Research Article

Identification of Macrophage Genotype and Key Biological Pathways in Circulating Angiogenic Cell Transcriptome

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Background. Circulating angiogenic cells (CAC) have been identified as important regulators of vascular biology. However, there is still considerable debate about the genotype and function of CAC. Methods and Results. Data from publicly available gene expression data sets were used to analyse the transcriptome of in vitro cultured CAC (CACiv). Genes and pathways of interest were further evaluated using qPCR comparing CACiv versus CD14+ monocytic cells. The CACiv transcriptome strongly related to tissue macrophages, and more specifically to regulatory M2c macrophages. The cytokine expression profile of CACiv was predominantly immune modulatory and resembled the cytokine expression of tumor-associated macrophages (TAM). Pathway analysis revealed previously unrecognized biological processes in CACiv, such as riboflavin metabolism and liver X receptor (LXR)/retinoid X receptor (RXR) and farnesoid X receptor (FXR)/retinoid X receptor (RXR) pathways. Analysis of endothelial-specific genes did not show evidence for endothelial transdifferentiation. Conclusions. CACiv are genotypically similar to regulatory M2c macrophages and lack signs of endothelial differentiation.

1. Background

Endothelial progenitor cell (EPC) therapy is an appealing strategy for the treatment of cardiovascular diseases. The concept of EPCs dates back to a landmark study published in 1997 by Asahara et al. [1], who isolated a ‘putative progenitor endothelial cell’ that could be found within the CD34+ mononuclear blood cell fraction. These EPCs were able to differentiate in vitro into an endothelial phenotype and induce neovascularization in vivo. After more than a decade of vigorous research, during which the early findings of Asahara et al. have been extended, the dogma that postnatal neovascularization relies solely on the proliferation, migration and remodeling of fully differentiated endothelial cells, has been largely revised. Nowadays, neovascularization is considered to be a dynamic process in which local endothelial cell proliferation and circulating progenitor cells join forces to engage in the restoration of tissue perfusion. However, there is still considerable debate about the phenotype and function of EPCs and much of the uncertainty is caused by a high degree of confusion about the definition of EPCs. First of all, over the years, different culture protocols have emerged, all claiming to produce EPCs from peripheral blood mononuclear cells. Furthermore, a variety of molecular marker combinations have been advocated for the characterization of circulating EPCs. Obviously, the ambiguity that surrounds the term ‘EPC’ has not facilitated the understanding and advancement of EPC biology.

In the present article, we have investigated an EPC sub-type that has been renamed as circulating angiogenic cell (CAC) [2], early EPC [3] or early pro-angiogenic cell (EPC) [4]. For clarity reasons, we will use the term CACiv for these in vitro cultured blood-derived mononuclear cells. The potential use of these cells to aid in the restoration of impaired neovascularization has been investigated [5]. We used gene expression profiling and transcriptome analysis
to identify the CAC<sub>iv</sub>-specific gene signature, to determine the CAC<sub>iv</sub> cytokine-cytokine receptor fingerprint and to investigate the biological processes that are important in CAC<sub>iv</sub> biology. This approach is not unprecedented, since, for instance in oncology, genetic profiling has revolutionized tumor characterization and yielded new insights into tumor biology [6]. Using transcriptome analysis, several groups were able to make considerable progress in redefining the relationships between the different culture-derived EPC subtypes and other hematopoietic and mesodermal lineage populations. For instance, EPCs cultured with the culture protocol of Hill et al. [7] could be requalified as T-lymphocytes on the basis of their gene signature [8].

In the present paper, we provide evidence that CAC<sub>iv</sub> strongly relate to tissue macrophages, and more specifically to regulatory M2 macrophages, without evidence for endothelial transdifferentiation.

The cytokine expression profile is predominantly immune modulatory and resembles the cytokine expression of tumor-associated macrophages (TAMs). Pathway analysis has revealed previously unrecognized biological processes in CAC<sub>iv</sub> such as riboflavin metabolism and liver X receptor (LXR)/retinoid X receptor (RXR) and farnesoid X receptor (FXR)/retinoid X receptor (RXR) pathways. Together, our findings provide novel insights into the field of CAC biology.

