10]. Hence, the present study aimed to appraise the distinct phenotype and functions of monocytes in different phases of CHI, identify the viral and host factors that could modulate the properties of these cells and study the interaction of monocytes with CD4⁺ T-cells. We also studied the impact of Tenofovir therapy on monocytes in CHB patients. This integrated knowledge would be vital for designing more effective therapies for CHB.

Methods

Detailed information regarding study subjects and experimental methodology is available in Supplementary information (SI).

Study subjects and samples

Treatment-naïve chronically HBV-infected patients were recruited from I.P.G.M.E.&R, Kolkata, India and grouped into IT, EP-CHB, IC and EN-CHB. Additionally, HBV-uninfected healthy individuals (HC)

were included. Blood samples and liver biopsy specimens were collected from study subjects with written informed consent. The study was approved by the Ethical Review Committee of I.P.G.M.E&R.

Characterization of monocytes

Frequencies of monocytes expressing TLR-2/TLR-4/CD64/LAP-TGF- β /CCR2/TLR-8/TLR-9/IL-6/IL-12/TNF- α /iNOS/IL-10/ β -catenin were analysed by flow-cytometry. Phagocytic ability was investigated using FITC-labelled zymosan and generation of reactive oxygen species (ROS) by 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA).

Serum Hepatitis B surface antigen (HBsAg) and cytokine quantification

Serum HBsAg and different cytokines in study subjects were quantified using Abbott Architect i1000sr platform and BD CBA Human Th1/Th2/Th17 Cytokine Kit respectively.

Treatment of sorted monocytes or PBMC

Monocytes were sorted from PBMC of HC with anti-CD14-coated magnetic-beads and treated with rHBsAg/ β -galactosidase (β -gal)/combination of rHBsAg and β -catenin/TCF inhibitor (iCRT3) in separate experiments. In all cases, the frequencies of monocyte-subsets and their TLR-2/IL-12/IL-10/CD64/iNOS/ β -catenin expression were determined by flow-cytometry. In addition, PBMC of HC was cultured in presence/absence of rIL-4/rTNF- α /combination of rIL-4 and iCRT3 and expression of IL-12/IL-10/ β -catenin on HLA-DR⁺CD14⁺-monocytes was investigated.

Differentiation of monocytes to macrophages

Purified monocytes were differentiated into macrophages with M-CSF followed by LPS and IFN- γ treatment to generate M1-macrophages or IL-4 to generate M2-macrophages and their intracellular TNF- α /IL-12/IL-10 levels were ascertained by flow-cytometry.

Co-culture of CD14⁺monocytes and monocyte-depleted PBMC

CD14⁺ monocytes were sorted from PBMC, co-cultured with anti-CD3/anti-CD28 treated monocyte-depleted PBMC and frequencies of CD4⁺CXCR3⁺ [Th1-cells], CD4⁺CCR4⁺CCR6⁻ [Th2-cells] and CD4⁺CD25⁺FOXP3⁺ [regulatory T-cells (Treg)] were determined by flow-cytometry.

Immunofluorescence staining

Incidence of β -catenin⁺CD14⁺-monocytes in liver biopsies of selected CHB/HC was analysed by immunohistochemical staining.

Assessment of virological/immunological parameters following Tenofovir-therapy

Blood samples were collected from 12 CHB patients treated with Tenofovir before therapy (=baseline) and after 12 months and frequency/phenotype/function of monocyte-subsets and serum HBV-DNA/ALT/HBsAg/cytokine levels were determined.

Statistical analysis

Statistical analysis was performed using GraphPad Prism5 software as relevant. $P < 0.05$ was considered statistically significant.

Results

Study population

A total of 65 chronically HBV-infected patients were categorised as IT (n=10), EP-CHB (n=15), IC (n=22) and EN-CHB (n=18) [Supplementary(S)Table S1]. In addition, 19 HC were included.

