immuno

MDPI

Article

Rosuvastatin Intervention in Patients with Chronic Hepatitis B (CHB) Expands CD14+ CD16− Classical Monocytes via Aryl Hydrocarbon Receptor (AHR)

Mina Rahmati [1], Mojtaba Zare Ebrahimabad [2], Alale Langari [2], Ali Najafi [3], Shohreh Taziki [4], Alireza Norouzi [3], Mehrdad Teimoorian [5], Milad Khorasani [6] and Saeed Mohammadi [3,7,*]

1 Biotechnology Research Center, Pasteur Institute of Iran, Tehran 1316943551, Iran; mnrahmati69@gmail.com
2 Metabolic Disorders Research Center, Golestan University of Medical Sciences, Gorgan 4934174515, Iran; mojtabazare1988@gmail.com (M.Z.E.); alale.langari@gmail.com (A.L.)
3 Golestan Research Center of Gastroenterology and Hepatology, Golestan University of Medical Sciences, Gorgan 4934174515, Iran; ali.najafi@goums.ac.ir (A.N.); alireza.norouzi@goums.ac.ir (A.N.)
4 Department of Pharmacology, School of Medicine, Golestan University of Medical Sciences, Gorgan 4913815739, Iran; dr_sh_taziki@yahoo.com
5 Stem Cell Research Center, Golestan University of Medical Sciences, Gorgan 4934174515, Iran; mehrdadtm@gmail.com
6 Department of Clinical Biochemistry, Neyshabur University of Medical Sciences, Neyshabur 1413993186, Iran; miladkh24@yahoo.com
7 Natural and Medical Sciences Research Center, University of Nizwa, Nizwa 616, Oman
* Correspondence: s.mohammadi@goums.ac.ir

Abstract: Chronic hepatitis B (CHB) poses treatment challenges, with treatment response and disease outcome often determined by the immune response, particularly mononuclear phagocytes. Monocytes can differentiate into various subpopulations influenced by AHR. Statins, known for inflammation modulation, may impact monocyte function via AHR activation. This study explored rosuvastatin (RSV)'s effects on monocyte subtypes, inflammatory markers, and AHR in CHB patients. Fifteen CHB patients were randomly assigned to receive either 20 mg RSV or a placebo daily for three months. Flow cytometry assessed CD14+ CD16− (classical), CD14+ CD16+ (intermediate), and CD14dim CD16+ (patrolling) monocyte subtypes, along with AHR levels in each subset. ELISA quantified cytokines IL-6, IFN-γ, IL-12, IL-10, TNF-α, TGF-β, and IL-1β. RSV expanded CD14+ CD16− classical and reduced CD14+ CD16+ intermediate monocytes in CHB patients while increasing AHR+ cell percentages in all subsets. RSV treatment upregulated key AHR target genes (Cyp1a1, Cyp1b1, and ARNT), indicating robust AHR signaling activation. It also reduced pro-inflammatory cytokine levels (IL-6, IFNγ, IL-12, TNF-α) and elevated anti-inflammatory cytokines (IL-10, TGF-β). Thus, RSV may modulate the immune response by altering monocyte subtypes in CHB patients via AHR activation.

Keywords: chronic hepatitis B (CHB); monocytes; aryl hydrocarbon receptor (AHR); rosuvastatin (RSV); CD14; CD16

Citation: Rahmati, M.; Zare Ebrahimabad, M.; Langari, A.; Najafi, A.; Taziki, S.; Norouzi, A.; Teimoorian, M.; Khorasani, M.; Mohammadi, S. Rosuvastatin Intervention in Patients with Chronic Hepatitis B (CHB) Expands CD14+ CD16− Classical Monocytes via Aryl Hydrocarbon Receptor (AHR). *Immuno* **2024**, *4*, 159–171. https://doi.org/10.3390/immuno4020011

Academic Editor: Ming-Lin Liu

Received: 21 March 2024
Revised: 11 May 2024
Accepted: 15 May 2024
Published: 17 May 2024

1. Introduction

The hepatitis B virus (HBV) may lead to a diffuse chronic infectious state, exhibiting diverse manifestations with life-threatening outcomes [1]. The vaccination of newborns is believed to be the most efficient solution against HBV [2]. However, some confining factors have blunted the vaccination plans, mostly in developing countries [3]. The effectiveness and integrity of the antiviral immune response can decisively alter the duration of the infection and prevent chronic hepatitis B (CHB). The elements of the body's natural defense system, which encompass mononuclear phagocytes (monocytes, macrophages, and Kupffer

cells), dendritic cells (DCs), and natural killer (NK) cells, and how they engage with the virus, significantly influence the outcome of CHB [4].

The secretion of HBV-mediated pro- and anti-inflammatory cytokines from effector immune cells, such as IFNγ, IFNα, IL1, IL10, IL6, etc., are deregulated during CHB [5–7]. The impairment of innate immunity [8], in addition to the consequent flawed maturation of adaptive immune cells [9], leaves the CHB patients vulnerable to the uncontrolled replication of HBV [10].

According to the indisputable role of immunity in the pathogenesis of CHB, numerous therapeutic strategies are focused on enhancing the immune response against HBV, especially via modulating innate immune cells [8]. While the present drugs used for CHB treatment, such as nucleotide analogs (like lamivudine, adefovir, tenofovir, and entecavir) [11] and interferons [12] can effectively inhibit viral replication and bolster the adaptive immune response [13], the safety of these medications remains a topic of debate [14,15], and their impact on innate immune cells is not thoroughly established.

Monocytes, macrophages, and Kupffer cells are innate immune cells directing the antiviral immune response, related to the mononuclear phagocyte system [16]. It has been suggested that the monocyte functions are disrupted during CHB [17,18], which could affect the function of phagocytes, while the principal origin of all immune cells within the mononuclear phagocyte system is circulating monocytes [19,20]. Monocytes are classified into three main subgroups based on their expression of CD14 and CD16, which include classical monocytes (CD14+ CD16−), intermediate monocytes (CD14+ CD16+), and patrolling (nonclassical) monocytes (CD14dim CD16+) [21,22]. While the percentages of these subpopulations are altered in different disease states, and each subset represents specific physiological functions [23], it is of great importance to study the alterations of monocyte subsets in CHB patients and monitor the responses of immune cells to each treatment. The CD14+ CD16− classical monocytes, as the most frequent subpopulation, are mainly involved in phagocytosis, antigen processing, and presentation [24]. The CD14+ CD16+ intermediate monocyte, as the least frequent subpopulation, is mostly involved in inflammatory responses (but may also exert proinflammatory effects), such as reactive oxygen species (ROS) production, and T-cell stimulation [25]. The patrolling monocytes are mobile monocytes, involved in tissue repair, exhibiting accelerated antiviral responses by producing proinflammatory cytokines [26].

Several signaling pathways have been identified with the ability to alter monocyte subsets or affect their maturation [27,28]. Recent studies revealed that the aryl hydrocarbon receptor (AHR) molecular pathway is able to modulate the function and maturation of immune cells [29], including monocytes [30], and the type of immune response [31]. AHR could be activated by diverse synthetic and natural ligands, mainly composed of polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and halogenated dioxins [32]. Statins competitively inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), leading to reduced blood cholesterol levels, while also demonstrating potent anti-inflammatory properties [33]. There have been indications that certain statins, such as rosuvastatin (RSV), might function as selective ligands for the aryl hydrocarbon receptor (AHR) [34]. While numerous contradictory studies have examined the impact of RSV on inflammation in different illnesses [35], as far as we know, no study has specifically examined the influence of RSV on altering monocyte subtypes in patients with CHB. Thus, our objective was to explore the effects of RSV on the alteration of monocyte subgroups in CHB patients, in correlation with AHR expression, and to evaluate cytokine levels in the sera of patients receiving RSV or placebo treatment.