2. Methods

2.1. Ethics Statement. The data that is reported in this manuscript used publicly available published data sets from other studies. The data collected from GSE2040 involved cell cultures of human volunteers and the data obtained from GSE5099 involved cell material from blood donor buffy coats. Both studies were in compliance with the Helsinki Declaration on research involving human subjects, human material or human data and under the approval of an appropriate local ethics committee. For a qPCR study of CAC<sub>iv</sub> we collected blood of healthy volunteers. The CAC<sub>iv</sub> culture protocols were reviewed and approved by the local ethics committee of the Antwerp University Hospital (EC number 12/10/101). Written informed consent was obtained from all participants.

2.2. Microarray and Pathway Analysis. To develop the CAC<sub>iv</sub> gene signature, a publicly available gene expression data set (GSE2040) (HG-U95Av2 microarray (Affymetrix Inc.), see supplementary data file (available here)), targeting 9,670 human genes as selected from the National Center for Biotechnology Information (NCBI) Gene Bank database, was retrieved from the NCBI website (https://www.ncbi.nlm.nih.gov). This gene expression data set contained 3 gene expression profiles of CAC<sub>iv</sub> and 3 gene expression profiles of CD14<sup>+</sup> monocytes, all of which were included in the analysis. Raw expression data were normalized using GCRMA and probe sets with a fluorescence intensity above 100 in at least 25% of the arrays were filtered for further analysis. The gene signature was generated using the nearest shrunken centroid method implemented in the R-package Prediction Analysis of Microarrays (PAM). Using a leave-one-out cross-validation procedure, a δ-value was selected in such a way that the misclassification error rate was minimal. The global clustering pattern of the CAC<sub>iv</sub> signature genes was evaluated using unsupervised hierarchical clustering (UHC) with the Euclidean distance as distance measure and complete linkage as the dendrogram drawing method. Using the global test [9] we evaluated global differences in expression for probe sets annotated to the KEGG pathway ‘cytokine-cytokine receptor interaction’ (map04060) between CAC<sub>iv</sub> and CD14<sup>+</sup> monocytes.

The Ingenuity Pathways knowledge base Analysis (IPA) (Ingenuity<sup>®</sup> Systems, http://www.ingenuity.com) software was used to identify biological networks, functions and canonical pathways important to CAC<sub>iv</sub> biology. To appreciate the genetic resemblance of the CAC<sub>iv</sub> gene expression profile in relation to other cell types of interest (i.e., macrophages, monocytes, endothelial cells), the expression of CAC<sub>iv</sub> signature-related genes was analyzed using the Reference database for gene Expression Analysis (RefExA, http://www.lsbm.org/databse), together with an extensive review of the literature.

The following strategy was adopted to evaluate the possible macrophage genotype of cultured CAC<sub>iv</sub>. A microarray data set (GSE5099, HG-U133A (Affymetrix Inc.), see supplementary data file) including a total of 44928 entries representing more than 33,000 human genes containing expression data on the differentiation of monocytes into macrophages, and of macrophages into an M1 or M2 macrophage subtype was downloaded from Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo). Raw expression data were normalized using GCRMA and probe sets with a fluorescence intensity above 100 in at least 25% of the arrays were filtered for further analysis. Using PAM, we generated gene centroids for the monocyte, macrophage, M1 macrophage and M2 macrophage cell fractions. The leave-one-out cross-validation procedure was used to select a δ-value in such a way that the misclassification error rate was minimal. The centroids were applied onto the GSE2040 data set using the nearest centroid classification routine. Samples were classified by correlating the centroid-specific gene expression profile of each sample in the data set with the shrunk centroids generated by the PAM algorithm. Positive correlation coefficients indicate resemblance of the tested sample to the cell fraction represented by the centroid. Mann–Whitney U tests were used to compare the resulting correlation coefficients between CAC<sub>iv</sub> and monocytes.

