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Hemoglobin induces monocyte recruitment and CD163-macrophage polarization in abdominal aortic aneurysm☆

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ABSTRACT

Background: Increased hemoglobin (Hb) accumulation was reported in abdominal aortic aneurysms (AAAs). CD163 is a macrophage receptor involved in tissue Hb clearance, however its role in AAA has not been reported. We investigated the role of Hb on monocyte recruitment and differentiation towards CD163 expressing macrophages *ex vivo*, *in vitro* and in human AAA.

Methods and results: CD163 mRNA and protein expression was significantly higher in human AAA (n = 7) vs. healthy wall (n = 6). CD163 was predominantly found in adventitia of AAA, coinciding with areas rich in hemosiderin and adjacent to neoangiogenic microvessels. Dual CD14/CD163 expression was observed in recently infiltrated monocytes surrounding microvessels. A higher release of soluble CD163 was observed in the conditioned medium from AAA (AAA-CM, n = 10), mainly in the adventitial layer. Similar to Hb, AAA-CM induced CD163-dependent monocyte chemotaxis, especially on circulating monocytes from AAA patients. Hb or AAA-CM promoted differentiation towards $CD163^{high}$/HLA-DR^{low}-expressing macrophages, with enhanced Hb uptake, increased anti-inflammatory IL-10 secretion and decreased pro-inflammatory IL-12p40 release. All these effects were partially suppressed when Hb was removed from AAA-CM. Separate analysis on circulating monocytes reported increased percentage of pre-infiltrating $CD14^{++}CD16^{+}$ monocytes in patients with AAA (n = 21), as compared to controls (n = 14). A significant increase in CD163 expression in $CD14^{++}CD16^{+}$ monocyte subpopulation was observed in AAA patients.

Conclusions: The presence of Hb in the adventitial AAA-wall promotes the migration and differentiation of activated circulating monocytes in AAA patients, explaining the existence of a protective CD163-macrophage phenotype that could take up the Hb present in the AAA-wall, avoiding its injurious effects.

1. Introduction

In abdominal aortic aneurysms (AAAs), arterial wall biological injuries leading dilatation and finally rupture are mainly represented by proteolysis and oxidation [1]. In this pathophysiological context, phagocytosis and endocytosis play an important role in the tissue clearance of products released by intraluminal thrombus (ILT) and outwardly convected towards adventitia [2]. In AAA, monocytes are recruited in the ILT from the aortic circulating blood and in adventitia from neoangiogenic microvessels [3,4], where they differentiate into tissue macrophages and assume, at least in part, the clearance of numerous released particles and molecules [5,6]. Although several molecules secreted by the adventitia have been proposed to be involved in monocyte recruitment to the adventitial layer in experimental models [7], mechanisms that drive their recruitment into the aneurysmal wall are still not well defined. AAAs are characterized by the presence of erythrocytes in ILT, where hemoglobin (Hb) and heme-iron are released from red blood cells and then outwardly convected towards the adventitia of AAA wall [2,8]. Thus, increased presence of Hb-derived iron was observed in adventitia of AAA patients [9]. Buttari et al. have recently reported that Hb triggers chemotaxis of human monocytes within

☆ All authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

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atherosclerotic plaque; however whether Hb may be involved in monocyte recruitment in AAA is not known [10].

Extracellular- and intracellular-free Hb is a strong oxidant catalyst that could lead to cell damage in the arterial wall [11,12]. To restrain these harmful effects, Hb is cleared by CD163, a scavenger receptor present on the surface of circulating monocytes and tissue macrophages [13]. A soluble form of CD163 (sCD163) has been also reported in plasma and cell culture after membrane proteolytic shedding under oxidant and pro-inflammatory conditions [14–16]. CD163-mediated Hb uptake not only decreases Hb concentration but also induces anti-inflammatory IL-10 release and heme oxygenase-1 (HO-1) synthesis [17], leading to a novel protective macrophage phenotype, as reported in hemorrhagic atherosclerotic plaques [18]. Under oxidant conditions, intracellular Hb dissociates into heme and globin. HO-1 transforms heme to biliverdin, a reaction that produces carbon monoxide and iron, which is subsequently stored in ferritin [19]. Therefore, the CD163/HO-1/ferritin system decreases cellular exposure to Hb and heme-derived products [19].

Monocytes and macrophages are heterogeneous for phenotype and function and different subsets are found in response to local microenvironment [20]. The majority of monocytes are $CD14^{++}CD16^{-}$ and are thought to represent classical monocytes that mediate inflammatory responses. A minor subpopulation of monocytes also expresses the cell surface activation marker CD16, identifying them as more mature than the $CD14^{++}CD16^{-}$ subpopulation. $CD16^{+}$ monocytes are thought to be the precursors of tissue-resident macrophages [21–25]. The $CD16^{+}$ subset of monocytes increases significantly in chronic phagocytic conditions, such as atherosclerosis and AAA [26,27]. $CD16^{+}$ consist of two subsets with distinct phenotypic and functional properties, namely, $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{+}$ cells. These subsets present with different CD163 levels [28–34]. CD163 is an important marker of monocyte activity in inflammatory diseases, including atherosclerosis, asthma, and acquired immune deficiency syndrome [32,34]. It has been suggested that mature $CD14^{+}CD16^{+}CD163^{+}$ blood monocytes may be potential precursors of infiltrating macrophages in resident tissues [33]. However, there are no data available on the role and expression of CD163 on circulating monocyte subsets in AAA patients.

In the present study we have examined whether Hb from human AAA samples could impact on monocyte migration and differentiation towards a protective CD163 macrophage phenotype. In addition, we have determined CD163 expression in circulating monocyte subsets from AAA patients and healthy controls.

2. Material and methods

2.1. Patients

2.1.1. Spanish population

Peripheral blood samples were obtained from 21 AAA-patients during surgical repair at IIS-Fundacion Jimenez Diaz hospital and 14 healthy donors. Blood was sampled on EDTA-coated tubes. Clinical characteristics of the two study groups are shown in Table 1. No significant differences for hypertension, hyperlipidemia, diabetes, cardiopathy, CKD and smoking habits were observed between healthy subjects and AAA patients. Healthy subjects showed higher blood Hb concentration than AAA patients [9]. All patients provided written informed consent prior to inclusion in the study. The study was approved by the Autonoma University Ethics Committee, and the investigation was conducted according to the principles outlined in the Declaration of Helsinki.

Table 1
Clinical characteristics.

	Controls (14)	AAA patients (21)	p
Age (years)	68.8 ± 3.5	75.1 ± 1.6	0.08
Gender (males), n (%)	10 (71.4)	19 (90.5)	0.14
Glucose (mg/dl)	114.6 ± 13.0	120.9 ± 7.0	0.65
Cholesterol (mg/dl)	169.3 ± 10.2	165.6 ± 10.8	0.81
Triglycerides (mg/dl)	116.6 ± 19.2	139.6 ± 23.8	0.68
eGFR (mg/dl)	76.2 ± 5.9	65.2 ± 4.2	0.15
Hemoglobin (g/dl)	14.7 ± 0.5	11.8 ± 0.6	**0.006**
Diabetes, n (%)	2 (14.3)	4 (19.0)	0.71
Hypertension, n (%)	8 (57.1)	13 (61.9)	0.78
Hyperlipidemia, n (%)	5 (35.7)	10 (47.6)	0.48
Cardiopathy, n (%)	7 (50.0)	7 (33.33)	0.32
COPD, n (%)	1 (7.1)	4 (19.0)	0.32
CKD, n (%)	2 (14.3)	8 (38.1)	0.13
Smoking, n (%)	8 (57.1)	16 (76.2)	0.23
Statins, n (%)	4 (28.6)	12 (57.1)	0.10

Entries in bold are statistically significant ($p < 0.05$) as compared to controls.

2.1.2. The Viborg Vascular (VIVA) screening trial

In an ongoing randomized population-based screening program for AAA, peripheral arterial disease (PAD), and hypertension in more than 50,000 men 65–74 years of age in the mid-region of Denmark [35], baseline plasma samples were obtained consecutively at diagnosis of 365 AAA patients and 164 healthy age-matched controls without AAA or PAD. AAA was defined as having maximal aortic diameter greater than 30 mm, and PAD was defined as an ankle-brachial index (ABI) lower than 0.90. Smoking status, coexisting diabetes mellitus, hypertension, medication, body-mass index (BMI) and systolic and diastolic blood pressure were also measured and recorded. Ankle systolic blood pressure also was measured as previously validated and reported [36], and a maximal anterior–posterior diameter of the infrarenal aorta was measured in the peak of the systole from the inner edge to inner edge of aorta. The interobserver variation of aortic diameter measurements was 1.52 mm [37]. Blood samples were centrifuged at 3000 ×*g* for 12 min, supernatants were removed and then stored at −80 °C until sCD163 analysis (R&D system 1630 DC) was performed. Written informed consent was obtained from all subjects before participation, and the study was approved by the Local Ethics Committee of the Viborg Hospital, Denmark, and performed in accordance with the Helsinki Declaration.