2. Materials and Methods

2.1. Participant Selection and Characteristics

The samples utilized in this study were obtained from a prior prospective, single-center, randomized, double-blinded, placebo-controlled clinical trial, which was registered and published in the Iranian Registry of Clinical Trials (IRCTID: IRCT20190602043789N1) [36].

In summary, 30 CHB patients, carefully matched for sex, age, and treatment methods, were selected from the Gastroenterology and Liver Clinic at Sayyad-e-Shirazi Hospital, Golestan University of Medical Sciences (GoUMS), Gorgan, Iran. The study protocol was thoroughly reviewed and approved by the GoUMS Ethics Committee in accordance with the principles outlined in the Declaration of Helsinki (Ethics Code: IR.GOUMS.REC.1397.342). All participants provided written informed consent after a comprehensive explanation of the study details. A skilled gastroenterologist verified the presence of CHB in all patients based on clinical examinations and laboratory indicators. Patients co-infected with hepatitis A virus (HAV), hepatitis C virus (HCV), human immunodeficiency virus (HIV), or hepatitis D virus (HDV) were excluded from the study. Exclusion criteria involved patients with chronic liver impairment due to non-viral factors, autoimmune disorders, acute renal failure (ARF), malignancies, drug and/or alcohol dependency, pregnant individuals, and those with a history of statin hypersensitivity.

The patients were randomly assigned in a blinded manner to two groups of 15 individuals each, in a 1:1 ratio, receiving either rosuvastatin or a placebo. Over a period of 12 weeks, all participants were orally administered rosuvastatin tablets (20 mg, Abidi Company, Tehran, Iran) or corresponding placebos once daily. In the event of any reported adverse effects, the treatment was halted under the supervision of the gastroenterologist. A total of 5 mL of peripheral blood (PB) was drawn from each participant under sterile conditions and sent to the Stem Cells Research Center laboratory at GoUMS.

2.2. Immunophenotyping of Monocytes by Flowcytometry

As previously outlined, we separated the peripheral blood mononuclear cells (PBMCs) through Ficoll-Paque (Baharafshan, Tehran, Iran) density gradient centrifugation [37]. After confirming the viability of PBMCs, we suspended 4–5 $\times$ 10^5 viable cells in warm PBS. These PBMCs were subsequently marked with APC-conjugated anti-human HLA-DR antibody (Catalogue # 307609; Biolegend, San Diego, CA, USA), and FITC-conjugated anti-human CD14 antibody (Catalogue # 301804; Biolegend). To examine the different subsets of monocytes in each group, we used FITC-conjugated anti-human CD14 antibody and PE/cyanine5-conjugated anti-human CD16 antibody (Catalogue # 302009; Biolegend). The proportions of CD14+ CD16− classical, CD14+ CD16+ intermediate, and CD14dim CD16+ patrolling monocytes were determined within the HLA-DR+ CD14+ monocyte population. Additionally, the monocytes were labeled with PE-conjugated human AHR antibody (Catalogue # 694503; Biolegend). The percentages of AHR+ events were assessed for each monocyte subpopulation. For each sample, cells were incubated with 5 µL of each antibody or marker for 30 min at room temperature, and dark, as suggested by the manufacturer [38].

To ensure the specificity of our analysis, we took precautionary measures to exclude any other cell types during flow cytometry, focusing exclusively on the targeted PBMCs. To enhance the accuracy of our fluorescence-based measurements, Fluorescence Minus One (FMO) control was employed, allowing us to establish baseline fluorescence levels and precisely delineate the boundaries of distinct monocyte subsets within the analyzed PBMC populations. All measurements were performed in triplicate. The immunophenotypes of the stained samples in each group were analyzed using the BD Accuri C6 flow cytometer (BD PharMingen, San Diego, CA, USA) and BD Accuri C6 plus software.

2.3. ELISA Cytokine Assay

Serum samples were obtained from all CHB patients receiving either RSV or placebo treatment. The levels of pro-inflammatory cytokines (IL-12, TNF-α, IL-1β, IL-6, and IFN-γ) and anti-inflammatory cytokines (IL-10 and TGF-β1) were evaluated using commercially available ELISA kits (Zellbio, Berlin, Germany) in accordance with the provided guidelines [39]. In brief, serum samples were added to microplate wells coated with specific antibodies for each cytokine and incubated for 2 h at room temperature to allow cytokine binding. Following incubation, wells were washed using provided washing buffers to

remove unbound substances. Enzyme-conjugated secondary antibodies were then added and incubated for 1 h at room temperature. After another round of washing, a substrate solution was added and incubated for 30 min at room temperature, and dark. The optical density (O.D) of each sample was measured at a wavelength of 450 nm using the StatFAX 2100 ELISA plate reader (Awareness Inc., Palm City, FL, USA). Each experiment for all samples was conducted in triplicate, and the results were expressed as picograms of cytokines per mL.

2.4. Gene Expression Analysis

Total RNA was isolated from PBMCs of CHB patients using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocols [40]. The isolated RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) to confirm its purity (A260/A280 ratio between 1.8 and 2.0) and concentration ($\geq$200 ng/µL). Complementary DNA (cDNA) was synthesized from the purified total RNA using the Yekta Tajhiz cDNA synthesis kit (Yekta Tajhiz, Tehran, Iran) according to the manufacturer's instructions [41]. Following cDNA synthesis, DNAse I treatment (Thermo Fisher Scientific, USA) was performed to eliminate any residual genomic DNA contamination. Real-time polymerase chain reaction (qPCR) was conducted using SYBR Green dye (Yekta Tajhiz, Iran) on a StepOne Plus PCR machine (Applied Biosystems, Waltham, MA, USA) [42]. The primer sequences used for amplification of target genes and the melting temperatures are listed in Table 1. The expression levels of genes of interest relevant to AHR activation, namely cytochrome P450 1A1 (Cyp1a1; NM_000410.4), cytochrome P450 1B1 (Cyp1b1; NM_000100.3), aryl hydrocarbon receptor nuclear translocator (ARNT; NM_000739.3), and indoleamine-2,3-dioxygenase 1 (IDO-1; NM_002164.4), were evaluated. To ensure accuracy and reliability, the expression levels were normalized to the endogenous control gene, 18S ribosomal RNA (18srRNA; NR_003278.3). The fold change in gene expression was determined using the comparative $2^{-\Delta\Delta Ct}$ method, using Livak method. The relative fold change in gene expression was calculated by comparing the experimental group (CHB patients treated with RSV) to the control group (CHB patients without RSV treatment) [43].

Table 1. List of primers used in gene expression quantification.