Distribution of monocyte-subsets in different phases of CHI

We first determined the frequency of total-monocytes and their subsets in different phases of CHI (Fig. 1a-b). The frequency of HLA-DR⁺CD14⁺-total-monocytes was comparable across all study groups. However, the proportion of HLA-DR⁺CD14⁺⁺CD16⁻[classical] monocytes was significantly diminished in IT (83.6±3.1%), EP-CHB (82.5±3.6%) and EN-CHB (82.7±2.4 %) relative to IC (92.5±1.7%) and HC (93.6±1.6%), while a stark increase was observed in HLA-DR⁺CD14⁺⁺CD16⁺[intermediate], and HLA-DR⁺CD14⁺CD16⁺⁺[non-classical] monocytes in IT (intermediate/non-classical-monocytes; 9.4±1.3/6.9±2.3%), EP-CHB (9.7±1.6/7.7±2.4%) and EN-CHB (9.7±1.5/7.4±1.7%) as compared to IC (3.8±1.0/3.6±0.9%) and HC (3.3±0.8/2.9±0.9%) (Fig. 1b).

TLR expression and cytokine production by monocytes

Recognition of PAMPs by TLRs of monocytes represent a critical step for the clearance of infecting microbes. We noted significant reduction in TLR-2⁺, TLR-4⁺ and TLR-9⁺-monocytes in IT and EP-/EN-CHB than IC and HC while no difference could be perceived in the incidence of TLR-8⁺-monocytes across the groups (Fig. 1c). All monocyte-subsets of IT and EP-/EN-CHB exhibited declining trends in TLR-2/4/9 expression (Fig. S1a). Simultaneously, marked diminution was observed in the percentages of total-monocytes (Fig. 1d) as well as all three-subsets (Fig. S1b) that expressed TLR-regulated pro-inflammatory cytokines TNF-α, IL-12 and IL-6 in IT and EP-/EN-CHB in comparison to IC/HC. While intramonocytic TNF-α and IL-6 level was equivalent in IT and EP-/EN-CHB, IL-12 was significantly low in EP-/EN-CHB than IT. Further, the frequencies of IL-12⁺-monocytes were less in IC than HC (Fig. 1d).

Additionally, we evaluated the production of inhibitory cytokines, TGF-β and IL-10 by the monocytes in different disease phases. We assessed the expression of TGF-β bound to the latency-associated peptide (LAP) [11] whereby lower cell-surface LAP-expression correlates with higher TGF-β secretion. IT as well as

EP-/EN-CHB patients displayed significant decline in proportion of LAP-TGF- β ⁺-monocytes, indicative of raised functional TGF- β levels, as opposed to IC and HC (Fig. 1e, S1c). Furthermore, an analogous expansion in IL-10⁺-monocytes (Fig. 1e, S1c) was apparent in IT and EP-/EN-CHB relative to IC/HC. Notably, monocytes of EP-/EN-CHB showed enhanced IL-10 expression than IT (Fig. 1e).

Phagocytic activity and oxidative response of monocytes in CHI

We analyzed the expression of Fc γ RI/CD64, the primary receptor for opsonic uptake of antigens on monocytes. In comparison to IC/HC, a deficit in phagocytic function of monocytes was noted in IT and EP-/EN-CHB, as evident from significantly low expression of CD64 in total-monocytes, including all subsets (Fig. 2a, S2a). Consistent with decreased CD64 expression, there was also substantial decline in the percentage of monocytes associated with zymosan-reporter signal in case of IT and EP-/EN-CHB, suggestive of poor zymosan uptake by these cells than those of IC/HC (Fig. 2a, S2b-S2c).

Phagocytosis leads to the generation of reactive oxygen and nitrogen species (ROS/RNS) within monocytes. The capacity of the monocytes (including all subsets) to produce ROS was significantly attenuated in IT and EP-/EN-CHB as inferred from decreasing DCF-fluorescence in these patients relative to HC and IC (Fig. 2b, S3a-S3b). RNS production is dependent upon nitric oxide that is generated by inducible *nitric oxide* synthase (iNOS). Compared to IC/HC, iNOS⁺ total-/monocyte-subsets were reduced in numbers in IT and EP-/EN-CHB, suggesting a decrease in iNOS-mediated RNS production (Fig. 2b, S3c). Additionally, IC harboured significantly lower frequency of iNOS-expressing monocytes compared to HC.