2.2. Cell culture

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats from healthy donors over a Lymphoprep (Nycomed Pharma, Oslo, Norway) gradient, according to standard procedures. Monocytes were purified from PBMCs by magnetic cell sorting using CD14 microbeads (Miltenyi Biotec, Auburn, CA, USA). Monocytes were cultured at 0.5×10^6 cells/ml for 7 days in RPMI 1640, supplemented with 10% FCS at 37 °C in a humidified atmosphere with 5% CO_2, containing 1000 U/ml GM-CSF or M-CSF (10 ng/ml; ImmunoTools GmbH, Friesoythe, Germany) to generate M1 and M2 monocyte-derived macrophages, respectively. Cytokines were added every 2 days. Human Hb (A_0, Sigma) and conditioned medium from adventitial human AAA (AAA-CM) or healthy aorta wall (HA-CM) were added together with cytokines. Potential endotoxin contamination in Hb was removed using polymyxin B-pre-packed columns (Detoxi Gel, Pierce, Rockford, USA) according to manufacturer's recommendation. Endotoxin contamination in eluted Hb was determined by the quantitative chromogenic limulus amebocyte lysate assay (Thermo Scientific), resulting in less than 0.03 EU/ml protein.

2.3. Flow cytometry

Fresh whole blood samples from healthy subjects and AAA patients or macrophages from cell culture experiments were incubated for 30 min at room temperature with 5 μl of the following mAb for flow cytometry analysis of surface molecules: CD14-APC, CD16-PerCP, CD209-APC, CD163-PE, human leukocyte antigen-DR (HLA-DR)-FITC (BD Biosciences). Blood was collected into EDTA-containing tubes for flow cytometry analysis since the use of both heparin and acid citrate dextran anticoagulant tubes may alter CD163 and CD16 protein expression [38,39]. Red blood cells were lysed for 15 min with FACS Lysing Solution (BD Biosciences). The remaining white blood cells were washed twice with phosphate-buffered saline. FITC/PE/APC-labeled, isotype-matched irrelevant antibodies were included as negative controls. Flow cytometry analysis was performed with the use of a FACSCanto II cytometer with FACSDiva software (BD Biosciences) or Infinicyt software, version 1.5 (Cytognos).

2.4. Human AAA tissues

AAA wall samples were collected during surgical repair and dissected into thrombus (luminal layer, at the interface with circulating blood, and abluminal layer), and wall (media and adventitia). AAA samples were obtained from patients undergoing surgery, enrolled in the RESAA protocol [40]. Control abdominal aortas were sampled from deceased organ donors with the authorization of the French Biomedicine Agency (PFS 09-007). These control aortic samples were macroscopically normal and devoid of lesions. Conditioned medium was obtained from incubation of small pieces (~5 mm^3) of healthy aortas and thrombus (luminal and abluminal)/wall (media and adventitia) layers from AAA in RPMI 1640 medium without FBS for 24 h at 37 °C (6 ml/g of wet tissue), as previously described [15]. Conditioned medium from healthy aortas or AAA samples were treated with HemogloBind™ reagent (Biotech Support Group LLC) according to the manufacturer's protocol for hemoglobin depletion. HemogloBind™ is a polyelectrolyte mixture designed to selectively bind hemoglobin from biological fluids.

2.5. Immunohistochemical analysis

AAA and control aortic tissue samples were fixed in 3.7% paraformaldehyde for 24 h and later in ethanol until paraffin embedded. Tissues were cross-sectioned into 5-μm-thick pieces, dewaxed and rehydrated, and incubated overnight with mouse anti-human CD68 (1:100 dilution; Dako), mouse anti-human CD163 (1:200 dilution; EDhu-1, Serotec),

mouse anti-human Glycophorin C (1:100; Dako), rabbit anti-human HO-1 (1:200 dilution; Assay Designs), or mouse anti-human ki-67 (1:100 dilution; Dako). Avidin/biotin blocking kit (Vector Laboratories) was used to inhibit endogenous avidin/biotin. Negative controls using the corresponding IgG were included to check for non-specific staining. Biotinylated secondary antibodies were applied for 1 h. Then avidin–biotin peroxidase complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA) was added for 30 min. Sections were stained with 3,3′-diaminobenzidine or 3-amino-9-ethyl carbazol (DAKO, Glostrup, Denmark) and counterstained with hematoxylin.

2.6. Multicolor confocal microscopy

The following antibodies were used: FITC-labeled anti-CD163 (Ber-Mac3, MBL International), anti-CD14 (BD) and anti-HLA-DR (DAKO). Thick sections of cryopreserved tissue were fixed with acetone and blocked with 1% human immunoglobulin, and then incubated with primary antibodies (1–5 μg/ml). Imaging was performed with an inverted confocal microscope (SP2, Leica Microsystems), using the glycerol immersion 20× PL-APO NA 0.7 and the 63× PL-APO NA 1.3 glycerol immersion objectives.

2.7. ELISA

Supernatants from macrophages and AAA conditioned mediums were tested for the presence of IL-10 or IL-12p40 using commercially available ELISA (BioLegend and BD, respectively), following the protocols supplied by the manufacturers. Concentrations of sCD163 were determined in AAA conditioned medium in duplicate with commercially available enzyme-linked immunosorbent assay kits (CD-1630 R&D). The presence of heme in conditioned medium was assessed by a chromogenic assay as described previously [41].

2.8. Quantification of Hb uptake by macrophages

Hb was labeled with the Alexa-555 protein labeling kit (Invitrogen) following the instructions of the manufacturer. All uptake assays were performed in cell culture medium without serum. Macrophages were then harvested and washed three times with phosphate-buffered saline. Uptake of the fluorescent ligand was then quantified by flow cytometry, as previously described [15].

