Supplementary material

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SECTION 1, S1

Methods

The exclusion criteria were: cortisol co-secretion; history of cancer; reduced glomerular filtration rate <45 ml/min/1.73 m^2; autoimmune diseases; pregnancy, HIV infection, active tuberculosis infection, HBV/HCV infection; oral antibiotic therapy undergone within the last 2 weeks or intravenous antibiotic therapy undergone within the last month; organ transplant; immuno-suppressive treatment.

Smoking status

Ever smoking status was defined as smoking in the past with minimum one year cessation, current smoking status was defined as active smoking.

Ambulatory blood pressure monitoring

In all patients, the ambulatory BP measurements (ABPM) were recorded using a SpaceLabs 90217 device (SpaceLabs, Redmond, WA). Average 24-hour systolic blood pressure (BP), diastolic BP and heart rate were analyzed. The nocturnal decrease in BP was quantified as the relative decrease in nocturnal BP for both systolic and diastolic BP. Subjects were classified as dippers if the proportional decrease from awake to asleep BP was ≥ 10% [1]. Hypertension was defined as day time BP ≥ 135/85 mmHg [2-3].

Evaluation for PA and PA subtyping

All included patients underwent a saline infusion test (intravenous infusion of 2 l of 0.9% saline for 4 hours) in a sitting position. Post-infusion plasma aldosterone level > 10 ng/dl confirmed

a diagnosis of PA [4]. Medication treatment was tailored according to current guidelines, including withdrawing spironolactone, diuretics and other drugs when appropriate before evaluations.

RAAS activity measurements

RAAS activity was assessed using radioimmunoassays according to the manufacturer's instruction. Aldosterone (ACTIVE® Aldosterone RIA DSL8600, Beckman Coulter) was measured in human serum. Renin (RENIN III GENERATION, CisbioBioassays) and angiotensin II (BÜHLMANN) were measured in human plasma.

Echocardiography

Standard transthoracic doppler tissue echocardiography was performed with the GE Vivid 7 using a 2.5-to-3.5 MHz transducer, on the same or next day when ABPM was recorded [5–6] All patients were examined at rest in the left-lateral decubitus position. The left ventricle mass (LVM) was calculated using the modified American Society of Echocardiography cube formula proposed by Devereux et al. and was indexed to body surface area to obtain the LVM index (LVMI) [5]. Left ventricular hypertrophy was defined as a LVMI ≥95 g/m^2 for women and ≥ 115 g/m^2 for men [5]. Left ventricular systolic function was evaluated by left ventricular ejection fraction (using Simpson method). Left ventricular diastolic function was evaluated by mitral inflow velocities.

Carotid ultrasonography

Carotid ultrasonography was conducted by certified sonographer. All examinations were verified by the same physician - with more than five years' experience. Both left and right carotid arteries were examined in the anterolateral and posterolateral directions with a high resolution ultrasound Doppler system (Philips ATL 5000), equipped with a linear probe at 7.5–12 MHz, at the levels of the common carotid artery and carotid bulb from transverse and longitudinal orientations. There were recorded multiple measurements on the distal wall of carotid artery. The region with the thickest intima-media thickness (IMT) was included in our analysis. Maximal IMT was measured at three points (3 mm apart) in two segments, i.e., 1 cm from the flow divider caudally (carotid bulb) and 1 cm from the beginning of the common carotid bulb (common carotid). The IMT value was calculated as an arithmetical mean of 24 measurements from bulb and common carotid segments on both sides.

Monocytes immunophenotype characteristics

Blood samples from patients with NDH (16), HTN (14), PHA (15) were collected in ethylen-ediaminetetraacetic acid (EDTA) tubes. Whole blood was centrifuged to separate plasma, then peripheral blood mononuclear cells (PBMC) were isolated from blood using Lymphocyte Separation Medium 1077 (LSM, PAA Laboratories GmbH, Austria) by means of standard gradient centrifugation. Isolated cells were washed twice with a solution of phosphate – buffered saline (PBS) with 1% heat inactivated fetal bovine serum (FBS) from Gibco (Life Technologies, USA) and suspended in this buffer solution. 5 x 10^5 cells were stained with the following monoclonal antibodies: anti-CD14-PerCP, anti-CD16-APC-H7, anti-HLA-DR-PE-Cy7, anti-CD86-APC, anti-CD192-FITC, anti-CD309-PE. All antibodies were obtained from BD Biosciences (San Jose, CA, USA) — except from antibody CD192 obtained from BioLegend. After 20 minutes of staining in the dark at 4° C, cells were washed with PBS + 1 % FBS and suspended in 200µl of this buffer solution. The cells thus obtained were collected using FACSVerse flow cytometer from BD Biosciences (San Jose, CA, USA). Data were analyzed using Flow Jo v10 (Ashland, OR, USA).