To evaluate the degree of resemblance between CAC<sub>iv</sub> and endothelial cells, we retrieved an endothelial-specific gene list reported by Bhasin et al. [10]. We used UHC analysis (Euclidean distance, complete linkage) to assess global differences between CAC<sub>iv</sub> and endothelial cells with respect to the endothelial-specific gene list. In addition, we calculated the average gene expression of the endothelial-specific genes and compared this level between CAC<sub>iv</sub> and CD14<sup>+</sup> monocytes to evaluate whether a difference in expression existed between both cell types with respect to the set of endothelial-specific genes.

The data that is reported in this manuscript used publicly available published data sets from other studies. The data
collected from GSE2040 involved cell cultures of human volunteers and the data obtained from GSE5099 involved cell material from blood donor buffy coats. Both studies were in compliance with the Helsinki Declaration on research involving human subjects, human material or human data and under the approval of an appropriate local ethics committee.

2.3. Cell Isolation and Cell Culture. Mononuclear cells were extracted out of blood specimens of healthy volunteers \((n = 4)\) by density gradient centrifugation using lymphocyte separation medium (Lonza). The CD14\(^+\) cell fraction was isolated by using CD14 MicroBeads (Miltenyi Biotec) according to the manufacturer’s instructions. CAC\(v\), were cultured out of the mononuclear cell fraction of blood specimens of healthy volunteers \((n = 4)\) according the method first described by Dimmeler et al. \[4\] In brief, \(1 \times 10^6\) mononuclear cells were plated on human fibronectin-coated 24-well culture dishes and maintained in EBM-2 basal medium with EGM-2-MV SingleQuots and 20% fetal bovine serum (FBS) (Lonza). After 3 days in culture, nonadherent cells were removed by washing with PBS and adherent cells were further incubated in fresh medium until day 7. Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza and cultured in EBM-2 basal medium with EGM-2 SingleQuots for 14 days \((n = 2\), technical replicates\). Cells from passage 6 and 7 were used.

2.4. RNA Extraction and Quality, cDNA Synthesis. RNA was isolated using the RNeasy mini kit (Qiagen) following the manufacturer’s instructions. On-column DNase treatment (Qiagen) was used to remove contaminating DNA leftovers. RNA concentration and purity were analyzed using Nanodrop spectrophotometer (Nanodrop technologies) readings at 260 and 280 nm. Complementary DNA (cDNA) was synthesized using Invitrogen superscript kit according to the manufacturer’s instructions and using random hexamer primers for reverse transcription. Reverse transcription was performed at 50°C for 55 minutes, followed by 5 minutes of incubation at 85°C to inactivate the reverse transcriptase enzyme. cDNA samples were placed on ice and stored at -20°C until further use.

2.5. qPCR. Taqman\(^\circledR\) gene expression assays (Applied Biosystems) were used for qPCR analysis on a LightCycler\(^\circledR\) 480 instrument (Roche). All primers were designed to be intron spanning. qPCR was performed using the LightCycler\(^\circledR\) Taqman\(^\circledR\) Master Mix (Roche) in a final reaction volume of 20 μl. We used the geNorm algorithm to determine an optimal combination of reference genes for internal normalization (i.e., GAPDH and HPRT). All qPCR reactions were carried out as follows: after an initial denaturation-activation step at 95°C for 10 min, amplifications consisted of 45 cycles of denaturation at 95°C for 10s, annealing at 60°C for 15 s and measurement of fluorescence at 72°C for 1 s. Cycle number \((C_q)\) was measured using the baseline-independent second derivative maximum method. Normalized relative gene expression was determined by the \(E^{ΔΔCq}\) method. Assay efficiency \((E)\) was measured by serial dilution of cDNA of pooled samples based on the slope of the standard dilution curve \((E = 10^{1/\text{slope}})\).