Regulation of monocyte functions by HBsAg and cytokines

We next sought to identify the viral antigen and systemic cytokines that might contribute to the altered monocyte functions during CHI. HBsAg is the most abundant viral protein in the sera of HBV-infected patients and it was found to be markedly high in IT ($5.2 \pm 0.9 \log_{10}$ IU/mL) and EP-/EN-CHB ($5.3 \pm 0.9 \log_{10}$ IU/mL) than IC ($3.2 \pm 0.5 \log_{10}$ IU/mL) (Fig. 3a). A positive correlation was observed between serum HBsAg levels and IL-10-expressing monocytes while HBsAg titers correlated inversely with frequencies of TLR2⁺/IL-12⁺/CD64⁺/iNOS⁺-monocytes, implying a potential role of HBsAg in monocyte dysfunction (Fig. 3a). Further, treatment of CD14⁺-monocytes, sorted from HC, with high concentration of rHBsAg resulted in reduced frequency of classical-monocytes and amplification of non-classical and intermediate-subsets, along with suppression of TLR-2/CD64/IL-12/iNOS and augmentation of IL-10 expression as compared to untreated and β -gal-treated cells (Fig. 3b, S4). On the other hand, even at low HBsAg concentration, the monocytes exhibited significant decrease in iNOS- and IL-12 expression over control setups (Fig. 3b).

Despite the similar HBsAg levels in IT and CHB, we noticed a decline in IL-12⁺ and heightened IL-10⁺-monocytes in CHB as compared to IT. We postulated that these functional variabilities could be related to the differences in local cytokines in these two phases. Significant increases were found for serum IL-

4 and TNF- α exclusively in EP/EN-CHB phases than other groups (Fig. 3c). In addition, we observed that treatment of monocytes with high concentration of rIL-4 conferred significant enhancement in IL-10⁺- and diminution in IL-12⁺-monocytes relative to untreated cells, while no discernible change was noted upon rTNF- α -treatment (Fig. 3c, S5a-S5b).

HBsAg and IL-4 activated β -catenin in monocytes

We next investigated the mechanism underlying HBsAg- or IL-4-mediated alteration in the properties of monocytes. Given that β -catenin could suppress TLR-triggered pro-inflammatory responses and induce anti-inflammatory cytokines [12, 13], we speculated that HBsAg/IL-4 could promote the aberrant monocyte function through activation of β -catenin. Our *in vitro* assays demonstrated that both HBsAg and IL-4 resulted in significant accumulation of β -catenin⁺-monocytes compared to untreated cells (Fig. 4a, S6a-S6b). Moreover, addition of β -catenin/TCF inhibitor led to enhanced frequency of classical-monocytes as well as that of TLR-2⁺/CD64⁺/iNOS⁺/IL-12⁺-monocytes but caused reduction in intermediate-/non-classical-subsets along with IL-10-expressing monocytes, relative to that observed when no inhibitor was added (Fig. 4b-c, S4, S5a). In parallel, β -catenin⁺ monocytes were significantly elevated in all chronically HBV-infected patients than HC and HBsAg titres correlated positively with percentages of β -catenin⁺-monocytes (Fig. 4a). Collectively, these findings signify that the functions of monocytes were compromised by induction of β -catenin by HBsAg/IL-4.

Characterization of *in vitro* differentiated macrophages

Monocytes from study subjects were differentiated *in vitro* to M1-/M2-macrophages and their cytokine production abilities were compared. HLA-DR⁺CD14⁺CD68⁺ M1-macrophages, particularly in CHB, and also IT were characterized by marked decline in IL-12 and TNF- α production relative to HC/IC (Fig. 5a). In addition, a heightened frequency of IL-10-expressing M1-macrophages was perceived in CHB followed by IT, while it was much lower in IC and HC (Fig. 5b). HLA-DR⁺CD14⁺CD68⁺ M2-macrophages from CHB and IT displayed superior abilities to produce IL-10 than IC/HC, although CHB showed higher IL-10 expression than IT (Fig. 5b). Irrespective of the study groups, all M2-macrophages expressed little IL-12 and TNF- α .

Monocyte-mediated differentiation pattern of CD4⁺T-cells-

Monocytes are known to drive the differentiation of CD4⁺T-cells into distinct functional populations [5]. We explored whether the monocytes promote any specific CD4⁺T-cell differentiation program in CHI. Co-culture of sorted CD14⁺-monocytes from CHB and IT with autologous anti-CD3/anti-CD28 stimulated monocyte-depleted PBMC resulted in enrichment of CD4⁺CD25⁺FOXP3⁺Treg by ~4.4- and ~4-folds respectively and an expansion of CD4⁺CCR4⁺CCR6⁺Th2-subset by ~2.6- and ~2-folds. However, an equivalent population of Treg or Th2-cells did not emerge in presence of monocytes sorted from HC/IC. Conversely, monocytes of HC and IC favor the amplification of CD4⁺CXCR3⁺Th1-cells by ~4 fold while in IT, ~2.4 fold rise in Th1-cells was also seen (Fig. 5c).