Cells were gated in an SSC (side scatter)/FSC (forward scatter) plot with the scatter gate for monocytes partially extending into lymphocytes. Cells containing all monocytes and a part of the lymphocyte population were then gated in an HLA-DR/CD14 plot to exclude HLA-DR-negative natural killer cells (which would otherwise contaminate the CD14dimCD16+ subpopulation) and finally were analyzed for CD14 and CD16 expression. Monocyte subsets were defined according to the expression of CD16 (Fcγ receptor type III) and CD14 (lipopolysaccharide receptor) as classical monocytes (CD14highCD16-), intermediate monocytes (CD14highCD16+), and nonclassical monocytes (CD14dimCD16+). The expression of HLA-DR, CD14, CD16, CD86, CD192, CD309 on monocyte subsets was quantified. Results are presented as percentages and mean fluorescence intensity (MFI). Fluorescence Minus One (FMO) controls were then used to determine the positivity of evaluated antigens.

SECTION 2 (S2)

Statistical analysis

P-value <0.05 was regarded as statistically significant. All the analyzes were performed with IBM SPSS Statistics software (version 25.0). All the graphs were drawn using GraphPad Prism v5.03 software. Heatmap was generated using ClustVis software [7].

SECTION 3 (S3)

Results and discussion

We observed that patients with PHA and HNT were diagnosed very often with diabetes mellitus type 2 (DM). It is well known that secondary and resistant hypertensives are more likely to suffer from insulin resistance and target organ damage. Hanslik G et al. have also observed a high percentage of patients with metabolic syndrome and DM type 2 in German Conn's Registry [8]. Hu Y and et. have reported that prevalence of PA in the new onset of DM type 2 among hypertensive patients was minimum 19% [9]. In the Japanese study, Wu et al. have reported that almost 30% prevalence of DM type 2 among PHA patients [10]. It might be postulated that aldosterone can contribute to the significant reduction in insulin secretion due to hypokalaemia. Thus, hyperaldosteronism plays a pivotal role in developing insulin resistance. Chronically elevated aldosterone levels are associated with the higher risk of metabolic syndrome development and poor hypertension control [11].

Additionally, patients with difficult to control hypertension, such as resistant hypertension subjects were also characterized by higher prevalence of DM 2 (about 45%) are more prone to develop obstructive sleep apnea and metabolic syndrome. In RESIST-POL registry the percentage of metabolic syndrome among 204 patients with resistant hypertension was about 65%. Moreover, in our study patients diagnosed with HTN presented higher BMI than patients with PHA. We did not observe any correlations between monocytes subsets and aldosterone, angiotensin II level in studied groups (data not shown).

In general, poor blood pressure control and comorbidities occurring in secondary hypertension forms such as obesity, ischemic heart disease, obstructive sleep apnea and DM type 2 promote an inflammatory state and augmented cytokine production creating a vicious circle. The chronic inflammatory state might induce an activation of peripheral blood mononuclear cells. In the current study we observed the higher expression of CD86 and CCR2 in HTN and PHA subjects in comparison to NDH. Interestingly, macrophages treated with high doses of glucose with LPS produced high levels of TNF-α [12]. In experimental models of diabetic nephropathy, the renin – angiotensin II – aldosterone system promoted the adhesion of monocytes and increased production of MCP-1 protein [13].

Our study has several limitations. The size of the study groups in our project is relatively small but other studies determining the role of inflammation in hypertension included similar numbers of patient [14-17]. Moreover, patients with PHA form a unique population of secondary hypertensive subjects. In addition, PHA group is often underdiagnosed. It can be estimated that PHA occurs in 4 – 12 % of all hypertensives and almost 20% of patients with uncontrolled

hypertension. Patients with PHA and HTN with target organ damage are usually older and are characterized with more comorbidities. It is also difficult to match a control group of NDH untreated subjects, who are usually younger and characterized by less comorbidities.