Gene	Gene ID	Forward Sequence (5′-3′)	Reverse Primer (5′-3′)	Product Length (bp)	Tm (°C)
Cyp1a1	NM_000410.4	GATTGAGCACTGTCAGGAGAAGC	ATGAGGCTCCAGGAGATAGCAG	138	61
Cyp1b1	NM_000100.3	GCCACTATCACTGACATCTTCGG	CACGACCTGATCCAATTCTGCC	129	61
ARNT	NM_000739.3	GGAATGCCTACTCCAGTCTTGC	CTTTGCCACTGCGACCAGACTT	109	61
IDO-1	NM_002164.4	GCCTGATCTCATAGAGTCTGGC	TGCATCCCAGAACTAGACGTGC	119	61
18srRNA	NR_003278.3	ACCCGTTGAACCCCATTCGTGA	GCCTCACTAAACCATCCAATCGG	159	61

2.5. Statistical Assessment

SPSS 23.0 (SPSS Inc., Chicago, IBM, IL, USA) and GraphPad Prism 8.4.2 software (GraphPad Software Inc., San Diego, CA, USA) were employed for data analysis and graphical representation. The data are presented as Means ± Standard Deviation (S.D.). A comparison of means between the two groups was conducted using the Mann–Whitney U test. Statistical significance was defined as p-values less than 0.05. The statistical procedures were validated by the Department of Research and Technology at GoUMS.

3. Results

3.1. The Administration of Rosuvastatin Led to an Increase in CD14+ CD16− Classical Monocytes and a Decrease in CD14+ CD16+ Intermediate Monocytes in CHB Patients

We identified the viable monocytes within PBMCs from both the placebo and rosuvastatin groups (Figure 1A,D) and evaluated the proportions of HLA-DR+ CD14+ monocytes in CHB patients (Figure 1B,E). Through an examination of the CD14 and CD16 markers on HLA-DR+ CD14+ monocytes, we determined the presence of CD14+ CD16− classical, CD14+ CD16+ intermediate, and CD14dim CD16+ patrolling monocytes in both sets of CHB patients (Figure 1C,F). Our results illustrated that rosuvastatin treatment substantially raised the percentages of CD14+ CD16− classical monocytes in CHB patients (p-value < 0.0001). Similarly, we observed a significant reduction in the percentages of CD14+ CD16+ intermediate cells following rosuvastatin intervention in comparison to the placebo group (p-value < 0.0001). The percentages of patrolling cells exhibited no significant changes with RSV treatment (Figure 1G).

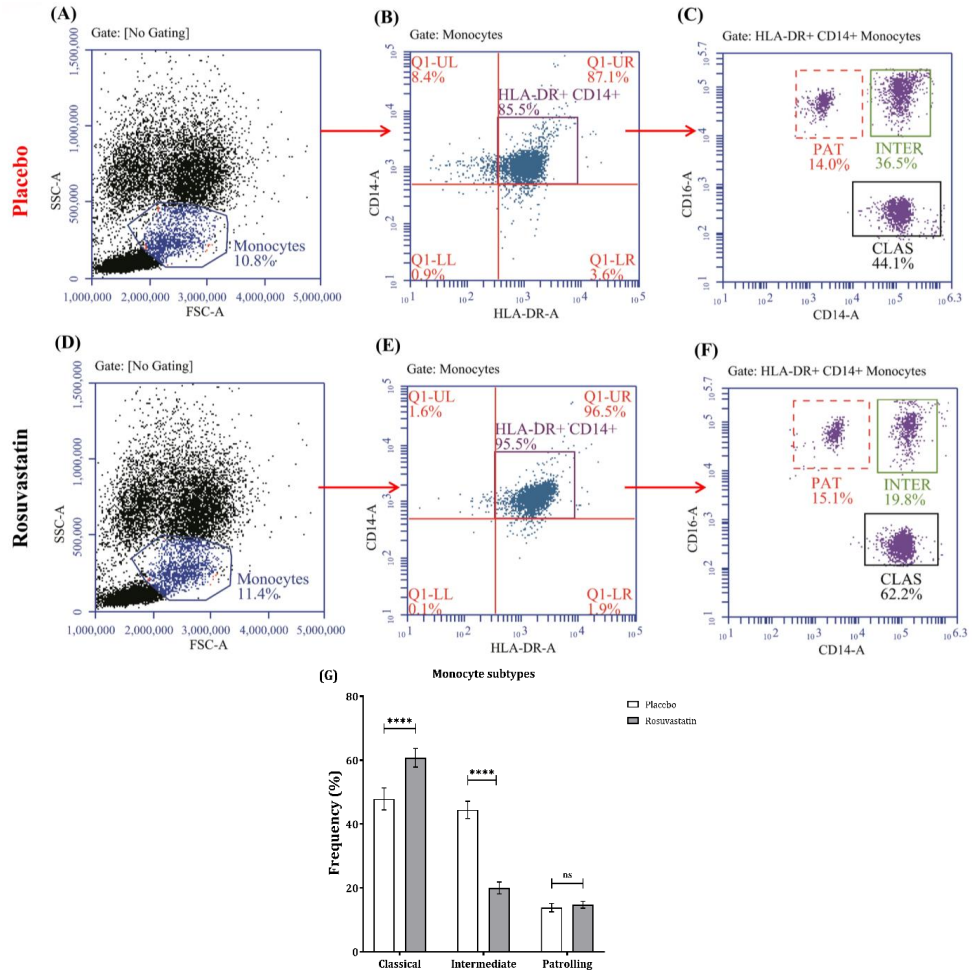

Figure 1. Immunophenotyping of HLA-DR+ CD14+ monocytes in chronic hepatitis B (CHB) patients treated with rosuvastatin or placebo. Monocytes (**A**,**D**) were gated according to staining by APC-conjugated anti-human HLA-DR antibody, and FITC-conjugated anti-human CD14 antibody (**B**,**E**). The percentages of CD14+ CD16− classical, CD14+ CD16+ intermediate, and CD14dim CD16+ patrolling monocytes were evaluated on HLA-DR+ CD14+ monocytes, by FITC-conjugated anti-human CD14 antibody, and PE/cyanine5-conjugated anti-human CD16 antibody (**C**,**F**). Rosuvastatin treatment increased the percentages of CD14+ CD16− classical monocytes and decreased the percentages of CD14+ CD16+ intermediate monocytes in CHB patients (**G**). Mann–Whitney U test was used to compare the means between two groups. Each bar represents Means ± Standard deviation. ns: not significant; **** p-value < 0.0001.

3.2. Rosuvastatin Treatment Resulted in Elevated Percentages of AHR+ Cells across All Monocyte Subgroups in CHB Patients

We assessed the proportions of AHR+ cells within the three subgroups of CD14+ CD16− classical, CD14+ CD16+ intermediate, and CD14dim CD16+ patrolling monocytes in CHB patients receiving either rosuvastatin or placebo treatment (Figure 2A–F). Rosuvastatin intervention notably raised the percentages of AHR+ cells among classical (p-value < 0.001), intermediate (p-value < 0.0001), and patrolling (p-value < 0.05) monocytes (Figure 2G).