Mobility traits of monocytes in CHI

CCR2 plays a key role in recruitment of monocytes to the liver [14]. CCR2⁺-monocytes, inclusive of all subsets, were found to be markedly elevated in both EP-/EN-CHB patients than other groups while IT showed a greater percentage of CCR2-expressing monocytes than IC/HC (Fig. 5d, S7). This suggests a higher potential of the monocytes in CHB as well as IT to home to the liver.

Assessment of intrahepatic β -catenin⁺CD14⁺-monocytes

We also studied the frequency of CD14 and β -catenin double-positive cells in liver biopsy sections of CHB patients and HC by immunohistochemical staining. Liver histology indicated prominent lymphocyte-predominant lobular and portal inflammation in CHB than HC. β -catenin⁺CD14⁺-cell density was found to be substantially high in the liver of CHB and such cells were barely perceptible in HC (Fig. 5e).

Frequency/phenotype/function of monocytes in Tenofovir-treated CHB patients

Tenofovir is recommended as first-line monotherapy for CHB patients [10] and we tested the effect of Tenofovir treatment on the frequency and expression of different functional markers of monocytes in 12 CHB patients that included 5 EP-CHB and 7 EN-CHB. We observed that all patients achieved <250 copies/ml of HBV-DNA and normalization of serum ALT after one-year of therapy (Fig. 6a) but no significant change was detected in the monocyte-subset distribution or the proportion of TLR2/IL12/IL-10/CD64/iNOS-expressing monocyte-subsets between pre- and post-treatment time-points (Fig. 6b). Moreover, the serum levels of HBsAg and IL-4 in these patients remained similar to baseline values (Fig. 6a).

Discussion

In this study we enumerated the broad spectrum of phenotypic and functional alterations in monocytes of chronically HBV-infected patients and studied the mechanisms underpinning the changes, which would add to the ongoing efforts of defining the process behind immune dysregulation in CHI and provide targets for development of new therapies aimed at reversing the defects.

An expansion of CD16⁺-compartment (intermediate- and non-classical-monocytes) along with concomitant decrease in classical-monocytes was perceived in IT and CHB patients relative to IC/HC. Similar shifts in monocyte-subset distribution had been previously reported in Chinese HBeAg-positive CHB patients [15] and also in other infectious diseases [6, 7]. The interaction of monocytes with the pathogen or pathogen-derived factors had been suggested to be the causal factor for this increase in CD16⁺-monocytes [16]. The secretory HBsAg is known to have an immunomodulatory effect and circulating CD14⁺-monocytes were found to harbour a detectable depot of HBsAg in CHB patients [17]. We demonstrated that at high concentration, HBsAg could stimulate the preferential generation of intermediate- and non-classical-monocytes and this concurred with the greater frequency of these two subsets in IT and CHB patients, who unlike IC, carried high serum HBsAg.

TLR-2 and TLR-4 of the monocytes sense the presence of virus via their proteins, whereas, TLR-7/8 binds single-stranded viral RNA and TLR-9 recognizes viral CpG DNA [3]. Previous studies on the TLR-expression on monocytes of CHB patients had revealed in many cases divergent data. Overexpression of TLR-2 and TLR-4 had been noted on CD14⁺-monocytes in HBV-infected Chinese patients [15] while a study conducted in Australia reported a marked reduction in TLR-2 but not TLR-4 expression on monocytes of HBeAg-positive CHB patients relative to HBeAg-negative CHB and controls [8]. In contrast, we observed that both TLR-2 and TLR-4 along with TLR-9 were downregulated in both EP- and EN-CHB and also in IT in comparison to IC/HC. These discrepancies in TLR-expression profile might partially result from investigating ethnically different patient populations or might be impacted by high exposure of a population to other pathogens causing tuberculosis, malaria, leprosy or to parasitic worms. TLR-signalling usually induce the expression of an array of inflammatory cytokines. We noted that the attenuation of TLR-expression in CHB/IT were reciprocated by greatly impaired production of TNF- α /IL-12/IL-6 by the monocytes while the levels of IL-10 and TGF- β were significantly enhanced. Intriguingly, higher expression of both TNF- α and IL-10 transcripts had been documented in the monocytes of Chinese CHB patients than healthy donors [18]. Different studies have established that monocyte-derived IL-12 could induce IFN- γ production from T-cells and also skew the naive CD4⁺T-cells toward the Th1-phenotype [5] that help in eliminating viral infection. Conversely, upregulation of IL-10 in monocytes coincides with impaired T-cell responses [3]. Hence, it appears that the inhibition of IL-12 and augmentation of IL-10 by monocytes in CHB/IT would limit the antiviral activities of T-cells and favour viral persistence.