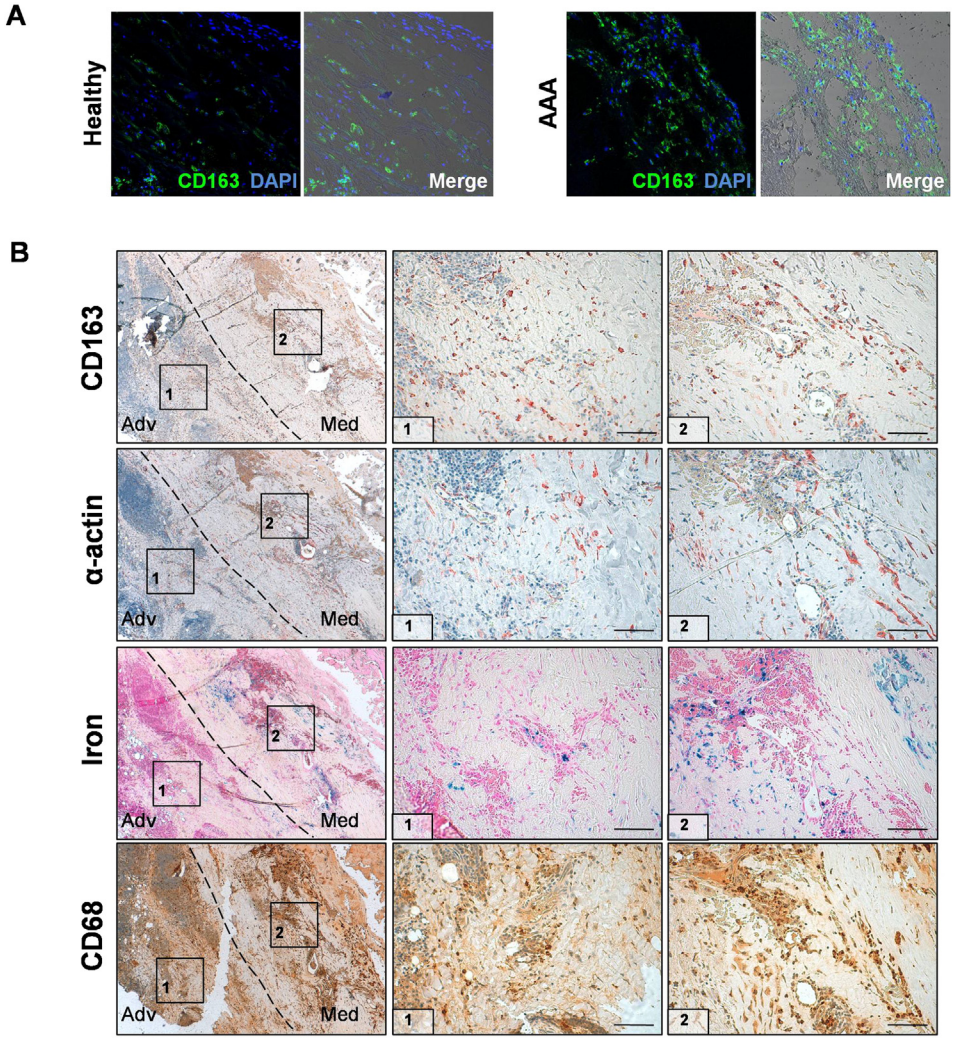


Fig. 1. CD163 is expressed in human abdominal aortic aneurysm (AAA). (A) Representative multicolor confocal microscopy images showing CD163 expression (green) in healthy aorta (left panel) and AAA (right panel). Nuclei were stained with DAPI (blue). (B) Representative serial micrographs showing different distribution of CD163, phagocytic cells (CD68), smooth muscle cells (alpha actin) and iron (Blue Perl's) in human AAA-wall. Left panel shows low-magnification images of AAA-wall (media and adventitia). The rectangles show the region of interest for which immunostaining are shown. Central- and right-panels show higher magnification of zones 1 (adventitia) and 2 (media) Scale bar, 100 μm. (C) Representative serial micrographs in AAA-wall showing CD163 staining in iron-rich areas, mainly localized in the vicinity of neocapillaries and within phagocytic cells expressing HO-1. Scale bar, 50 μm. (D) Representative confocal microscopy images showing co-localization of CD163 (green) in CD68-phagocytes (red, left panel) and HO-1 (red, right panel) expressing cells within adventitia. Nuclei were stained with DAPI (blue). Orange arrows indicate the dual presence of CD68 or HO-1 in CD163 macrophages. (E) Representative confocal microscopy images showing co-localization of CD14 (red) and CD163 (green) macrophages within adventitia. Nuclei were stained with DAPI (blue). Microvessels (v) were marked as a rounded and white circle that contained erythrocytes in vessel lumen. Orange arrows indicate the dual presence of CD14 and CD163 in macrophages, whereas green arrows show CD163 positive macrophages with no CD14 staining. (F) Representative serial micrographs showing CD163 positive cells and the proliferation marker (Ki-67) in human AAA-wall. Orange arrows indicate the presence of proliferating cells, whereas white arrows show CD163 positive macrophages. Scale bar, 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

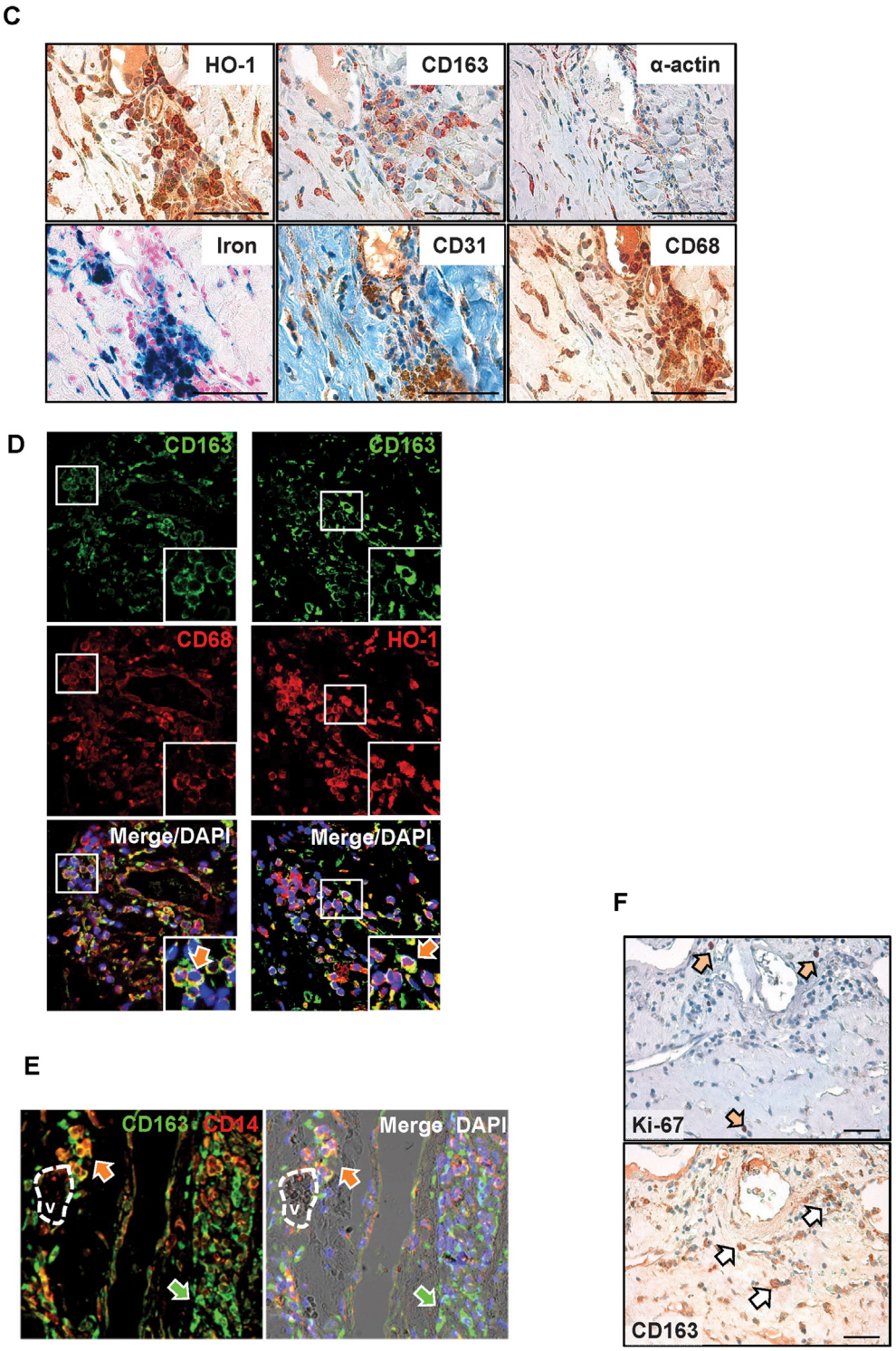


Fig. 1 (*continued*).

2.9. RNA extraction and real-time PCR

Human AAA wall tissues were snap frozen in N_2 liquid and homogenates (0.2 g) were divided and resuspended for mRNA analysis. Total RNA from AAA (media + adventitia) or healthy tissues was obtained by TRIzol method (Life Technologies) and quantified by absorbance at 260 nm in duplicate. Real-time PCR was performed on a TaqMan ABI 7700 Sequence Detection System using heat-activated TaqDNA polymerase (Amplitaq Gold). After an initial hold of 2 min at 50 °C and 10 min at 95 °C, the samples were cycled 40 times at 95 °C for 15 s and 60 °C for 60 s. 18S rRNA served as housekeeping gene and was amplified in parallel with the genes of interest. The expression of target genes was normalized to housekeeping transcripts. All TaqMan probes were purchased from Applied Biosystems and optimized according to the manufacturer's protocol. All measurements were performed in duplicate. The amount of target mRNA in samples was estimated by the 2ΔCT relative quantification method.

2.10. Cell migration experiments

Chemotaxis assays were performed using the transwell system (pore size 5 μM, Millipore) as previously reported [42] with some modifications. Freshly isolated

monocytes (2.5×10^5 cells) were pretreated or not with anti-CD163 monoclonal antibody (Ab Serotec) for 1 h in RPMI at 37 °C to block this receptor in 24-well microchamber. Monocytes were allowed to migrate in the presence of Hb (5–500 μg/ml), healthy aorta conditioned medium (HA-CM), AAA-CM (2.5% v/v), hemoglobin depleted AAA-CM or HA-CM (2.5% v/v) for 2 h. CCL2 (500 ng/ml) served as the positive control. After migration, cells in the lower chamber were collected and the total number of migrated cells was calculated by flow cytometry (120 second counts). All samples were run in duplicate and averaged. Results are expressed as percent increase in migration relative to migration towards the control medium.

2.11. Statistical analysis

Statistical analysis was performed using SPSS 11.0 statistical software. Significance at the $p < 0.05$ level was assessed by Student's t-test for two groups of data and ANOVA for three or more groups for normal variables. When statistical significance was found, Tukey's post hoc comparison test was used to identify group differences. Mann–Whitney test was used to compare results between two groups of non-parametric variables. Pearson or Spearman rank correlation test were used to determine correlations between two variables. Dichotomous variables were expressed as proportions and compared by the chi-square test. Regression analysis was performed to describe the relationships between the percentage of the different monocyte subsets, age, glucose levels and CKD presence in AAA patients and healthy controls. In vitro experiments were replicated three times for each incubation period. Results are expressed as mean ± SD. Statistical significance was accepted for $p < 0.05$.