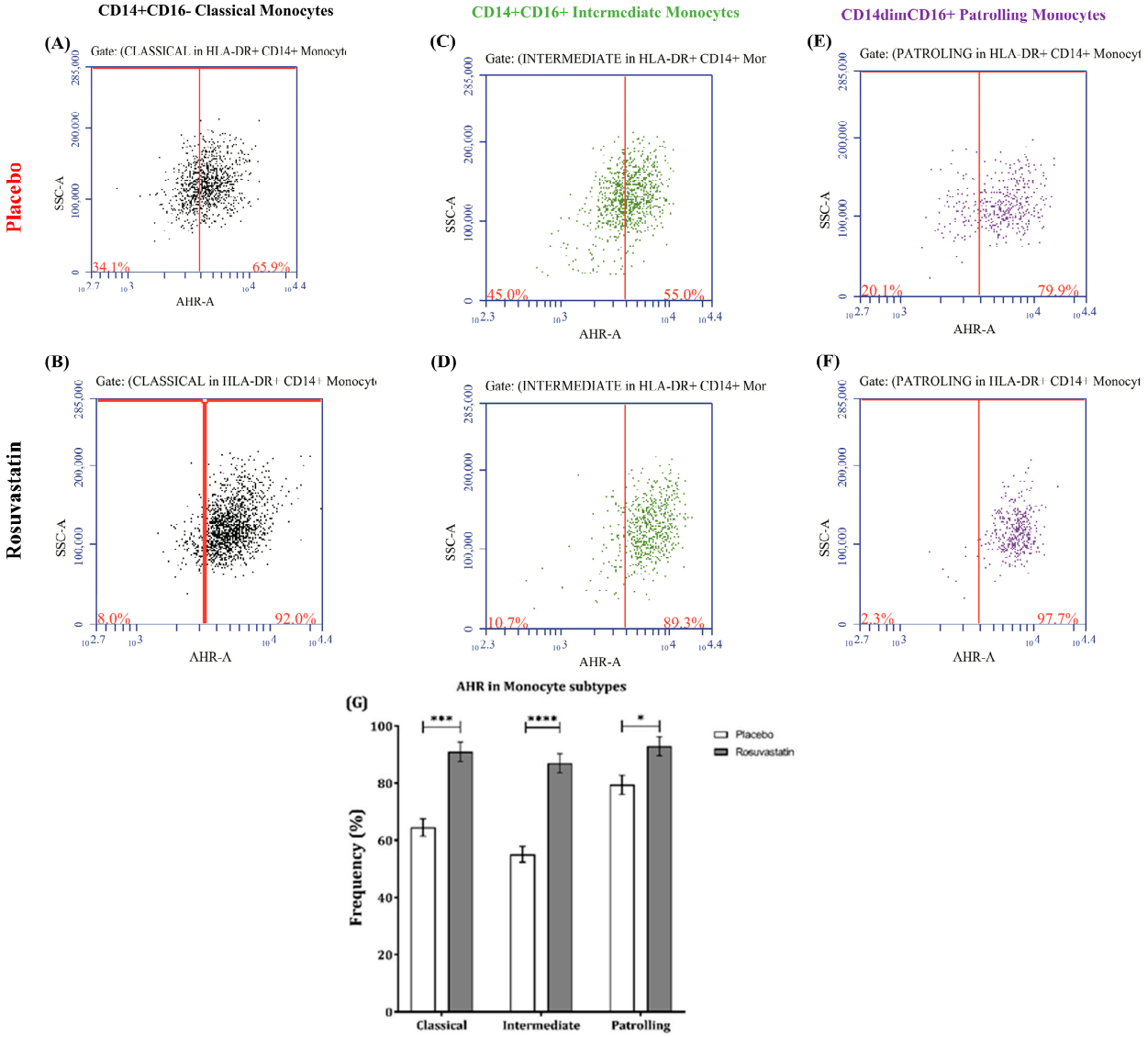

Figure 2. Evaluating the percentages of AHR+ cells among each subpopulation of monocytes in CHB patients treated with rosuvastatin or placebo. PE-conjugated anti-human AHR antibody was used to stain monocytes in each subpopulation (**A**–**F**). We found that RSV intervention could significantly increase the percentages of AHR+ cells among classical, intermediate, and patrolling monocytes (**G**). Mann–Whitney U test was used to compare the means between two groups. Each bar represents Means ± Standard deviation. **** p-value < 0.0001; *** p-value < 0.001; * p-value < 0.05.

3.3. Rosuvastatin Treatment Modified the Levels of Pro- and Anti-Inflammatory Cytokines

We conducted ELISA cytokine assays on the serum samples from CHB patients treated with either RSV or placebo to evaluate the secretion levels of pro- and anti-inflammatory cytokines. The results revealed that in response to RSV, IL-6 was significantly reduced in CHB patients (p-value < 0.001) (Figure 3A). The pro-inflammatory cytokine IFNγ exhibited a

significant decrease in RSV-treated CHB patients (p-value < 0.001) (Figure 3B). Additionally, the serum levels of IL-12 were downregulated in CHB patients who received RSV (p-value < 0.01) (Figure 3C). On the other hand, the anti-inflammatory cytokine IL-10 showed a notable increase in RSV-treated patients (p-value < 0.05) (Figure 3D). TNF-α demonstrated a decrease in response to RSV (p-value < 0.01) (Figure 3E), whereas TGF-β showed a significant increase (p-value < 0.01) (Figure 3F). The levels of IL-1β did not exhibit significant changes between the two groups of patients (Figure 3G).

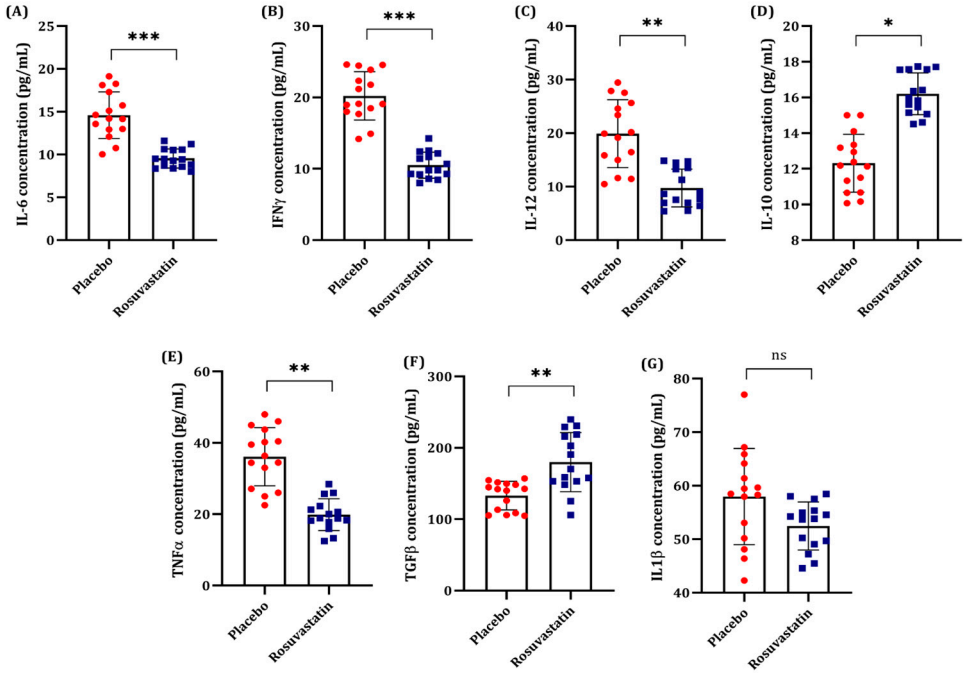

Figure 3. ELISA cytokine assay was used to evaluate the pro- and anti-inflammatory cytokine levels in the sera of CHB patients treated with placebo or rosuvastatin (RSV), related to the activation of monocytes. IL-6 was significantly downregulated in response to RSV (**A**). The pro-inflammatory cytokine of IFNγ was significantly decreased in RSV-treated CHB patients (**B**). IL-12 serum expression was downregulated in CHB patients who received RSV (**C**). The anti-inflammatory cytokine of IL-10 was markedly increased in RSV-treated patients (**D**). TNF-α was decreased in response to RSV (**E**), while TGF-β was significantly increased (**F**). IL-1β was not significantly changed between two groups of patients (**G**). Mann–Whitney U test was used to compare the means between two groups. Each bar represents Means ± Standard deviation. p-values lower than 0.05 were considered statistically significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: not statistically significant.