We cannot precisely indicate which monocytes subsets are altered depending on onset of hypertension or hypertension related target organ damage (TOD). TOD develops simultaneously with hypertensive responses. In addition, we cannot exclude the important influence of hypotensive drugs on peripheral blood mononuclear cells phenotype. The main drugs used in the hypertension treatment are ACE, ARB and CCA. It is well-known that ARBs and CCAs can inhibit both the expression of CCL2 and its receptor CCR2 [18]. Dai et al. have shown that AT1R antagonists attenuated the expression of CCL2 and CCR2 in the aorta and peripheral blood monocytes and lowered the serum level of CCL2 [19]. Downregulated CCL2 expression was also observed in previously untreated patients with essential hypertension after treatment with telmisartan [18-19]. The study conducted by Marketou et al. indicated that the administration of the CCA amlodipine did not change the expression of CCL2 by peripheral monocytes of patients with essential hypertension [20]. Animal study in hyperlipidaemic mice model demonstrated that ACEI reduced the recruitment of DCs to the infarct site, leading to a higher survival rate and improved function – lower level of CD86 at splenic DCs [21]. Moreover, ACEI drugs have a potential to inhibit dendritic cells (DC) maturation and maintain their tolerogenic property, which is closely associated with DC anti-atherosclerosis activity in rats. Berezin et al. have found the positive influence of valsartan in escalation doses on bone marrow-derived endothelial progenitor cells such as CD14+ CD309+ and CD14+CD309+Tie2+ in DM type 2 patients with known asymptomatic coronary artery disease [22].

In general, the influence of hypotensive drugs on peripheral mononuclear cells' phenotype is very complex. Most data demonstrating the changes in inflammatory state come from atherosclerotic animal model and humans with coronary artery disease. In the present study, we observed the differences in CD86, CD192 and CD309 expression in monocytes despite the intensive pharmacotherapy in our patients.

Table S1. Clinical Characteristics of patients with newly diagnosed hypertension (NDH), established hypertension (HTN) and primary hyperaldosteronism (PHA)

Clinical features	**NDH (n=16)**	**HTN (n=14)**	**PHA (n=15)**	***P*–value**
Male sex, n (%)	14 (87.5)	10 (71.4)	9 (60)	0.22
Age, years, median (IQR)	55.5 (53–59.5)	56.5 (48–62)	59 (56–64)	0.20
BMI, kg/ m^2, mean (SD)	28.8 (3.7)	31.5 (5.5)	31.9 (5.6)	0.17
Blood pressure characteristics				
24 – h ABPM (systolic), mmHg, mean (SD)	145.3 (13.5)	146.4 (19.4)	145.9 (14.9)	0.98
24 – h ABPM (diastolic), mmHg, mean (SD)	90.9 (10.1)	83.7 (15.0)	83.9 (8.6)	0.15
Aldosterone, pg/ml, mean (SD)	142.6 (78.8)	164.6 (95.9)	345.3 (227.7)	0.001
Angiotensin II, pg/ml, median (IQR)	6.14 (3.85–8.73)	3.26 (2.32–5.76)	2.18 (1.17–3.72)	0.002
Aldosterone - renin ratio (ARR), median (IQR)	9.25 (7.7–14.8)	10.2 (5.48–20.1)	56.9 (40–131)	<0.001
Renal function characteristics				
GFR, ml/min per 1.73 m,^{2} median (IQR)	88.35 (70.33–90)	120.86 (97.8–151.2)	86.9 (81.4–90)	0.001
Albuminuria in daily urine sample, mg/24h, median (IQR)	12.85 (11.15–24.82)	18.97 (6.25–67.72)	25.3 (16.3–46.3)	0.04
Cardiovascular risk characteristics				
Current smoking, n (%)	0 (0%)	3 (21.4%)	2 (13.3%)	0.17
Ex smoking, n (%)	5 (31.3%)	4 (28.6%)	7 (46.7%)	0.54
Total cholesterol, mmol/l, mean (SD)	5.5 (1.0)	4.7 (1.4)	4.5 (1.0)	0.05
LDL – cholesterol, mmol/l,	3.5 (1.2)	2.7 (1.3)	2.5 (0.8)	0.03