3. Results

3.1. CD163 is expressed in human AAA

In order to determine CD163 gene expression in human AAA, mRNA was extracted from AAA-wall (media + adventitia) ($n = 7$) and healthy aorta wall ($n = 6$). Real-time PCR showed that CD163 gene expression was increased in human AAA-wall as compared with healthy aorta (7.41 ± 0.53 vs. 1.00 ± 0.77, $p < 0.05$, respectively). These results were confirmed by immunohistochemistry, where increased CD163-positive cells were observed in AAA-wall as compared to healthy aortic wall (Fig. 1A). In serial AAA sections, CD163 expression was mainly found surrounding areas with Hb-derived iron deposits (Perl's blue staining) and associated with phagocytes (CD68) in the close vicinity of microvessels (CD31) in the outer media or full adventitia (Fig. 1B–C). HO-1 staining was also associated with adventitial $CD163^+$ positive cells and iron-rich areas, indicating enhanced heme-catabolism in response to uptake of large amounts of Hb by phagocytes (Fig. 1B–C). In this line, dual confocal immunofluorescence confirmed that CD163 positive cells were CD68-phagocytes that expressed HO-1 simultaneously (Fig. 1D). Furthermore, increased HO-1 and ferritin mRNA expression was observed in AAA-wall as compared to healthy aorta (HO-1: 6.37 ± 4.97 vs. 1.00 ± 1.24, and ferritin: 5.67 ± 3.48 vs. 1.00 ± 0.84, $p < 0.05$, respectively). In addition to monocyte-derived macrophages, stromal cells might be also involved in heme-uptake since iron deposits and HO-1 expression were observed in vascular smooth cells from the media and adventitial fibroblasts (Fig. 1B–C). Furthermore, we observed dual CD14 and CD163 expression in recently infiltrated and shape-rounded monocytes surrounding microvessels (Fig. 1E); however low CD14 staining was detected in CD163 positive phagocytes situated more distant to microvessels. Immunostaining for proliferation marker Ki-67 reported that a low number of CD163 positive cells were proliferating in areas surrounding microvessels, suggesting that the origin of adventitial CD163 macrophages was mainly due to recruited monocytes, although *in situ* proliferation cannot be excluded (Fig. 1F). In contrast to adventitia, a lower presence of CD163 was shown in the intraluminal thrombus, a tissue characterized by massive erythrocyte accumulation (Glycophorin C) and neutrophil retention (CD15) (Supplemental Fig. 1).

3.2. CD163 is released in human AAA

We then assessed Hb and sCD163 levels in the conditioned medium from total AAA-wall ($n = 10$) and healthy aortic walls ($n = 6$). As expected, Hb and sCD163 were released in a higher extent in AAA wall as compared to healthy aorta ($p < 0.05$) (Fig. 2A–B). In a further step, we analyzed Hb and sCD163 levels released by the layers of AAA. As expected, increased Hb levels were observed in the conditioned medium from luminal thrombus and adventitial layers (Fig. 2D). It is noteworthy that tissue-conditioned media from both adventitia and media layers released higher levels of sCD163 than the intraluminal thrombus (luminal or abluminal layer) ($p < 0.05$) (Fig. 2C). Interestingly, a positive association was observed between the amount of Hb and sCD163 concentrations in the conditioned medium from AAA ($r = 0.486$; $p < 0.001$, Fig. 2E).

IL-10 is a cytokine secreted by CD163-expressing macrophages and has been involved in macrophage polarization [18], therefore we determined the levels of this cytokine in the conditioned medium from human AAA tissues (Fig. 2F). Conditioned medium from media- and adventitia-AAA contained higher levels of IL-10 than healthy walls. In relation to the different AAA layers, we found that adventitia and luminal layer secreted more IL-10. Finally, we observed a direct relationship between IL-10 and sCD163 release in the conditioned medium, corroborating the association between these two molecules in AAA (Fig. 2G).

3.3. Conditioned medium obtained from human adventitial-AAA layer promotes CD163 expression, increases Hb clearance and IL-10 secretion in vitro

A previous study reported the presence of a novel anti-oxidant macrophage phenotype (HA-mac) associated with intraplaque hemorrhages characterized by high levels of CD163, low levels of HLA-DR, increased capacity for Hb clearance and enhanced IL-10 release [18]. By dual-color immunostaining, we also confirmed the existence of this HA-mac macrophage phenotype in the AAA-wall (Supplemental Fig. 2). Thus, we observed the presence of CD163 positive macrophages with low levels of HLA-DR in the adventitia layer of AAA. In order to determine whether the microenvironment of the adventitial AAA wall also promotes this macrophage phenotype in infiltrating monocytes, we performed *in vitro* experiments in human monocytes purified from healthy donors. Monocytes were grown in the presence of either GM-CSF or M-CSF, to induce macrophage differentiation, in combination with Hb or conditioned medium obtained from adventitial AAA wall (AAA-CM). As previously reported, the mean fluorescence intensities (MFI) of CD163 and Hb uptake were increased in M-CSF macrophages as compared to GM-CSF macrophages, a phenotype that correlates with classical M2 and M1 polarization, respectively (Fig. 3A). Interestingly, flow-cytometry analysis show that addition of both Hb and AAA-CM induced the expression of CD163 and decreased HLA-DR levels in M-CSF stimulated macrophages, leading to a $CD163^{high}$ and HLA-DR^{low} phenotype (Fig. 3A–C). This striking phenotypic switch was less evident in GM-CSF-macrophages, suggesting that this cytokine partially suppressed the Hb triggering effects (Fig. 3A–C). Increased expression of CD209 (M2 marker) was also observed in Hb or AAA-CM stimulated macrophages, corroborating the anti-inflammatory phenotype of these cells (Supplemental Fig. 3).

Since a major function of CD163 is to take up Hb, we reasoned that Hb- or AAA-CM-induced CD163 expression might facilitate this cardinal feature of phagocytes. Thus, we measured uptake of AlexaFluor555-labeled Hb after addition to macrophage cultures. As compared to non-treated cells, addition of Hb or AAA-CM increased Hb uptake, mainly in M-CSF derived macrophages (Fig. 3A–B). These results were confirmed by confocal microscopy showing increased intracellular accumulation of Alexa Fluor 555-labeled Hb within cells treated by Hb or AAA-CM (Fig. 3B). We found a positive association between CD163 expression and Hb uptake ($r = 0.83$, $p < 0.05$). It is important to note that Hb uptake and $CD163^{high}$/HLA-DR^{low} phenotypic differentiation were partially reversed when Hb was selectively removed from AAA-CM, suggesting that Hb present in the AAA-wall specifically promotes HA-mac macrophage polarization and Hb-uptake in these cells (Fig. 3D).