2.6. Statistical Analysis. Statistical analysis was performed in PASW\(^\circledR\) statistics 18 (IBM Corp.). Graphs were created in GraphPad Prizm\(^\circledR\). Data are expressed as mean ± SEM. Student’s t-test was used for statistical analysis of relative expression data after logarithmic transformation because of non-normality of data subsets. A two-sided \(p\)-value of <0.05 indicated statistical significance.

3. Results

3.1. CAC\(iv\) Gene Signature Closely Resembles M2 Macrophage Transcriptome, with Little Evidence of Endothelial Cell Transdifferentiation. We composed a gene signature that discriminates between CAC\(iv\) and CD14\(^+\) monocytes using a publicly available gene expression data set (GSE2040) that has previously been used to investigate the neovascularization capacity \[11\] and production of cytokines by CAC\(iv\) \[12\] and to validate the proteomic characterization of CAC\(iv\) \[4\]. Using the nearest shrunken centroid method, we identified 70 genes that were significantly upregulated versus 107 genes that were significantly downregulated in CAC\(iv\) versus CD14\(^+\) mononuclear cells \((\Delta = 2.85\), misclassification error rate = 0) (Figure 1).

Comparison of the CAC\(iv\) signature with the cell specific expression data in the RefExA database yielded abundant evidence for macrophage-associated gene expression or genes that were related to monocyte/macrophage differentiation, such as GPNMB, APOE, APOC1, ACP2 and CYP27A1. In total, 43 of the 70 CAC\(iv\) signature genes showed to be highly expressed and/or specific for macrophages. Considering the downregulated genes, repression of the monocytic differentiation of macrophages, such as RGS2, NR4A2, FCN1, IL1B and SELLI. Furthermore, the CAC\(iv\) signature revealed the upregulation of several genes associated with the differentiation and function of DCs (CD40, MARCO, FZD2, LILRB4 and LGMN) and osteoclasts (ACP5, CTSK, LPXN, ATP6V1A and MITF) (Figure 2).

Interestingly, the expression profile of the CAC\(iv\) evidenced a high resemblance to that of ‘alternatively activated’ M2 macrophages, and more specifically to the M2c subtype that is induced by the anti-inflammatory cytokine IL10 and is characterized by the upregulation of CD163 and CCL18 \[13\]. Both CD163 and CCL18, together with other markers specific for M2 differentiation of macrophages, such as SLC20B1, were identified in the CAC gene signature. The upregulation of the M2c trait was further substantiated with qPCR gene expression analysis of CD163, CCL18 and SLC20B1 in CAC\(iv\) from healthy volunteers compared to CD14\(^+\) monocytes and HUVEC (Figure 3(a)).

Additionally, the expression of M1 inflammatory macrophage differentiation markers, such as ATF3, IL1B and CCL3, was found to be significantly downregulated in the microarray data. Interestingly, we identified several genes in the CAC\(iv\) signature that are known to be implicated in lipid processing (PLTP, NR1H3) and plaque remodeling by atherosclerotic plaque-associated macrophages (CTSK, LGMN...
Concerning M1 versus M2 differentiated monocytes, respectively. We highlighted those genes of the CAC iv signature that are specific to no evidence for endothelial cell (EC) differentiation, respectively. We found that the CAC iv expression profile was discordant with the monocytic cell signature (p = 0.001) but significantly correlated with the signature of macrophages (p = 0.011). Concerning M1 versus M2 differentiation, CAC iv exhibited a predominant M2 expression profile (Figure 4).