3.4. RSV Treatment Activates AHR Signaling in CHB Patients

To investigate the activation of AHR by RSV in CHB patients, we analyzed the expression of four AHR target genes (Cyp1a1, Cyp1b1, IDO-1, and ARNT). The fold change in mRNA expression was compared between CHB patients treated with RSV and those receiving placebo using qPCR. Compared to the placebo group (fold change = 1.031), RSV treatment significantly upregulated Cyp1a1 expression with a fold change of 1.872 (Figure 4A). This 82% increase suggests a robust activation of AHR pathways by RSV in CHB patients. Similar to Cyp1a1, Cyp1b1 expression was significantly higher in the RSV group (fold change = 3.856) compared to the placebo group (fold change = 1.041) (Figure 4B). This threefold increase further supports the activation of AHR signaling following RSV exposure. Consistent with the trends observed for Cyp1a1 and Cyp1b1, ARNT expression demonstrated a significant upregulation in the RSV group (fold change = 3.413) compared to the placebo group (fold change = 1.013) (Figure 4C). This increase in ARNT, a key component of the AHR heterodimer, indicates a robust activation of the AHR complex and downstream signaling pathways. The RSV treatment led to a slight increase in IDO-1

expression (fold change = 1.625) compared to the placebo group (fold change = 1.007) (Figure 4D).

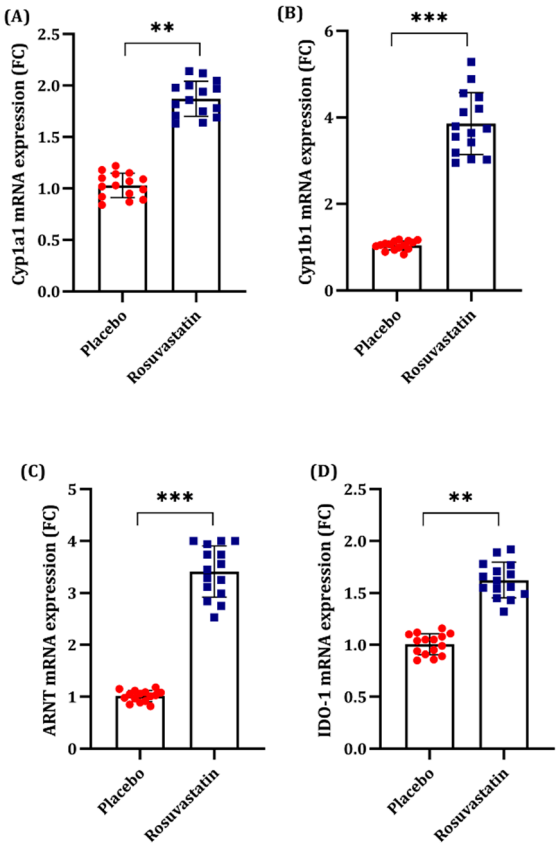

Figure 4. Activation of AHR target genes in CHB patients following RSV treatment. CHB patients were treated with RSV or placebo, and the mRNA expression of four AHR target genes (Cyp1a1, Cyp1b1, IDO-1, and ARNT) was measured using qPCR. Data are presented as mean fold change relative to the placebo group (set to 1) with error bars representing standard deviation (SD) from three independent replicates. Significant differences between groups were determined using Mann–Whitney U test. (**A**) Cyp1a1 expression was significantly upregulated in the RSV group compared to the placebo group (fold change = 1.872 vs. 1.031, $p < 0.05$). (**B**) Similar to Cyp1a1, Cyp1b1 expression was significantly higher in the RSV group compared to the placebo group (fold change = 3.856 vs. 1.041, $p < 0.01$). (**C**) Consistent with the trends observed for Cyp1a1 and Cyp1b1, ARNT expression demonstrated a significant upregulation in the RSV group compared to the placebo group (fold change = 3.413 vs. 1.013, $p < 0.01$). (**D**) The RSV treatment led to a slight increase in IDO-1 expression compared to the placebo group (fold change = 1.625 vs. 1.007). ** $p < 0.01$, *** $p < 0.001$.

4. Discussion

Hepatitis B virus (HBV) infection remains a significant health concern, with roughly 45% of the global population residing in regions with high prevalence [44]. Vaccination is currently the most efficient and cost-effective approach to prevent HBV infection [5]. Individuals with positive HBV serology for six months or more are considered chronic hepatitis B (CHB) patients [6,20]. In some cases, chronic infection, which is associated with deregulated immune responses, may lead to irrecoverable damage to the liver [6]. CHB is divided into HBeAg positive or negative states, where the HBeAg positive patients are usually more prone to higher rates of viral replication and thus increased infection [45].

The initial defense against pathogens, such as HBV, involves the body's innate immunity, including mononuclear phagocytes [10,17,46]. Viral infections trigger the production of type I interferons (IFN-α and IFN-β) and other inflammatory cytokines via Toll-like receptors (TLR3, 7, 8, and 9) and the retinoic acid-inducible gene I (RIG-I) [17]. Whether the HBV

infection clears or persists into a chronic state is largely determined by the host's innate immune responses, including monocytes [14]. The monocyte functions are disrupted during CHB [18,19], which could alter the fate of the disease and response to treatment [20,22]. The monocytes are classified into three primary subgroups based on their expression of CD14 and CD16: classical monocytes (CD14+ CD16−), intermediate monocytes (CD14+ CD16+), and patrolling (nonclassical) monocytes (CD14dim CD16+) [21]. Although the monocyte subtypes are altered in different infectious diseases, there is relatively little information about the consequences of chronic HBV infection, and treatment strategies, on monocyte subtypes. According to a recent study conducted by Dey et al., classical monocytes (CD14+ CD16−) were decreased, while intermediate monocytes (CD14+ CD16+) and patrolling monocytes (CD14dim CD16+) were increased in CHB patients compared to the control group. Additionally, the study found that one year of tenofovir therapy did not restore the functions and populations of monocytes to normal levels [47]. Accordingly, novel or repurposed drugs should also target the normalization of monocytes, where current drugs failed to.

The aryl hydrocarbon receptor (AHR) molecular pathway is able to modulate the function and maturation of immune cells [29], including monocytes [30], and the type of immune response [31]. AHR could be activated by diverse synthetic and natural ligands, of which statins, including rosuvastatin (RSV) may act as specific AHR ligands [34]. In this study, our objective was to examine how RSV affects alterations in monocyte subgroups among CHB patients, with a focus on AHR expression, while also evaluating cytokine levels in the serum of patients treated with either RSV or placebo. Our findings revealed that RSV treatment led to an increase in CD14+ CD16− classical monocytes and a decrease in CD14+ CD16+ intermediate monocytes in CHB patients. However, there was no significant alteration in the proportions of patrolling monocytes following RSV administration. While certain studies have reported conflicting impacts of statins on monocyte subsets in atherosclerosis, no study, as far as we are aware, has highlighted the effects of RSV on monocyte subtypes in CHB patients. While de Carvalho et al. showed higher percentages of classical monocytes and lower patrolling monocytes in statin-treated patients, which was in accordance with our findings [48], Krychtiuk et al. indicated that monocyte subset distribution was skewed toward an increase in CD14+ CD16+ intermediate cells [49]. Kauerova et al. established that the utilization of statin therapy elevated the ratio of anti-inflammatory macrophages, concurrently reducing the ratio of pro-inflammatory macrophages, consistent with our own findings [50]. Since the CD14+ CD16− classical monocytes are mainly involved in phagocytosis and antigen processing, expanding the classical monocytes could be in favor of increasing the antiviral immune response. Eberhardt et al. showed that statins can increase the rate of efferocytosis, by reducing the CD47-mediated "do not eat me" signal [51]. Contrary to the immunomodulatory impacts of statins, certain in vitro research has indicated that statins might induce pro-inflammatory signaling in mononuclear phagocytes. Specifically, the activation of NF-κB and AP-1 through LPS-induced TLR4 activation in macrophages can favor a pro-inflammatory immune response [52,53].