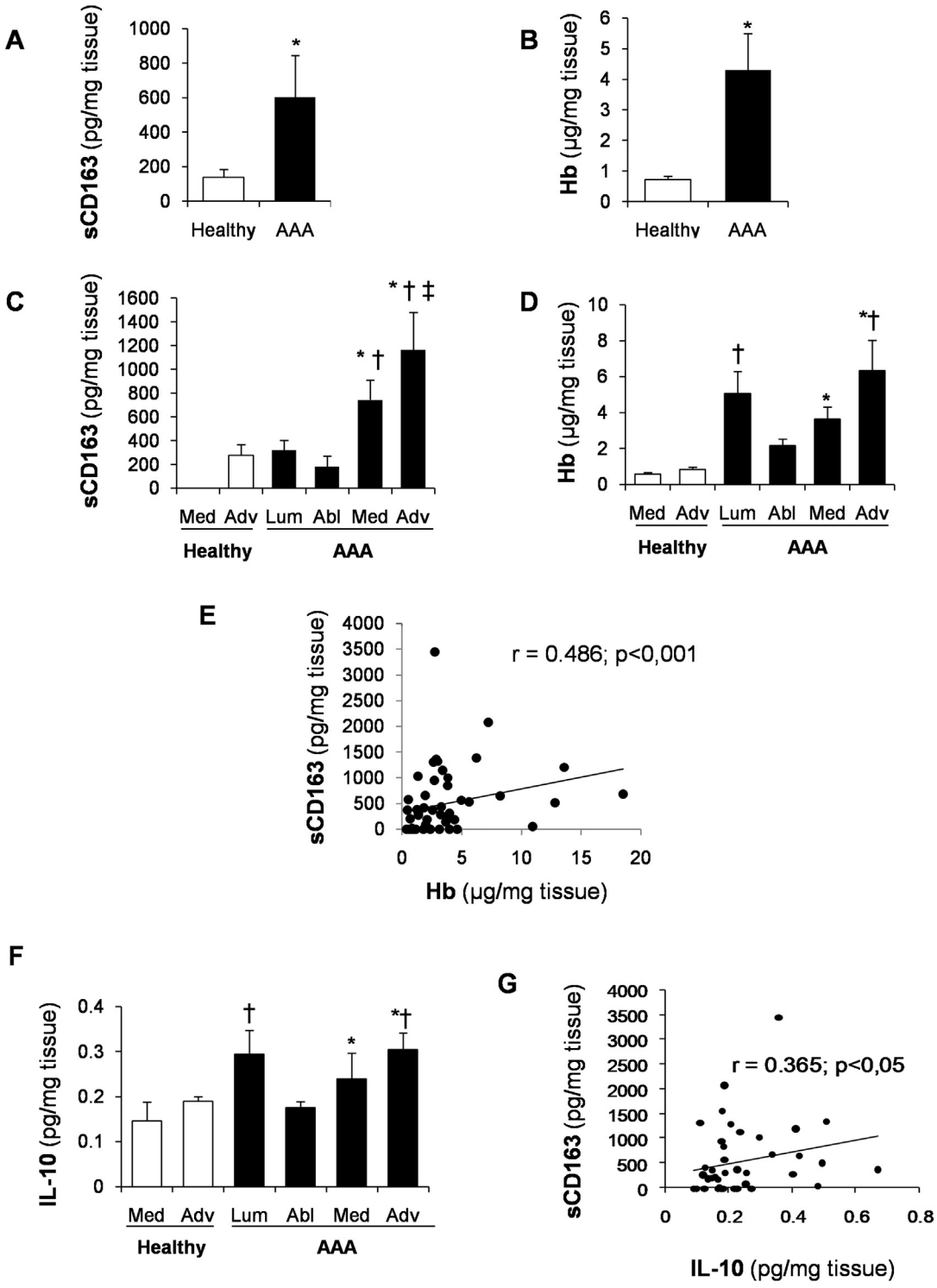
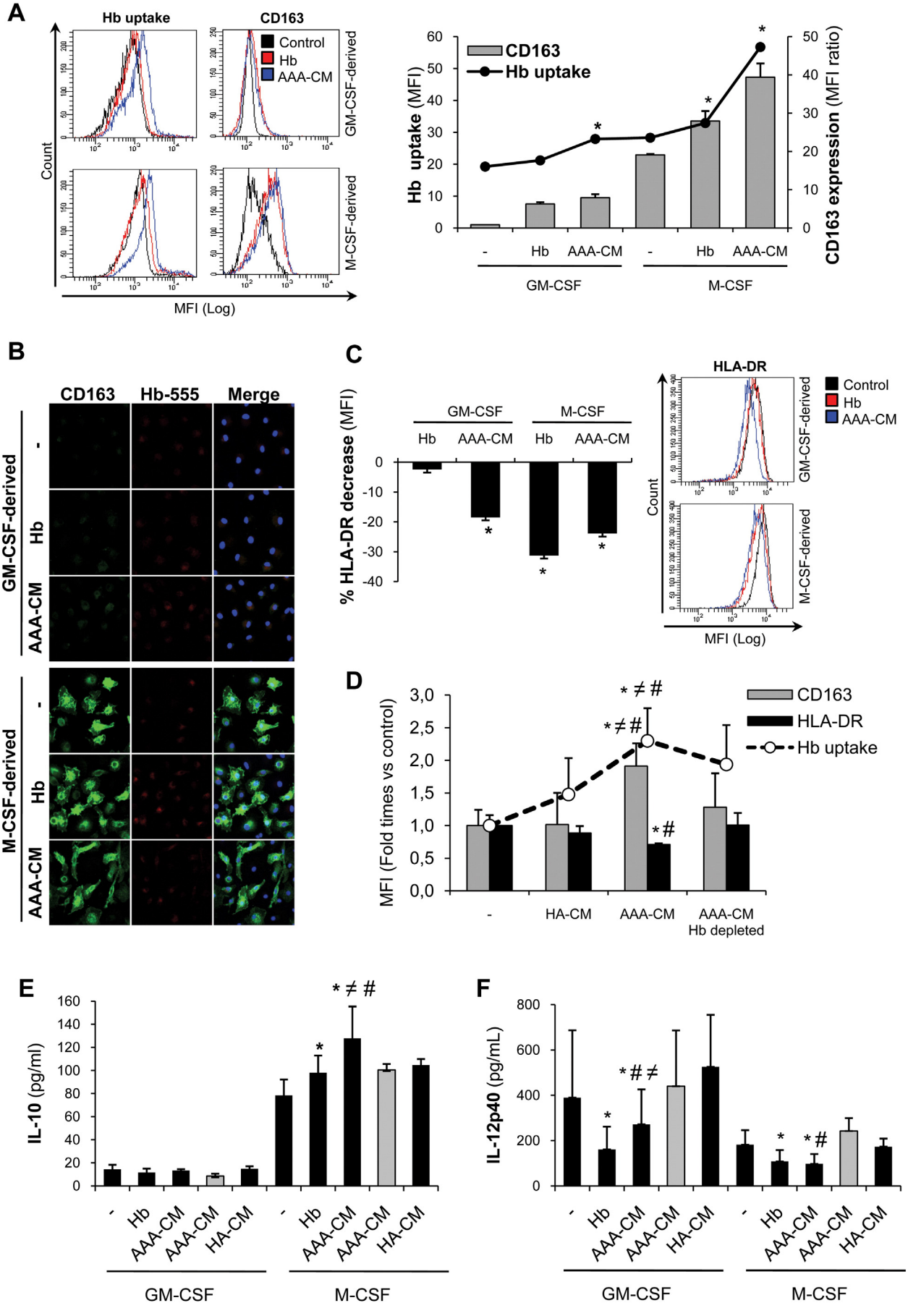


Fig. 2. sCD163 release is increased in human AAA. (A–B) Soluble CD163 (sCD163) and hemoglobin (Hb) were quantified in the conditioned medium obtained from total human AAA-wall (n = 10) and healthy aorta wall (n = 10). Human AAA samples were then dissected separating the thrombus layers (luminal (Lum) and abluminal (Abl)) from the aortic wall (media (Med) and adventitia (Adv) layers). Conditioned medium obtained from each layer were then analyzed for sCD163 (C), Hb (D) and IL-10 (F). Correlation between sCD163 and Hb (E) or sCD163 and IL-10 (G) in AAA conditioned medium. Median Hb, IL-10 and sCD163 concentrations were normalized by the wet weight of the tissue sample used to obtain conditioned medium. Results are expressed as means ± SD. *p < 0.05 as compared with healthy aorta. †p < 0.05 as compared with abluminal AAA. ‡p < 0.05 as compared with luminal AAA.

Finally, we investigated the anti-inflammatory phenotype of the *in vitro* differentiated macrophages. Hb or AAA-CM increased IL-10 release in M-CSF derived macrophages; however no difference in IL-10 secretion was shown in GM-CSF treated cells (Fig. 3E). On the contrary, Hb or AAA-CM decreased pro-inflammatory IL-12p40 release in both M-CSF and GM-CSF derived macrophages (Fig. 3F). AAA-CM mediated effects on both IL-10 and IL-12p40 release were also partially suppressed when Hb was removed from conditioned medium (Fig. 3E–F).


A
Hb uptake
CD163
Control
Hb
AAA-CM
GM-CSF-derived
M-CSF-derived
Count
MFI (Log)
CD163
Hb uptake
Hb uptake (MFI)
CD163 expression (MFI ratio)
GM-CSF
M-CSF
B
CD163
Hb-555
Merge
GM-CSF-derived
M-CSF-derived
C
% HLA-DR decrease (MFI)
HLA-DR
D
MFI (Fold times vs control)
HA-CM
AAA-CM
AAA-CM Hb depleted
E
IL-10 (pg/ml)
F
IL-12p40 (pg/mL)

3.4. Increased migration of monocytes in response to Hb and conditioned medium obtained from adventitial human AAA

To test the role of Hb and conditioned medium obtained from adventitial human AAA layer (AAA-CM) on monocyte chemotactic responses, we performed *in vitro* experiments on human monocytes. As reported in Fig. 4A, Hb promoted the migration of monocytes in a dose-dependent manner. No chemotactic responses were seen with heat-inactivated Hb or when Hb was added in the presence of EDTA, in accordance with the known calcium dependence of the Hb binding to CD163 [43]. To investigate whether Hb-induced chemotaxis was specifically mediated by CD163, we repeated these experiments in the presence of an anti-CD163 blocking antibody or a non-specific Ab. Surprisingly, pre-treatment of monocyte with the anti-CD163 antibody increased Hb-induced migration, suggesting that cross-linking of CD163 could induce these chemotactic effects (Fig. 4B). Finally, we observed that the addition of conditioned medium from AAA wall (AAA-CM) increased monocyte migration as compared with conditioned medium from healthy aorta wall (HA-CM) or Hb alone (Fig. 4C). It is important to note that Hb concentration in AAA-CM was around 25 μg/ml whereas Hb used in these experiments was 50 μg/ml, suggesting that additional factors might be responsible for the increased migration observed in AAA-CM. Importantly, AAA-CM mediated chemotaxis was partially suppressed when Hb was selectively removed from AAA conditioned medium (Fig. 4C), indicating that Hb present in the AAA-wall could play an important role on monocyte migration.

3.5. Hb induces an enhanced migratory response in monocytes from AAA patients

We next tested whether AAA patients showed an enhanced chemotactic response in the presence of Hb relative to healthy controls. As shown in Fig. 4D, circulating monocytes from AAA patients displayed a higher migratory capacity than those isolated from healthy controls, suggesting a pre-activating state of monocytes in AAA. Interestingly, addition of Hb significantly increased the migration of monocytes from both healthy and AAA patients, however Hb-mediated chemotactic effect was higher on circulating monocytes of AAA patients.

3.6. Differential distribution of CD163 in $CD14^{++}CD16^{+}$ monocyte subsets in AAA patients

It has been suggested that mature and/or activated $CD14^{+}CD16^{+}$-$CD163^{+}$ blood monocytes may be potential precursors of infiltrating macrophages within tissues [33]. To identify whether this subset of monocytes is more represented in AAA patients, we applied three-color flow cytometry to analyze the co-expression of CD163 by monocyte subsets in whole blood from healthy donors and AAA patients. Monocyte subsets were defined as $CD14^{++}CD16^{-}$, $CD14^{+}CD16^{+}$ and $CD14^{++}CD16^{+}$ as reported in Fig. 5A.