A salient feature of the monocytes is their capability to phagocytose foreign organisms and generate ROS and RNS by specialized enzymes, NADPH oxidase and iNOS that can irreversibly oxidize and damage the cellular structures of the pathogens. Significant reduction in phagocytic activity, intracellular ROS production and suppression of iNOS was manifested by the monocytes of EP-/EN-CHB and IT when compared with IC/HC. Our finding was in congruity to that of Prieto *et al.* who had reported diminished phagocytosis by monocytes of CHB patients relative to HBeAg-negative/anti-HBe positive patients and HC [19]. This overall inadequate antimicrobial activity of monocytes in CHB/IT contribute to the chronicity of infection and high viral load in these phases.

Monocytes give rise to macrophages in tissues and the heterogeneity in monocytes underlie that of macrophages. To determine if MDM-subsets are functionally altered in CHI, we investigated the cytokine profiles of these macrophages, which can confer a better appreciation of functional polarization than that of cell-surface receptor expression. Our results revealed that M1-macrophages from CHB/IT acquired an M2-like anti-inflammatory state characterized by decreased production of TNF- α and IL-12 and increased IL-10. Similar cytokine secretion features of M1-cells had been reported in chronic HCV patients [7]. However, unlike M2-subsets from HCV-infected individuals, which secrete more pro-inflammatory cytokines than controls [7], the M2-macrophages in all phases of CHI produced very low concentrations of IL-12 and TNF- α . Moreover, M2-cells generated from CHB and IT displayed remarkable enhancement in IL-10 production in comparison to IC/HC. Thus, the aberrant functions of MDM in CHB/IT and their shifts

towards M2-phenotype are likely to influence T-cell activation and function that would contribute towards viral perpetuation and pathogenicity in CHI.

We next identified the HBV antigen and host cytokines that dictate the functional modification of monocytes during CHI. We demonstrated that high concentration of HBsAg encountered in IT and EP/EN-CHB, potentiated the reduced expression of TLR-2/CD64/iNOS/IL-12 and heightened expression of IL-10 by the monocytes. Moreover, HBsAg, even at low concentration, could inhibit IL-12 and iNOS expression and thus could explain the observed decreased production of these molecules by monocytes in IC relative to HC. The role of HBsAg in inhibiting TLR-2 ligand-induced IL-12 production in monocytes/macrophages had also been previously highlighted by Wang *et al.* [9]. In addition, *in vitro* assays depicted that IL-4, detected at high levels exclusively in EP-/EN-CHB phase, could constrain IL-12 but stimulate IL-10 expression in monocytes and thus could account for the greater immunosuppressor trait of monocytes in CHB patients. Similar IL-4-mediated inhibition of IL-12 production had been observed in murine peritoneal macrophages [20].

We dissected the molecular mechanisms through which HBsAg and IL-4 could affect the phenotype/function of monocytes and showed that both pathways impinge on the activation of β -catenin. An increased accumulation of β -catenin was seen in HBsAg or IL-4 treated monocytes whereas, pharmacological inhibition of β -catenin could potentially normalize the immune functions in these cells, signifying a crucial regulatory role of β -catenin in monocyte function during CHI. We also observed a positive correlation between HBsAg level and frequency of β -catenin⁺-monocytes in chronically HBV-infected patients. It seems plausible that high or low β -catenin concentration exert distinct effects on the expression of different functional markers of monocytes and thus could account for the difference in monocyte properties in CHB and IC.