mean (SD)				
Diabetes mellitus type 2, n (%)	1 (6.3)	6 (42.9)	8 (53.3)	0.01
Inflammatory parameters				
hsCRP, mg/dl, median (IQR)	0.23 (0.13–0.45)	0.14 (0.09–0.29)	0.22 (0.08–0.63)	0.66
WBC, cell/µl, mean (SD)	6.2 (1.2)	6.7 (2.0)	6.8 (2.0)	0.63
Atherosclerosis and CAD				
Prior myocardial infarction, n (%)	0	3 (21.4)	1 (6.7)	0.112
Ischemic heart disease, n (%)	0	5 (35.7)	5 (33.3)	0.03
Stroke, n (%)	0	0	1 (6.7)	0.36
Medications				
ACE inhibitors, n (%)	0	6 (42.8)	8 (53.3)	0.014
Angiotensin receptor blockers, n (%)	0	8 (57.1)	3 (20)	0.004
B-blockers, n (%)	0	14 (100)	12 (80)	<0.001
Calcium channel blockers, n (%)	0	12 (85.7)	14 (93.3)	<0.001
Alpha – blockers, n (%)	0	6 (42.8)	8 (53.3)	0.003
Diuretics, n (%)	**0**	**14 (100)**	**9 (60)**	**<0.001**
Statins, n (%)	**0**	**9 (64.3)**	**8 (53.3)**	**<0.001**

Continuous variables are shown as mean (SD) or median (IQR). Whereas categorical variables are shown as n (%). All values except medication are shown as mean. ABPM indicates ambulatory blood pressure

Abbreviations: ACE, angiotensin-converting enzyme; BMI, body mass index; GFR, glomerular filtration rate; hsCRP, high sensitivity C-reactive protein; HTN, established hypertension; IHD, ischemic heart disease; NDH, newly diagnosed hypertension; NS, non-significant; PHA, primary hyperaldosteronism; WBC, white blood cell

Table S2. Echocardiographic and Carotid Ultrasonography Characteristics in patients with newly diagnosed hypertension (NDH), established hypertension (HTN) and primary hyperaldosteronism (PHA)

Echocardiography characteristics	**NDH (n = 16)**	**HTN (n = 14)**	**PHA (n = 15)**	***P*–value**
LVEF, %, mean (SD)	66.6 (6.1)	66.3 (5.4)	65.8 (6.4)	0.75
IVSD, mm, mean (SD)	12.1 (1.4)	12.5 (2.6)	12.5 (2.9)	0.57
PWD, mm, mean (SD)	11.1 (1.5)	12.7 (2.7)	12.8 (2.1)	0.05
LVMI, g/ m^2, mean (SD**)**	102.5 (34.4)	137.8(43.9)	130.8 (30.6)	0.02
LAVI, ml/ m^2 , mean (SD)	30.9 (7.0)	37.5 (14.6)	41.8 (14.0)	0.15
GLS, mean (SD)	18.4 (2.5)	16.9 (3.7)	18.2 (2.9)	0.37
Carotid ultrasonography characteristics				
Mean carotid IMT, cm, mean (SD)	0.067 (0.009)	0.076 (0.012)	0.083 (0.015)	0.01

Abbreviations: GLS, global longitudinal strain; HTN, established hypertension; IMT, intima media thickness; IVSD, intraventricle septum diameter; LVEF, left ventricular ejection fraction; LVMI, left ventricular mass index; NDH, newly diagnosed hypertension; PHA, primary hyperaldosteronism; PWD, posterior wall diameter

Table S3. Table showing the percentage (%) and MFI (mean fluorescence intensity) of selected markers in monocytes in patients with NDH — newly diagnosed, untreated hypertension, HTN — established hypertension and PHA — primary hyperaldosteronism

Monocytes	**NDH**	**HTN**	**PHA**	***P*-value**
CD86+ (%), median (IQR)	85.2 (60.8–95.4)	98.8 (85.5–99.5)	96.7 (77.4–97.4)	0.07
CD86+ (MFI), median (IQR)	490.5 (404.3–581)	795 (496–935)	653 (457–916)	0.08
CD192+ (%), median (IQR)	86 (77.5– 89.8)	94 (90.5–94.5)	93.4 (90.2–95.1)	0.02
CD192+ (MFI), median (IQR)	508 (411–602)	1034 (650–1282)	999 (588–1325)	0.003
CD309+ (%), median (IQR)	0.6 (0.5–1)	1.25 (0.4–2.4)	0.84 (0.42–1.4)	0.58

The FDR (false discovery rate) correction was applied when testing differences in all cell characteristics between NDH, RHT and PHA subpopulations, by Kruskal–Wallis test. All values are shown as median (IQR)