AAA patients showed increased total blood leukocyte number relative to healthy controls (Table 2). Specifically, neutrophils number was higher in AAA as compared with controls, whereas lymphocytes count was decreased. In relation to the distribution of monocyte subsets, our results show that the percentage of circulating $CD14^{++}CD16^{+}$ monocytes was significantly higher (22.2 ± 2.8 vs 12.9 ± 2.2, $p < 0.05$) and $CD14^{++}CD16^{-}$ monocytes were lower (70.7 ± 2.9 vs 80.0 ± 2.6, $p < 0.05$) in AAA patients, as compared to healthy controls (Fig. 5A–B). No significant differences were observed on percentage of $CD14^{+}CD16^{+}$ monocytes between AAA and healthy individuals (7.1 ± 0.9 vs 6.9 ± 0.9, NS).

We next compared CD163 protein expression on circulating monocytes from AAA patients and healthy controls (Fig. 5C). In both AAA patients and healthy controls, the highest levels of CD163 were found on $CD14^{++}CD16^{-}$ and $CD14^{++}CD16^{+}$ monocytes, whereas the lowest ones were reported on $CD14^{+}CD16^{+}$ monocytes. CD163 surface expression tended to be higher in total monocytes from AAA patients than in controls, but this tendency did not reach statistical significance. However, we found a different distribution of CD163 surface expression among monocyte subsets in AAA patients (Fig. 5C). Thus, CD163 membrane expression was statistically higher in $CD14^{++}CD16^{+}$ monocyte subset from AAA patients (34.7 ± 2.7 vs 26.6 ± 1.9, $p < 0.05$). We also analyzed $CD163^{+}$ populations in each monocyte subset. We observed that $CD14^{++}CD16^{+}CD163^{+}$ monocyte subset were expanded in AAA patients as compared with controls (86.4 ± 2.0 vs 79.4 ± 2.9, $p < 0.05$), remaining statistically significant even after adjusting by classical confounding factors, including age, glucose levels and CKD presence ($p = 0.048$) [44–46]. Nevertheless, $CD163^{+}$ cells did not show significant changes between AAA patients and controls in either $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{+}$ subsets (Fig. 5D).

Finally, given the role of soluble CD163 as a marker for inflammation, we analyzed sCD163 concentration and its relation with AAA size in 164 controls and 365 AAA patients from the VIVA screening trial. No changes in sCD163 serum concentration were observed in AAA patients, compared with AAA-free controls (Supplemental Table I). Moreover, we observed no association between sCD163 and maximal aortic diameter ($r = 0.035$, $p = 0.52$).

4. Discussion

In the current study we demonstrated the increased presence of CD163-positive macrophages in the adventitial wall of AAA. We also show that the release of sCD163 is increased in aneurysm samples relative to healthy aortas and is associated with higher Hb-content in ILT and adventitial layer. By *in vitro* studies, we show that the presence of Hb in conditioned medium from adventitial aneurysm promotes migration and differentiation of monocyte towards $CD163^{high}$ and HLA-DR^{low}-expressing macrophages, which showed enhanced Hb uptake and increased anti-inflammatory IL-10 release and decreased pro-inflammatory IL-12p40 secretion. Finally we observed an increased percentage of pre-infiltrating $CD14^{++}CD16^{+}CD163^{+}$ monocytes in blood circulating monocytes of patients with AAA as compared with healthy individuals. These findings suggest a potentially important role of CD163 in AAA pathophysiology by triggering Hb uptake, avoiding its deleterious effects.

Beside haptoglobin binding, one of the primary mechanisms protecting against the deleterious effects of free Hb is governed by the direct scavenging capacity of CD163 [13]. CD163 is a scavenger receptor

Fig. 3. Conditioned medium from adventitial-AAA layer induces CD163 expression in monocyte-derived macrophages. CD14+ monocytes were purified from healthy donors and then grown in the presence of either GM-CSF or M-CSF, to induce M1 or M2 macrophage differentiation, in combination with Hb or conditioned medium obtained from AAA wall (AAA-CM, 2.5% v/v) for 7 days. Fluorescence-labeledhemoglobin (Hb, 50 μg/ml) was added to differentiated macrophages for 60 min. (A) Cell-surface expression of CD163 and Hb uptake in differentiated macrophages by flow cytometry. The experiment was done on four different donors, and a representative example is shown. (B) CD163 expression (green) and Hb uptake (red) in macrophages as determined by confocal microscopy. Nuclei were stained with DAPI (blue). (C) Flow cytometry analysis showing cell-surface expression of HLA-DR and percentage of HLA-DR decrease relative to non-stimulated cells in each macrophage subtype. The experiment was done on three different donors, and a representative example is shown. (D) Conditioned mediums from AAA were incubated with HemogloBind™ reagent for hemoglobin depletion. Complete conditioned medium from healthy aorta (HA-CM) and complete- or Hb-depleted conditioned medium from AAA (AAA-CM) were added to M-CSF macrophages to analyze CD163 and HLA-DR expression or Hb uptake, as previously described (N = 4/group). Quantification of IL-10 (E) and IL-12p40 (F) in macrophage supernatants by ELISA. Complete conditioned medium (black bars) or Hb-depleted conditioned medium (gray bars) were added to cells and cytokines release was analyzed. Results are expressed as means ± SD. *$p < 0.05$ as compared with non-treated cells within GM-CSF or M-CSF-treated groups. ≠$p < 0.05$ as compared with HA-CM. #$p < 0.05$ as compared with Hb-depleted conditioned medium. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