The monocytes in CHB/IT displayed enhanced CCR2-expression and thus appeared to have high propensity to migrate to inflamed liver. Interestingly, in regorafenib-resistant cancer cells, β -catenin was found to be a direct transcriptional activator of CCR2-expression [14] and it is conceivable that the upregulation of CCR2 in the monocytes of CHB/IT is related to β -catenin activation. In support to this conclusion, we also noticed a greater prevalence of β -catenin-expressing monocytes in liver tissues of CHB patients compared to HC.

Apart from the innate effector functions, monocytes can also act as a bridge to the adaptive immune system. Colocalization of CD14⁺-cells with clusters of CD4⁺ T-cells had been reported at sites of inflammation [5], suggesting an interaction between these cells *in vivo*. We showed that monocytes from CHB/IT cause preferential skewing of CD4⁺ T-cell compartment towards Treg- and Th2-dominated phenotype and this together with profound suppression of Th1-polarization facilitate the chronicity of infection.

Finally, we tested whether Tenofovir therapy could modulate the monocyte phenotype/function in CHB patients. Our results highlighted that the monocyte-subsets continued to retain their baseline

characteristics after one-year therapy and no reduction in HBsAg or IL-4 level was perceived. The persistence of immune suppressive cascade in CHB patients even after Tenofovir treatment may represent a pivotal risk factor for the advancement of liver diseases.

Taken together, this study has illustrated a multitude of functional defects in monocytes in different phases of CHI. It thus appears that therapeutic targeting of intramonocytic β -catenin or reducing the circulating HBsAg levels or modulation of the cytokine milieu in chronically HBV-infected patients might be successful in restoring the monocyte function, clearance of HBV and cure of HBV-induced liver disease.

Abbreviations

HBV, Hepatitis B Virus;

CHI, Chronic HBV infection;

HBsAg, Hepatitis B surface antigen;

rHBsAg, Recombinant Hepatitis B surface antigen;

ALT, Alanine aminotransferase;

AST, Aspartate aminotransferase;

HBeAg, Hepatitis B e-antigen;

FBS, Fetal bovine serum;

IT, Immune-tolerant;

CHB, Chronic hepatitis B;

EP-CHB, HBeAg-positive CHB;

IC, Inactive carrier;

EN-CHB, HBeAg-negative CHB;

TLR, Toll-like receptor;

PAMP, Pathogen-associated molecular pattern;

LPS, Lipopolysaccharide;

Fc γ R, Fc-gamma receptor;

iNOS, Inducible nitric oxide synthase;

TNF- α , Tumor Necrosis Factor- α ;

DCFH-DA, 2',7',-dichlorodihydrofluorescein diacetate;

DCF, Dichlorofluorescein;

CCR2, Chemokine Receptor Type 2;

CCR4, Chemokine Receptor Type 4;

CCR6, Chemokine Receptor Type 6;

TGF- β , Transforming Growth Factor- β ;

LAP, Latency associated peptide;

mAb, Monoclonal antibody;

ROS, Reactive oxygen species;

RNS, Reactive nitrogen species;

NO, Nitric oxide;

MDM, Monocyte derived macrophage,

HC, Healthy control;

PBMC, Peripheral blood mononuclear cells;

RPMI-1640, Roswell Park Memorial Institute medium 1640;

rTNF- α , Recombinant TNF- α ;

β -gal, β -Galactosidase;

HCC, Hepatocellular Carcinoma;

SD, Standard Deviation;

ANOVA, Analysis of variance;

IL-4, Interleukin-4;

rIL-4, recombinant IL-4;

IL-12, Interleukin-12;

IL-6, Interleukin-6;

IL-10, Interleukin-10;

HLA-DR, Human Leukocyte Antigen-DR isotype;

HIV, Human immunodeficiency virus;

FITC, Fluorescein isothiocyanate;

Th1, Type 1 T helper;

Th2, Type 2 T helper;

Treg, Regulatory T cell;

CXCR3, CXC chemokine receptor 3,

NF- κ B, Nuclear Factor kappa-light-chain-enhancer of activated B cells;

STAT4, Signal transducer and activator of transcription 4;

MHC, major histocompatibility complex;

HCV, Hepatitis C virus;

iCRT3,2-[[[2-(4-ethylphenyl)-5-methyl-4-oxazolyl]methyl]thio]-N-(2 phenylethyl)acetamide;

FACS, fluorescence-activated cell sorting

Declarations

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Author Contributions

SD, AC and DD were involved in study concept and design. DD, SP and S. Bhadra executed different assays and performed flow-cytometry. RG, BCC and DD performed immunohistochemistry procedure. DD and SD participated in analysis and interpretation of data. SMA and AC scrutinized the clinical parameters of the patients and helped in the patient recruitment procedure. SD and DD drafted the manuscript and S. Banerjee helped in revising it critically. All authors read and approved the final version of the manuscript.