We also showed that RSV intervention may expand the AHR+ cells in all subpopulations of monocytes in CHB patients. Manni et al. demonstrated that RSV can activate AHR in a dose-dependent manner. Moreover, AHR-deficient bone marrow-derived macrophages (BMDMs) expressed lower levels of HMG-CoA as the main target protein of statin [54].

Our findings offered compelling evidence for RSV-mediated activation of AHR signaling in CHB patients. The significant upregulation of Cyp1a1, Cyp1b1, and ARNT mRNA expression strongly suggests RSV triggers robust AHR pathway activation. These results align with previous studies demonstrating RSV's ability to induce Cyp1a1 and Cyp1b1 expression in human endothelial cells [55]. The observed upregulation of ARNT, a critical AHR coactivator, further corroborates the notion of enhanced AHR complex formation and downstream signaling. Our findings within the context of CHB require further exploration to elucidate the interplay between RSV, AHR, and IDO-1 regulation. Future studies employ-

ing larger sample sizes and exploring additional AHR target genes could provide deeper insights into the intricate mechanisms underlying RSV-AHR interactions in CHB patients.

Additionally, our study exhibited that RSV treatment in CHB patients reduced the serum concentrations of pro-inflammatory cytokines IL-6, IFNγ, IL-12, and TNF-α, while elevating the levels of anti-inflammatory cytokines IL-10 and TGF-β. Consistently, Iwata et al. demonstrated that statins might exert anti-inflammatory effects by decreasing cytokine production, aligning with our observations [56]. In line with our findings, Fu et al. revealed that statin-stimulated monocytes may produce TNFα, IL6, and IL1β. Moreover, monocyte-derived macrophages (MDMs), which are prepared in the absence of statins, may lose their capacity for cytokine production and phagocytosis [57].

5. Conclusions

The administration of rosuvastatin led to an increase in CD14+ CD16− classical monocytes and a decrease in CD14+ CD16+ intermediate monocytes among CHB patients, suggesting a favorable antiviral immune response. Additionally, RSV treatment raised the proportions of AHR+ cells across all monocyte subgroups, highlighting RSV as an effective AHR agonist. RSV intervention in CHB patients reduced the serum levels of pro-inflammatory cytokines IL-6, IFNγ, IL-12, and TNF-α, while elevating the levels of anti-inflammatory cytokines IL-10 and TGF-β. Overall, our results position RSV as an immune response modulator in CHB patients through the alteration of monocyte subtypes via AHR. Nonetheless, further investigations are warranted.

Author Contributions: Conceptualization, M.R. and S.M.; methodology, M.R., M.Z.E., A.L. and S.M.; software, M.R.; validation, M.R., M.K. and S.M.; formal analysis, M.R. and S.M.; investigation, M.R. and A.N. (Ali Najafi); resources, A.N. (Alireza Norouzi), S.T. and S.M.; data curation, S.M.; writing—original draft preparation, M.R. and S.M.; writing—review and editing, M.R., M.T. and S.M.; visualization, M.R.; supervision, S.M.; project administration, M.R.; funding acquisition, S.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Department of Research and Technology at Golestan University of Medical Sciences, Gorgan, Iran, grant number 57-112070.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Golestan University of Medical Sciences (protocol code IR.GOUMS.REC.1400.207 and 21-9-2021).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All data can be accessed upon a reasonable request directed to S.M. via s.mohammadi@goums.ac.ir.

Acknowledgments: Grateful acknowledgment is extended to the staff of the Golestan Research Center of Gastroenterology and Hepatology at Sayyad Shirazi Hospital in Gorgan for their assistance during the collection of data and samples. Furthermore, we extend our special gratitude to Jaba Gamrekelashvili from Hannover Medical School for his valuable technical and methodological guidance throughout the project's design and implementation.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Seto, W.-K.; Lo, Y.-R.; Pawlotsky, J.-M.; Yuen, M.-F. Chronic hepatitis B virus infection. *Lancet* **2018**, *392*, 2313–2324. [CrossRef] [PubMed]
2. Mohebbi, A.; Mohammadi, S.; Memarian, A.J.V. Prediction of HBF-0259 interactions with hepatitis B Virus receptors and surface antigen secretory factors. *VirusDisease* **2016**, *27*, 234–241. [CrossRef]
3. Zampino, R.; Boemio, A.; Sagnelli, C.; Alessio, L.; Adinolfi, L.E.; Sagnelli, E.; Coppola, N. Hepatitis B virus burden in developing countries. *World J. Gastroenterol.* **2015**, *21*, 11941. [CrossRef]
4. Bertoletti, A.; Gehring, A.J. The immune response during hepatitis B virus infection. *J. Gen. Virol.* **2006**, *87*, 1439–1449. [CrossRef]
5. Dembek, C.; Protzer, U.; Roggendorf, M. Overcoming immune tolerance in chronic hepatitis B by therapeutic vaccination. *Curr. Opin. Virol.* **2018**, *30*, 58–67. [CrossRef] [PubMed]