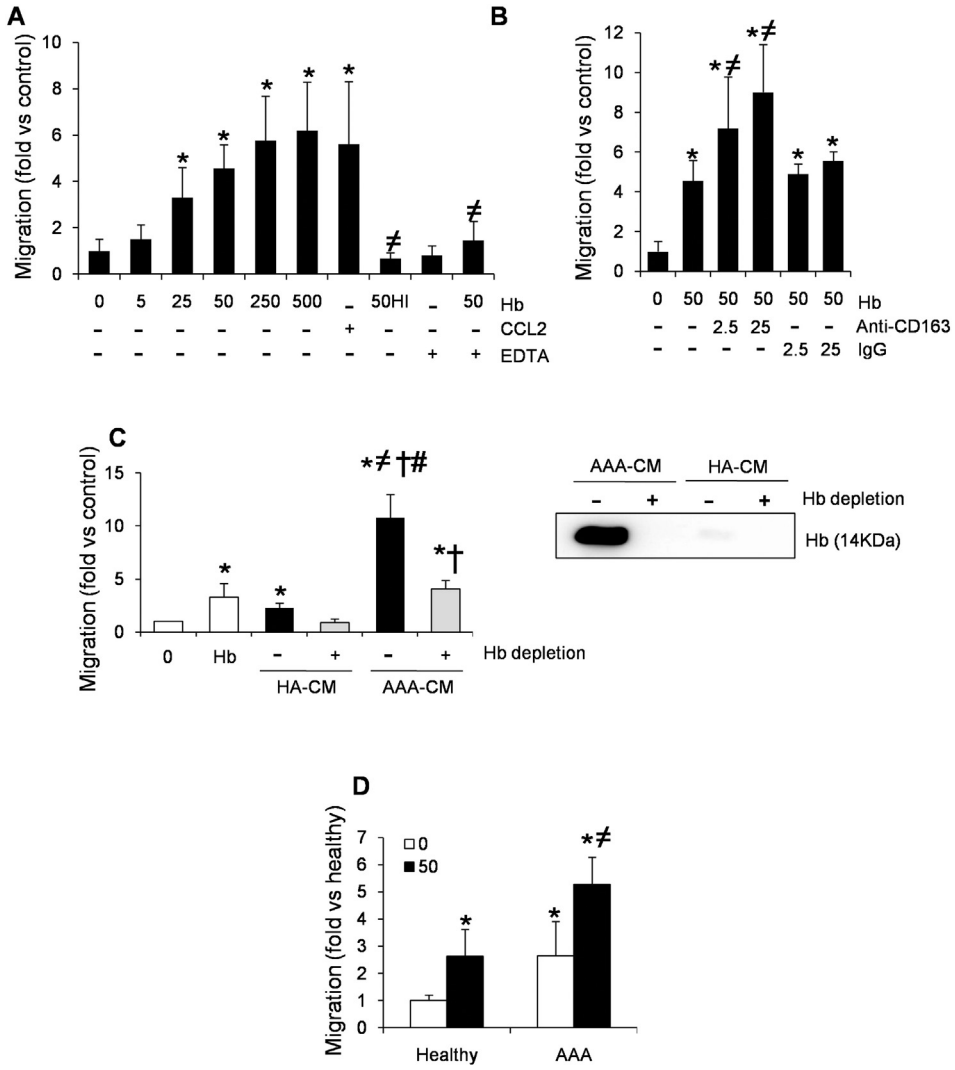


Fig. 4. In vitro migration of human monocyte in response to Hb or conditioned medium from adventitial-AAA. (A) Monocytes from healthy individuals were allowed to migrate within 2 h towards different concentrations of Hb (0–500 μg/ml), put in the lower chambers, in the presence or absence of EDTA. CCL2 (500 ng/ml) served as the positive control. Heat inactivated (HI) Hb served as the negative control. (B) Monocytes were pre-incubated for 1 h with anti-CD163 Ab (2.5–25 μg/ml) or irrelevant IgG Ab (2.5–25 μg/ml) and then allowed to migrate (N = 6). (C) Monocytes were then allowed to migrate in the presence of Hb (50 μg/ml) and conditioned medium from human healthy aortas (HA-CM, 2.5% v/v) or AAA (AAA-CM, 2.5% v/v). Complete conditioned medium (black bar) or Hb-depleted conditioned medium (gray bar) from both healthy aortas or AAA were used in this experiment (N = 4/group). For hemoglobin depletion, conditioned mediums were incubated with HemogloBind™ reagent. As reported by Western blot (right figure), Hb was effectively removed from HA-CM or AAA-CM, resulting in Hb-depleted HA-CM or Hb-depleted AAA-CM. *$p < 0.05$ as compared with non-treated cells, ≠$p < 0.05$ as compared with cells stimulated with Hb, †$p < 0.05$ as compared with HA-CM, #$p < 0.05$ as compared with Hb-depleted conditioned medium. (D) Monocytes from healthy individuals (N = 3) or patients with AAA (N = 3) were migrated in the presence of Hb (50 μg/ml). *$p < 0.05$ as compared with non-treated monocyte from healthy individuals. ≠$p < 0.05$ as compared with Hb-treated monocytes from healthy individuals. Results are expressed as mean ± SD and represent the increase in percent of migration relative to non-treated cells.

present on the surface of circulating monocytes and tissue macrophages [13]. In agreement with previous studies, where CD163 expression was observed in coronary and aortic lesions [47,48], we observed that CD163 was also highly expressed in the adventitia of human AAA, as compared to healthy aortas. Increased retention of Hb-derived iron has been also reported in adventitia of AAA patients [9]. In our study, the major proportion of $CD163^+$ phagocytes was located near areas rich in Hb-derived iron in AAA adventitia. It thus seems reasonable to speculate that these adventitial macrophages might be implicated in the clearance of Hb and heme-iron accumulated in the AAA-wall as consequence of radial convection of Hb from hemolyzed erythrocytes in the luminal thrombus [2,8]. In favor of this hypothesis, we observed both a positive association between sCD163 and Hb in the conditioned medium obtained from AAA-wall and induced differentiation of macrophages towards a CD163 expressing phenotype in the presence of conditioned medium from adventitial AAA layer. In this line, Hb-derived iron endocytosis induced CD163 expression by human macrophages *in vitro*[18]. CD163-mediated Hb uptake not only decreases Hb concentration but also induces anti-inflammatory pathways by increasing heme oxygenase-1 (HO-1) synthesis and IL-10 release [17]. It has been proposed that IL-10 mediates an autocrine feedback mechanism to promote Hb-induced CD163-macrophage polarization. Furthermore, the presence of a novel anti-oxidant macrophage phenotype (HA-mac) has been recently shown in hemorrhagic plaques characterized by high levels of CD163 and decreased HLA-DR expression, an increased capacity for Hb clearance and enhanced IL-10 secretion [18]. In our study, we also reported the presence of $CD163^{high}$ and HLA-DR^{low} macrophages in the AAA-wall, showing for the first time the existence of the HA-mac phenotype in adventitia from human AAA. By *in vitro* studies we have reported that conditioned medium from AAA wall induced macrophages polarization to this HA-mac phenotype. Altogether, these results indicate that Hb accumulation in the adventitial layer of AAA directs monocyte/macrophage differentiation towards an anti-oxidant and protective phenotype by stimulation of IL-10 release and CD163 expression, therefore

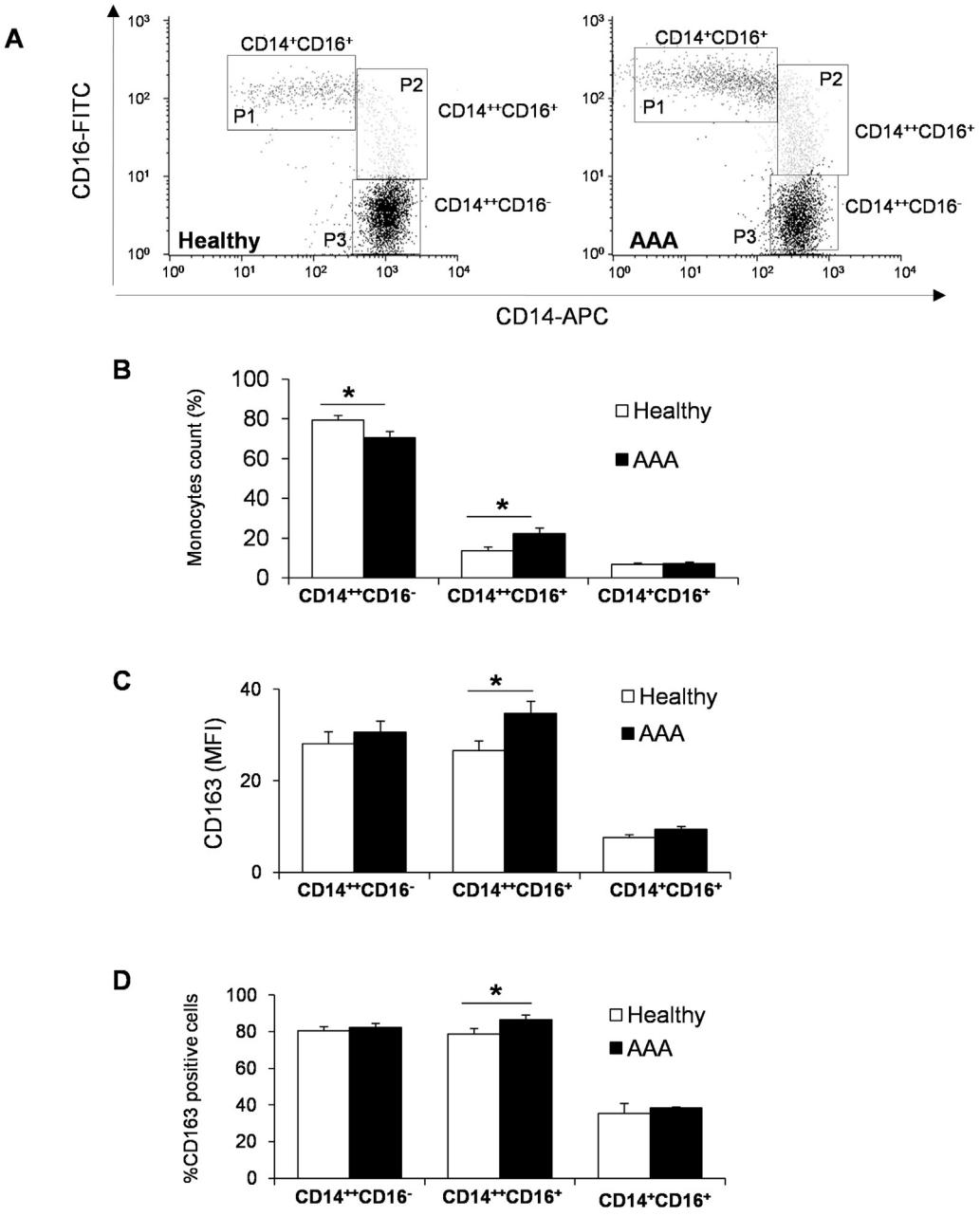


Fig. 5. Distribution of CD163 in different monocyte subsets in healthy individuals and AAA patients. (A) Representative example of the monocyte subset analysis in healthy (left) and AAA patients (right). Monocytes from whole blood were identified by forward and side scatter properties and expression of CD14 vs. CD16 with subsets defined as $CD14^{++}CD16^{-}$ (P3, lower right), $CD14^{++}CD16^{+}$ (P2, upper right) and $CD14^{+}CD16^{+}$ (P1, upper left). (B) Distribution of different monocyte subsets according to CD14 and CD16 expression in healthy (N = 14) and AAA subjects (N = 21). Cell-surface expression of CD163 (C) and percentage of CD163 positive cells (D) on each monocyte subset. Results are expressed as mean ± SD. *$p < 0.05$ as compared with healthy individuals.

Table 2
Blood leukocyte populations and CD163 expression.