Conflict of interest

The authors have no conflicts of interest to disclose in connection with the submitted manuscript.

Consent to Participate

The study was approved by the Ethical Review Committee of Institute of Post Graduate Medical Education and Research (I.P.G.M.E&R), Kolkata, India. Written informed consent was obtained from each participant or from parents or legal guardians of minors prior to study inclusion.

Consent for Publication

Not applicable.

Animal research

This article does not contain any studies with animals performed by any of the authors.

Availability of data and plant reproducibility

All data and materials are included in the manuscript and supplementary information.

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Figures

Figure 1

Frequency, phenotype and function of monocytes in different phases of chronic HBV infection (a) Sequential gating strategy for identification of HLA-DR⁺CD14⁺ total, HLA-DR⁺CD14⁺CD16⁻ classical, HLA-DR⁺CD14⁺CD16⁺ intermediate and HLA-DR⁺CD14⁺CD16⁺ non-classical monocytes using flow cytometry. (b) The relative abundance of total, classical, intermediate and non-classical monocytes in Immune-tolerant (IT), HBeAg-positive chronic hepatitis B (EP-CHB), Inactive carriers (IC), HBeAg-negative CHB (EN-CHB) and healthy controls (HC). Percentages of total monocytes expressing (c) TLR-2, TLR-4,

TLR-8, TLR-9, (d) TNF- α , IL-12, IL-6 and (e) LAP-TGF- β , IL-10 in different study groups. Mean $\pm$ SD are given. Means among groups were compared by One-way ANOVA with Tukey's Multiple comparison. (* P < 0.05, ** P < 0.005 and *** P < 0.0001).

Figure 2

Phagocytosis and oxidative responses by monocytes during chronic HBV infection Bar diagrams demonstrating frequencies of (a) CD64-expressing monocytes and those with phagocytosed zymosan-FITC particles, (b) ROS-generating and iNOS⁺ monocytes in Immune-tolerant (IT), HBeAg-positive chronic hepatitis B (EP-CHB), Inactive carriers (IC), HBeAg-negative CHB (EN-CHB) and healthy controls (HC). Mean $\pm$ SD are given. Means among groups were compared by One-way ANOVA with Tukey's Multiple comparison. (** P < 0.005 and *** P < 0.0001).

Figure 3

Effect of viral and host factors on functional properties of monocytes (a) Serum levels of Hepatitis B surface antigen (HBsAg) in Immune-tolerant (IT), Chronic hepatitis B (CHB) patients and Inactive carriers (IC). Correlation analysis between percentages of TLR-2, IL-12, IL-10, CD64 and iNOS expressing monocytes and level of HBsAg in chronically HBV infected patients. Statistical significance was assessed by one way ANOVA followed by Tukey's Multiple Comparison Test and linear regression analysis (** $P < 0.005$ and *** $P < 0.0001$). (b) The proportion (%) of different monocyte subsets and the expression of TLR-2, IL-12, IL-10, CD64, iNOS in sorted CD14⁺ monocytes of healthy control (HC) treated with or without β-galactosidase (β-gal) (20µg/ml), recombinant Hepatitis B surface antigen (rHBsAg) (10µg/ml and

20µg/ml) for 48 hours. Statistical significance was assessed by one way ANOVA followed by Tukey's Multiple Comparison Test (** $P < 0.005$ and *** $P < 0.0001$). (c) Concentration of serum IL-4 and TNF-α in HC, IT, CHB and IC and grouped bar diagram of relative percentages of HLADR⁺CD14⁺ monocytes expressing IL-12 and IL-10 in PBMC of HC treated with or without rIL4 (5ng/ml or 25ng/ml) and TNF-α (50ng/ml). Statistical significance was assessed by one way ANOVA followed by Tukey's Multiple Comparison Test and two way ANOVA (** $P < 0.005$ and *** $P < 0.0001$).