Table S4. Multiple regression estimates of the HTN and PHA vs. NDH effect on selected monocytes characteristics. Estimates were derived from linear regression adjusted for sex, BMI, age and current smoking status. MFI (Mean fluorescence intensity)

Cell type	HTN			PHA		
	B	**SE**	***P*-value**	**B**	**SE**	***P*-value**
Total monocytes, CD192+ (%)	8.6	3.03	0.007	6.9	2.9	0.02
Total monocytes, CD192+ (MFI)	532.2	123.8	<0.001	405.7	120.3	0.002
CD14highCD16-CD192+ (%)	3.3	1.1	0.003	2.8	1.04	0.01
CD14highCD16-CD192+ (MFI)	554.5	128.7	<0.001	420.4	125	0.002
CD14highCD16+CD192+ (MFI)	307.3	77.2	<0.001	201.8	75	0.01
CD14dimCD16+CD86+ (MFI)	607.4	203.7	0.005	462	198	0.03
CD14dimCD16+CD309+ (%)	0.9	0.8	0.237	0.7	0.7	0.36

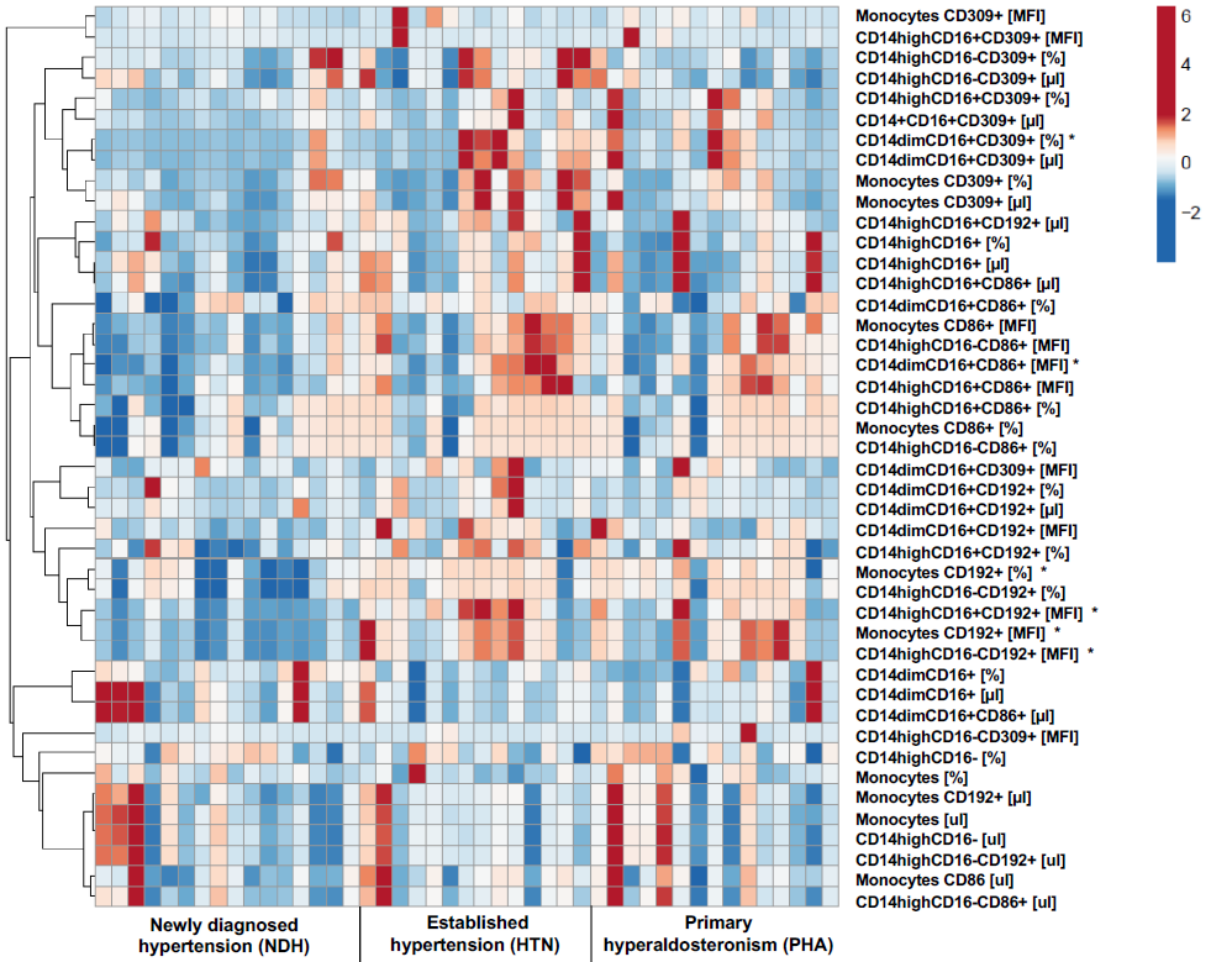