	Controls (14)	AAA patients (21)	p
Blood leukocyte populations			
Leukocytes (per μl)	5490 ± 1130	9121 ± 936	**0.025**
Neutrophils (per μl, %)	3323 ± 728 (59.8)	7523 ± 947 (78.6)	**0.001**
Lymphocytes (per μl, %)	1655 ± 346 (30.7)	1241 ± 140 (13.7)	0.21
Eosinophils (per μl, %)	98 ± 24 (1.8)	87 ± 17 (1.1)	0.70
Monocytes (per μl, %)	389 ± 80 (7.2)	449 ± 58 (5.2)	0.55
Basophils (per μl, %)	24 ± 5 (0.4)	51 ± 21 (0.6)	0.35
CD163 expression			
Neutrophils (MFI)	16.7 ± 1.8	13.5 ± 1.4	0.16
Lymphocytes (MFI)	2.4 ± 0.2	2.4 ± 0.2	0.94
Monocytes (MFI)	25.8 ± 2.3	29.8 ± 2.4	0.27

Entries in bold are statistically significant ($p < 0.05$) as compared to controls.

promoting Hb clearance and tissue repair through TGF-β secretion, a function for which these macrophages are particularly competent [49,50].

It has been reported that Hb may induce monocyte chemotaxis within human atherosclerotic plaque [10]. Thus, we tested whether Hb accumulation and release in AAA may represent an explanation for the increased monocyte recruitment in the AAA-wall. As previously reported, we observed that Hb promoted monocyte chemotaxis in a dose-dependent-manner. In our study, Hb-induced chemotactic effects were higher in monocyte isolated from AAA patients than in those isolated from healthy individuals, suggesting a pre-activated state of monocytes in AAA. Interestingly, our study show for the first time that the addition of conditioned medium from AAA wall increased monocyte migration, whereas a low chemotactic response was observed with the conditioned medium from

healthy wall. AAA tissues contain soluble peptides capable of specifically directing the migration of mononuclear phagocytes, such as CCL2, IL-6, and IL-8 [51,52]. However, AAA-CM mediated chemotaxis was partially suppressed when Hb was selectively removed from AAA-CM, highlighting the important role of Hb from the AAA-wall on monocyte migration. To investigate whether Hb- or AAA-CM induced chemotaxis was specifically mediated by Hb-CD163 interaction, we repeated these experiments in the presence of EDTA or an anti-CD163 blocking antibody (clone EDHu1). No chemotactic responses were observed when Hb was added in the presence of EDTA, in agreement with the known calcium dependence of the Hb binding to CD163 [43]. Surprisingly, pre-treatment of monocyte with the anti-CD163 antibody increased both Hb- and AAA-CM induced migration, indicating that cross-linking of CD163 could induce these chemotactic effects. The CD163 (EDHu1) antibody binds to the domain 3 of CD163, the functional domain responsible of Hb-CD163 interaction [43], therefore it is possible that this antibody activates CD163 receptor, inducing the same cellular response as Hb. In favor of this hypothesis, binding of CD163 with EDHu1 antibody induced a protein tyrosine kinase-dependent signal that resulted in slow-type calcium mobilization, inositol triphosphate production, and secretion of IL-6 and GM-CSF [29]. Another possible explanation may be related to a higher Hb consumption mediated by CD163. Thus, in medium with Hb alone, migrated monocyte may uptake Hb, decreasing the Hb concentration and lowering the Hb-gradient. However, addition of the CD163-blocking antibody inhibits Hb-uptake, maintaining the Hb concentration, and therefore favoring monocyte chemotaxis, as we have observed. On the other hand, our results differentiated to those reported by Buttari et al., where an anti-CD163 antibody (clone GH1/61) reduced the Hb-induced monocyte migration [10]. The CD163 (GH1/61) antibody binds to the domain 7 of the CD163 protein [53]. However, the functional domain responsible of Hb-CD163 interaction is the domain 3 of CD163 [43], and no blocking effects have been reported with the CD163 (GH1/61) antibody [53].

Monocytes participate in pro- or anti-inflammatory conditions depending upon their state of differentiation and activation [54]. The majority of monocytes express cell surface CD14 ($CD14^{+}CD16^{-}$), whereas a minor subpopulation of monocytes also express the cell surface activation marker CD16, identifying them as more mature than the $CD14^{+}CD16^{-}$ subpopulation. In agreement with previous studies [27], our data show that human peripheral blood monocyte subsets might be differentially distributed in AAA patients. Thus, $CD14^{++}CD16^{+}$ monocytes were expanded, whereas proportion of $CD14^{++}CD16^{-}$ monocytes was decreased in AAA-patients as compared with healthy individuals. The $CD14^{++}CD16^{+}$ subset of monocytes increases significantly in a number of chronic inflammatory conditions [26]. However, the functional significance of $CD14^{++}CD16^{+}$ monocytes in AAA is unknown. $CD14^{++}CD16^{+}$ monocytes have a highest capacity to activate $CD4^{+}$ T-cells proliferation and are predisposed for antigen presentation since they express MHC II molecules (CD74 and HLA-DR) [20]. Furthermore, $CD14^{++}CD16^{+}$ monocytes have been documented to have proinflammatory activity, as they produce IL-12 [55] and IL-1β and tumor necrosis factor-alpha after lipopolysaccharide stimulation [56]. These cytokines are involved in B-cell differentiation and immunoglobulin secretion and $CD8^{+}$ T-cell differentiation [57]. Our current findings may suggest that the relative expansion of $CD14^{++}CD16^{+}$ monocytes in AAA might play specialized immunologic functions in this pathology and possibly to contribute to its progression due to the key role of adaptive immunity in AAA [58].

High levels of CD163 have been detected on monocytes during the resolution phase of tissue hemorrhages [59]. In our study, CD163 expression was detectable on the majority of $CD14^{++}CD16^{-}$ and in approximately half of $CD14^{++}CD16^{+}$. However, there are contradictory data regarding CD163 protein expression on monocyte subsets [28,29]. Some studies reported increased CD163 expression in $CD16^{+}$ monocytes [30,31] while others observed highest expression on $CD14^{++}CD16^{-}$ monocytes [32,34]. These discrepancies in the reported expression of CD163 expression may be related to several technical issues, including type of anticoagulant for blood sampling, monocyte isolation process, and antibodies used for analysis [32–34,53]. Interestingly, this is the first report indicating that CD163 expression in $CD14^{++}CD16^{+}$ monocytes was higher in AAA patients as compared with controls, while the opposite trend was reported in $CD14^{++}CD16^{-}$ cells. Several studies have proposed that neovascularization in adventitia might permit infiltration of leukocytes in AAA [4,60]. It has been suggested that mature and/or activated $CD14^{+}CD16^{+}CD163^{+}$ blood monocytes may be potential precursors of infiltrating macrophages within tissues [33]. In this line, our immunofluorescence analysis showed a colocalization of CD14 and CD163 in macrophages of the AAA adventitia, principally in areas surrounding neovessels. This fact may indicate that monocyte-derived macrophages may be recruited, at least in part, from vasa vasorum vessels in the adventitia. We also showed that CD163 is expressed not only near blood vessels but also in other areas of the adventitial layer. In agreement with this observation, Dutertre et al. reported a greater percentage of proliferation in macrophages along the entire aneurysmal wall, suggesting that local proliferation may be also involved in macrophage accumulation during AAA progression [61]. Therefore, adventitial macrophage accumulation may be due to both recruited monocytes and resident macrophages proliferation.

High levels of sCD163 can be detected in plasma in a wide range of inflammatory diseases, including carotid- [62], peripheral- [16] and coronary-artery disease [63], as well as type 2 diabetes [48] and human immunodeficiency virus [64]. In our study, circulating sCD163 levels were similar between patients with AAA and control individuals, and no association was found between sCD163 and maximal aortic diameter, a surrogate marker of AAA growth. These data suggest that sCD163 is not a diagnostic biomarker of AAA, indicating that increased presence of CD163 in the aortic wall from AAA patients is not enough to produce systemic sCD163 elevation. This observation does not exclude a potentially important role of CD163 in AAA pathophysiology by triggering Hb uptake and, consequently, preventing Hb-mediated oxidative and inflammatory harmful effects in the AAA-wall, as reported in our article. Regarding the potential therapeutic role of our findings, it is important to note that CD163 is a promising therapeutic target for selective intracellular delivery of anti-inflammatory drugs to macrophages. Thus, two different strategies, i.e. dexamethasone-rich liposomes targeting CD163 or CD163-antibody-dexamethasone complexes, specifically inhibited pro-inflammatory cytokines production in two animal sepsis models [65,66]. Interestingly, these anti-inflammatory effects were comparable to a 50-fold higher dose of non-conjugated dexamethasone, avoiding glucocorticoids systemic side effects. Whether these experimental approaches may modulate macrophage activity in chronic inflammatory diseases, such as AAA, is not known. In addition, due to the potential protective role of CD163 by scavenging Hb, studies conducted to increase its expression in macrophages in pathological disorders may be of interest. Therefore, new studies are necessary to address these hypothesis.

In conclusion, in the current study we demonstrated that hemoglobin promotes migration and differentiation of monocyte *in vitro*, explaining the increased presence of CD163-positive macrophages in the adventitia of the AAA. These macrophages could take up hemoglobin present in the AAA avoiding its pro-inflammatory and pro-oxidants deleterious effects.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijcard.2015.08.053.

Conflict of interest

There are no conflicts of interest.

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