Figure 4

Induction of intramonocytic β-catenin by HBsAg and IL-4 and its impact on monocyte function (a) Frequency of HLA-DR⁺CD14⁺β-catenin⁺ cells following treatment of sorted CD14⁺ monocytes of healthy

controls (HC) with β -galactosidase (β -gal) (20 μ g/ml), rHBsAg (20 μ g/ml) and treatment of PBMC of HC with rIL-4 (25ng/ml). Bar diagram demonstrating β -catenin⁺ total monocytes of HC, inactive carriers (IC), immunetolerant (IT)/ chronic hepatitis B (CHB) patients. Correlation analysis between percentages of β -catenin expressing monocytes and level of HBsAg in chronically HBV infected patients. Statistical significance was assessed by one way ANOVA followed by Tukey's Multiple Comparison Test and Student's t-test and linear regression analysis (** $P < 0.005$ and *** $P < 0.0001$). (b) Grouped dot plots showing proportion (%) of monocyte-subsets and expression of TLR-2, IL-12, IL-10, CD64, iNOS on monocytes treated with rHBsAg (20 μ g/ml) or combination of rHBsAg (20 μ g/ml) with iCRT3 (25 μ M). (c) Percentages of IL-12⁺ and IL-10⁺ monocytes following treatment with rIL-4 (25ng/ml) or combination of rIL-4 (25ng/ml) with iCRT3 (25 μ M). Statistical significance was assessed by Repeated Measures ANOVA (** $P < 0.005$ and *** $P < 0.0001$).

Figure 5

Cytokine profile of monocyte-derived macrophages, monocyte-mediated CD4⁺ T-cell differentiation, frequency of CCR2⁺ circulating monocytes and incidence of intrahepatic CD14⁺β-catenin⁺ monocytes during chronic HBV infection Percentages (%) of (a) IL-12⁺, TNF-α⁺ and IL-10⁺ HLA-DR⁺CD14⁺CD68⁺ monocyte-derived M1-macrophages and (b) IL-12⁺, TNF-α⁺ and IL-10⁺ HLA-DR⁺CD14⁺CD68⁺ monocyte-derived M2-macrophages in Immune-tolerant (IT), Chronic hepatitis B (CHB) patients, Inactive carriers (IC) and healthy controls (HC). Mean ± SD are given. Means among groups were compared by One-way

ANOVA with Tukey's Multiple comparison. (* $P < 0.05$ and *** $P < 0.0001$). (c) Grouped bar diagram representing fold changes of CD4⁺CXCR3⁺ Th1 cells, CD4⁺CCR4⁺CCR6⁻ Th2 cells and CD4⁺CD25⁺FOXP3⁺ Treg cells following co-culture of sorted CD14⁺ monocytes with autologous monocyte depleted PBMC derived from HC, IT, CHB and IC. For all comparisons, p value was calculated by the Paired Student's t-tests (*** $p < 0.0001$). (d) Frequencies of total circulating monocytes expressing CCR2 in HC, IT, HBeAg-positive CHB (EP-CHB), IC and HBeAg-negative CHB (EN-CHB). Comparisons were done using one way ANOVA followed by Tukey's Multiple Comparison (** $P < 0.005$ and *** $P < 0.0001$). (e) Representative images of Haematoxylin and Eosin (H&E) staining of liver tissues from HC and CHB. Sections were stained with anti-CD14-FITC, anti- β -catenin-PE and DAPI. Immunofluorescence images at 63X magnification showing CD14⁺ monocytes (green), their β -catenin expression (red) and their co-localization.

Figure 6

Monocyte frequencies, functions and clinical parameters in tenofovir treated CHB patients (a) Serum HBV DNA load, serum ALT levels, serum HBsAg and IL-4 levels and (b) proportions of monocyte-subsets of CHB patients at baseline and at the end of 12-months of tenofovir therapy and percentages of TLR-2, IL-12, IL-10, CD64 and iNOS expressing monocyte-subsets in CHB patients before and after tenofovir treatment. Paired Student's *t* test and Repeated Measures ANOVA were performed for statistical analysis (****P* <0.001).

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