Figure S1. Heatmap presenting the percentage of monocytes and their subsets and MFI (mean fluorescence intensity) of cellular markers in 16 patients with newly diagnosed, untreated hypertension (NDH), 14 patients with established hypertension (HTN), 15 patients with primary hyperaldosteronism (PHA). Heatmap was generated using ClustVis software. The FDR *(false discovery rate)* correction was applied when testing differences in all cell characteristics between NDH, HTN and PHA subpopulations. FDR adjusted *$P < 0.05$ by Kruskal–Wallis test

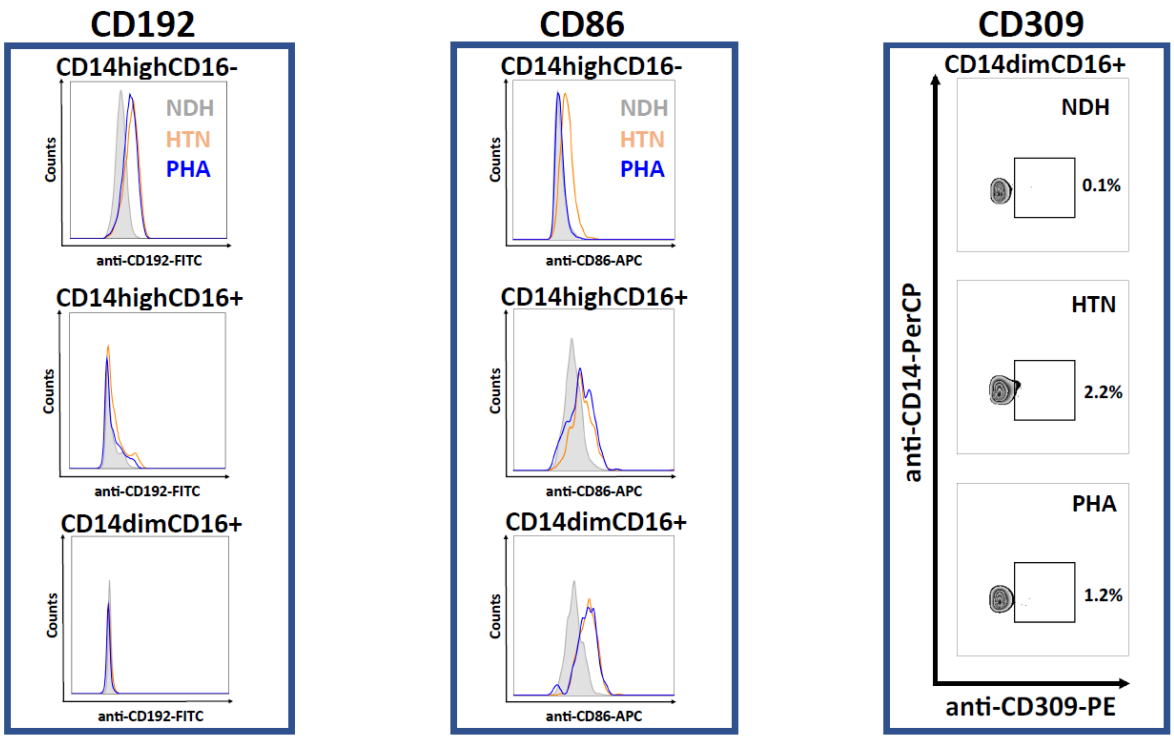

Figure S2. Flow cytometric examples of selected markers in monocyte subsets in patients with hypertension. Representative flow cytometric examples of CD192, CD86 and CD309 expression in monocyte subsets including: CD14highCD16-, CD14highCD16+ and CD14dimCD16+ cells are shown in patients with NDH, HTN and PHA

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