6. Li, T.-Y.; Yang, Y.; Zhou, G.; Tu, Z.-K. Immune suppression in chronic hepatitis B infection associated liver disease: A review. *World J. Gastroenterol.* **2019**, *25*, 3527. [CrossRef] [PubMed]
7. Sprengers, D.; Janssen, H. Immunomodulatory therapy for chronic hepatitis B virus infection. *Fundam. Clin. Pharmacol.* **2005**, *19*, 17–26. [CrossRef] [PubMed]
8. Suslov, A.; Wieland, S.; Menne, S. Modulators of innate immunity as novel therapeutics for treatment of chronic hepatitis B. *Curr. Opin. Virol.* **2018**, *30*, 9–17. [CrossRef] [PubMed]
9. Shokoohifar, N.; Ahmady-Asbchin, S.; Besharat, S.; Roudbari, F.; Mohammadi, S.; Amiriani, T.; Khodabakhshi, B.; Norouzi, A.; Shahabinasab, I. The Impaired Balance of CD4+/CD8+ Ratio in Patients with Chronic Hepatitis B. *Hepat. Mon.* **2020**, *20*, e96799. [CrossRef]
10. Kumar, A. Innate immune responses in hepatitis B virus (HBV) infection. *Virol. J.* **2014**, *11*, 22.
11. Xu, X.-W.; Chen, Y.-G. Current therapy with nucleoside/nucleotide analogs for patients with chronic hepatitis B. *Hepatobiliary Pancreat. Dis. Int. HBPD INT* **2006**, *5*, 350–359. [PubMed]
12. Tan, G.; Song, H.; Xu, F.; Cheng, G. When hepatitis B virus meets interferons. *Front. Microbiol.* **2018**, *9*, 1611. [CrossRef]
13. Chen, G.F.; Wang, C.; Lau, G. Treatment of chronic hepatitis B infection-2017. *Liver Int.* **2017**, *37*, 59–66. [CrossRef] [PubMed]
14. Minutti, C.M.; Jackson-Jones, L.H.; García-Fojeda, B.; Knipper, J.A.; Sutherland, T.E.; Logan, N.; Ringqvist, E.; Guillamat-Prats, R.; Ferenbach, D.A.; Artigas, A.J.S. Local amplifiers of IL-4Rα–mediated macrophage activation promote repair in lung and liver. *Science* **2017**, *356*, 1076–1080. [CrossRef]
15. Li, T.; Liang, Y.; Zhang, M.; Liu, F.; Zhang, L.; Yang, B.; Wang, L. Nucleoside/nucleotide analog consolidation therapy in hepatitis B e-antigen positive chronic hepatitis B patients: Three years should be preferred. *Hepatol. Res.* **2021**, *51*, 633–640. [CrossRef]
16. Babania, O.; Mohammadi, S.; Yaghoubi, E.; Sohrabi, A.; Sadat Seyedhosseini, F.; Abdolahi, N.; Yazdani, Y. The expansion of CD14+ CD163+ subpopulation of monocytes and myeloid cells-associated cytokine imbalance; candidate diagnostic biomarkers for celiac disease (CD). *J. Clin. Lab. Anal.* **2021**, *35*, e23984. [CrossRef]
17. Khanam, A.; Chua, J.V.; Kottilil, S. Immunopathology of chronic hepatitis B infection: Role of innate and adaptive immune response in disease progression. *Int. J. Mol. Sci.* **2021**, *22*, 5497. [CrossRef] [PubMed]
18. Riordan, S.M.; Skinner, N.; Kurtovic, J.; Locarnini, S.; Visvanathan, K. Reduced expression of toll-like receptor 2 on peripheral monocytes in patients with chronic hepatitis B. *Clin. Vaccine Immunol.* **2006**, *13*, 972–974. [CrossRef]
19. Li, N.; Li, Q.; Qian, Z.; Zhang, Y.; Chen, M.; Shi, G. Impaired TLR3/IFN-β signaling in monocyte-derived dendritic cells from patients with acute-on-chronic hepatitis B liver failure: Relevance to the severity of liver damage. *Biochem. Biophys. Res. Commun.* **2009**, *390*, 630–635. [CrossRef]
20. Balmasova, I.P.; Yushchuk, N.D.; Mynbaev, O.A.; Alla, N.R.; Malova, E.S.; Shi, Z.; Gao, C.-L. Immunopathogenesis of chronic hepatitis B. *World J. Gastroenterol. WJG* **2014**, *20*, 14156. [CrossRef]
21. Kapellos, T.S.; Bonaguro, L.; Gemünd, I.; Reusch, N.; Saglam, A.; Hinkley, E.R.; Schultze, J.L. Human Monocyte Subsets and Phenotypes in Major Chronic Inflammatory Diseases. *Front. Immunol.* **2019**, *10*, 2035. [CrossRef] [PubMed]
22. Tavakoli, S.; Schwerin, W.; Rohwer, A.; Hoffmann, S.; Weyer, S.; Weth, R.; Meisel, H.; Diepolder, H.; Geissler, M.; Galle, P.R. Phenotype and function of monocyte derived dendritic cells in chronic hepatitis B virus infection. *J. Gen. Virol.* **2004**, *85*, 2829–2836. [CrossRef] [PubMed]
23. Wong, K.L.; Tai, J.J.-Y.; Wong, W.-C.; Han, H.; Sem, X.; Yeap, W.-H.; Kourilsky, P.; Wong, S.-C. Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. *Blood J. Am. Soc. Hematol.* **2011**, *118*, e16–e31. [CrossRef] [PubMed]
24. Patel, A.A.; Zhang, Y.; Fullerton, J.N.; Boelen, L.; Rongvaux, A.; Maini, A.A.; Bigley, V.; Flavell, R.A.; Gilroy, D.W.; Asquith, B. The fate and lifespan of human monocyte subsets in steady state and systemic inflammation. *J. Exp. Med.* **2017**, *214*, 1913–1923. [CrossRef]
25. Tak, T.; Drylewicz, J.; Conemans, L.; de Boer, R.J.; Koenderman, L.; Borghans, J.A.; Tesselaar, K. Circulatory and maturation kinetics of human monocyte subsets in vivo. *Blood J. Am. Soc. Hematol.* **2017**, *130*, 1474–1477. [CrossRef] [PubMed]
26. Tak, T.; Van Groenendael, R.; Pickkers, P.; Koenderman, L. Monocyte subsets are differentially lost from the circulation during acute inflammation induced by human experimental endotoxemia. *J. Innate Immun.* **2017**, *9*, 464–474. [CrossRef] [PubMed]
27. Robinson, A.; Han, C.Z.; Glass, C.K.; Pollard, J.W. Monocyte regulation in homeostasis and malignancy. *Trends Immunol.* **2021**, *42*, 104–119. [CrossRef] [PubMed]
28. Ghasemi, H.; Jamshidi, A.; Ghatee, M.A.; Mazhab-Jafari, K.; Khorasani, M.; Rahmati, M.; Mohammadi, S. PPARγ activation by pioglitazone enhances the anti-proliferative effects of doxorubicin on pro-monocytic THP-1 leukemia cells via inducing apoptosis and G2/M cell cycle arrest. *J. Recept. Signal Transduct.* **2022**, *42*, 429–438. [CrossRef]
29. Mohammadi, S.; Memarian, A.; Sedighi, S.; Behnampour, N.; Yazdani, Y. Immunoregulatory effects of indole-3-carbinol on monocyte-derived macrophages in systemic lupus erythematosus: A crucial role for aryl hydrocarbon receptor. *Autoimmunity* **2018**, *51*, 199–209. [CrossRef]
30. Goudot, C.; Coillard, A.; Villani, A.C.; Gueguen, P.; Cros, A.; Sarkizova, S.; Tang-Huau, T.L.; Bohec, M.; Baulande, S.; Hacohen, N.; et al. Aryl Hydrocarbon Receptor Controls Monocyte Differentiation into Dendritic Cells versus Macrophages. *Immunity* **2017**, *47*, 582–596.e586. [CrossRef]
31. Gutiérrez-Vázquez, C.; Quintana, F.J. Regulation of the immune response by the aryl hydrocarbon receptor. *Immunity* **2018**, *48*, 19–33. [CrossRef] [PubMed]

32. Larigot, L.; Benoit, L.; Koual, M.; Tomkiewicz, C.; Barouki, R.; Coumoul, X. Aryl hydrocarbon receptor and its diverse ligands and functions: An exposome receptor. *Annu. Rev. Pharmacol. Toxicol.* **2022**, *62*, 383–404. [CrossRef] [PubMed]
33. Satny, M.; Hubacek, J.A.; Vrablik, M. Statins and inflammation. *Curr. Atheroscler. Rep.* **2021**, *23*, 80. [CrossRef] [PubMed]
34. Denison, M.S.; Nagy, S.R. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu. Rev. Pharmacol. Toxicol.* **2003**, *43*, 309–334. [CrossRef] [PubMed]
35. Matsuo, S.; Saiki, Y.; Adachi, O.; Kawamoto, S.; Fukushige, S.; Horii, A.; Saiki, Y. Single-dose rosuvastatin ameliorates lung ischemia–reperfusion injury via upregulation of endothelial nitric oxide synthase and inhibition of macrophage infiltration in rats with pulmonary hypertension. *J. Thorac. Cardiovasc. Surg.* **2015**, *149*, 902–909. [CrossRef] [PubMed]
36. Norouzi, A.; Taziki, S.; Najafipasandi, A.; Mohammadi, S.; Roshandel, G. Rosuvastatin Intervention Decreased the Frequencies of the TIM-3+ Population of NK Cells and NKT Cells among Patients with Chronic Hepatitis B. *Iran. J. Immunol.* **2022**, *19*, 255–262. [CrossRef] [PubMed]
37. Mohammadi, S.; Sedighi, S.; Memarian, A.; Yazdani, Y. Overexpression of interferon-γ and indoleamine 2, 3-dioxygenase in systemic lupus erythematosus: Relationship with the disease activity. *LaboratoriumsMedizin* **2017**, *41*, 41–47. [CrossRef]
38. Park, L.M.; Lannigan, J.; Jaimes, M.C. OMIP-069: Forty-color full spectrum flow cytometry panel for deep immunophenotyping of major cell subsets in human peripheral blood. *Cytom. Part A* **2020**, *97*, 1044–1051. [CrossRef]
39. Pouresmaeil, V.; Mashayekhi, S.; Sarafraz Yazdi, M. Investigation of serum level relationship anti-glutamic acid decarboxylase antibody and inflammatory cytokines (IL1-β, IL-6) with vitamins D in type 2 diabetes. *J. Diabetes Metab. Disord.* **2022**, *21*, 181–187. [CrossRef]
40. Mannhalter, C.; Koizar, D.; Mitterbauer, G. Evaluation of RNA isolation methods and reference genes for RT-PCR analyses of rare target RNA. *Clin. Chem. Lab. Med.* **2000**, *38*, 171–177. [CrossRef]
41. Rafat, A.; Asl, K.D.; Mazloumi, Z.; Movassaghpour, A.A.; Talebi, M.; Shanehbandi, D.; Farahzadi, R.; Nejati, B.; Charoudeh, H.N. Telomerase inhibition on acute myeloid leukemia stem cell induced apoptosis with both intrinsic and extrinsic pathways. *Life Sci.* **2022**, *295*, 120402. [CrossRef] [PubMed]
42. Naji, E.; Fadajan, Z.; Afshar, D.; Fazeli, M. Comparison of reverse transcription loop-mediated isothermal amplification method with SYBR green real-time RT-PCR and direct fluorescent antibody test for diagnosis of rabies. *Jpn. J. Infect. Dis.* **2020**, *73*, 19–25. [CrossRef] [PubMed]
43. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *Methods* **2001**, *25*, 402–408. [CrossRef] [PubMed]
44. Akyıldız, M.; Ahıskalı, E.; Zeybel, M.; Yurdaydın, C. Regional epidemiology, burden, and management of hepatitis B virus in the Middle East. *Clin. Liver Dis.* **2019**, *14*, 212. [CrossRef] [PubMed]
45. Raptopoulou, M.; Papatheodoridis, G.; Antoniou, A.; Ketikoglou, J.; Tzourmakliotis, D.; Vasiliadis, T.; Manolaki, N.; Nikolopoulou, G.; Manesis, E.; Pierroutsakos, I. Epidemiology, course and disease burden of chronic hepatitis B virus infection. HEPNET study for chronic hepatitis B: A multicentre Greek study. *J. Viral Hepat.* **2009**, *16*, 195–202. [CrossRef] [PubMed]
46. Bonnardel, J.; Da Silva, C.; Henri, S.; Tamoutounour, S.; Chasson, L.; Montañana-Sanchis, F.; Gorvel, J.-P.; Lelouard, H. Innate and adaptive immune functions of peyer's patch monocyte-derived cells. *Cell Rep.* **2015**, *11*, 770–784. [CrossRef] [PubMed]
47. Dey, D.; Pal, S.; Chakraborty, B.C.; Baidya, A.; Bhadra, S.; Ghosh, R.; Banerjee, S.; Ahammed, S.K.M.; Chowdhury, A.; Datta, S. Multifaceted Defects in Monocytes in Different Phases of Chronic Hepatitis B Virus Infection: Lack of Restoration after Antiviral Therapy. *Microbiol. Spectr.* **2022**, *10*, e0193922. [CrossRef] [PubMed]
48. de Carvalho, D.C.; Fonseca, F.A.H.; Izar, M.C.O.; Silveira, A.; Tuleta, I.D.; do Amaral, J.B.; Neves, L.M.; Bachi, A.L.L.; França, C.N. Monocytes presenting a pro-inflammatory profile persist in patients submitted to a long-term pharmacological treatment after acute myocardial infarction. *Front. Physiol.* **2022**, *13*, 1056466. [CrossRef] [PubMed]
49. Krychtiuk, K.A.; Kastl, S.P.; Hofbauer, S.L.; Wonnerth, A.; Goliasch, G.; Ozsvar-Kozma, M.; Katsaros, K.M.; Maurer, G.; Huber, K.; Dostal, E.; et al. Monocyte subset distribution in patients with stable atherosclerosis and elevated levels of lipoprotein(a). *J. Clin. Lipidol.* **2015**, *9*, 533–541. [CrossRef]
50. Kauerova, S.; Bartuskova, H.; Muffova, B.; Janousek, L.; Fronek, J.; Petras, M.; Poledne, R.; Kralova Lesna, I. Statins Directly Influence the Polarization of Adipose Tissue Macrophages: A Role in Chronic Inflammation. *Biomedicines* **2021**, *9*, 211. [CrossRef]
51. Eberhardt, N.; Giannarelli, C. Statins boost the macrophage eat-me signal to keep atherosclerosis at bay. *Nat. Cardiovasc. Res.* **2022**, *1*, 196–197. [CrossRef]
52. Henriksbo, B.D.; Tamrakar, A.K.; Phulka, J.S.; Barra, N.G.; Schertzer, J.D. Statins activate the NLRP3 inflammasome and impair insulin signaling via p38 and mTOR. *Am. J. Physiol.-Endocrinol. Metab.* **2020**, *319*, E110–E116. [CrossRef]
53. Sheridan, A.; Wheeler-Jones, C.P.D.; Gage, M.C. The Immunomodulatory Effects of Statins on Macrophages. *Immuno* **2022**, *2*, 317–343. [CrossRef]
54. Manni, G.; Gargaro, M.; Turco, A.; Scalisi, G.; Matino, D.; Pirro, M.; Fallarino, F. Statins regulates inflammatory macrophage phenotype through the activation of AhR. *J. Immunol.* **2018**, *200*, 167.19. [CrossRef]
55. Conway, D.E.; Sakurai, Y.; Weiss, D.; Vega, J.D.; Taylor, W.R.; Jo, H.; Eskin, S.G.; Marcus, C.B.; McIntire, L.V. Expression of CYP1A1 and CYP1B1 in human endothelial cells: Regulation by fluid shear stress. *Cardiovasc. Res.* **2009**, *81*, 669–677. [CrossRef] [PubMed]

56. Iwata, A.; Shirai, R.; Ishii, H.; Kushima, H.; Otani, S.; Hashinaga, K.; Umeki, K.; Kishi, K.; Tokimatsu, I.; Hiramatsu, K.; et al. Inhibitory effect of statins on inflammatory cytokine production from human bronchial epithelial cells. *Clin. Exp. Immunol.* **2012**, *168*, 234–240. [CrossRef]
57. Fu, H.; Alabdullah, M.; Großmann, J.; Spieler, F.; Abdosh, R.; Lutz, V.; Kalies, K.; Knöpp, K.; Rieckmann, M.; Koch, S.; et al. The differential statin effect on cytokine production of monocytes or macrophages is mediated by differential geranylgeranylation-dependent Rac1 activation. *Cell Death Dis.* **2019**, *10*, 880. [CrossRef]