Surprisingly, however, our CAC iv signature revealed little to no evidence for endothelial cell (EC) differentiation. We compared the CAC iv signature to the RefExA database and two published data sets of endothelial restricted genes [10, 14]. Except for RNASE1, we found no evidence for induction of endothelial-specific gene expression in the CAC iv. Furthermore, comparison of the CAC iv signature to a published gene signature of tumor-derived endothelium (ovarian carcinoma) yielded only one tumoral vascular marker, GPNMB, which was upregulated in both profiles [15]. qPCR analysis of freshly cultured CAC iv from healthy volunteers confirmed RNASE1 and VEGFB upregulation in CAC iv versus CD14+ monocytes and HUVEC. However TIE-2 (TEK) expression pertained exclusively to HUVEC whereas both CAC iv and CD14+ monocytes failed to show any TIE-2 expression (Figure 3(b)). This finding restricts CAC iv from transdifferentiating into the endothelial cell lineage.

To understand whether the CAC iv transcriptome showed higher resemblance to endothelial cells than CD14+ monocytes, we performed UHC analysis using an endothelial cell-specific gene set (derived from [10]) to cluster CAC iv versus CD14+ monocytes. Overall, CAC iv did not demonstrate higher differentiation capacity towards the endothelial profile compared to CD14+ monocytes (Figure 5). RNASE1, also present in the EC signature, stood out in both the UHC and qPCR analysis as highly upregulated gene. This is interesting and may point to an important function of this gene in CAC biology.

Together, these findings do not seem to support the hypothesis that CAC iv give rise to cells with an endothelial genotype, but would rather suggest that CAC iv closely resemble M2c macrophages.

3.2 CAC iv Express a Cytokine Profile Compatible with Regulatory M2 Macrophages and TAM. To gain further insight into CAC iv biology, we investigated their cytokine and cytokine receptor expression. Using a global test, we evaluated differences in cytokine-cytokine receptor expression for genes annotated to the KEGG pathway ‘Cytokine-

![Figure 1: CAC iv gene signature. 70 genes were significantly upregulated among 107 genes were significantly downregulated in CAC iv versus CD14+ monocytes. The upregulated (red) and downregulated (blue) genes express a close lineage relationship with macrophages and monocytes, respectively. We highlighted those genes of the CAC iv signature that are specific for either macrophages or monocytes. *: very specific. Mo: macrophage; Mo: monocyte.](image-url)
cytokine receptor interaction and were able to demonstrate clear differences in interleukin, chemokine and chemokine receptor expression between CAC iv and monocytes. Monocytes exhibited a classical inflammatory genotype, with expression of inflammatory cytokines, such as IL1β, (IL6), IL8, (IL12α), IL12β and TNF (Figure 6(a)). CAC iv, on the other hand, showed significant upregulation of the anti-inflammatory cytokine IL10 and the matrix remodeling, pro-angiogenic cytokine IL23α. Increased IL10 expression combined with reduced expression of inflammatory cytokines, is characteristic of the M2c macrophage subtype, in the literature referred to as regulatory macrophages [16].

CAC iv significantly expressed the chemokines CCL2 and CCL18 (Figure 6(b)). Other chemokines, such as CCL17 and CCL22, showed a trend towards increased expression in CAC iv. Interestingly, the chemokine combination of

| Symbol | Mφ | Mo | EC | DC | Ocl |
|--------|----|----|----|----|-----|
| GPNMB  | *  |    |    |    |     |
| APOE   |    |    |    |    |     |
| DFNA5  |    |    |    |    |     |
| LILRB4 | *  |    |    |    |     |
| ACP2   | *  |    |    |    |     |
| FLP   |    |    |    |    |     |
| CYR27A1 | * |    |    |    |     |
| CD163  |    |    |    |    |     |
| RNASE1 |    |    |    |    |     |
| APOC1  | *  |    |    |    |     |
| ACP5   | *  |    |    |    |     |
| SLC2O2B1 | * |    |    |    |     |
| CTX2   |    |    |    |    |     |
| PLG2G7 | *  |    |    |    |     |
| CMKLR1 |    |    |    |    |     |
| AMPD3  |    |    |    |    |     |
| PLD3   | *  |    |    |    |     |
| LGMN   |    |    |    |    |     |
| FZD2   |    |    |    |    |     |
| LPXN   |    |    |    |    |     |
| ENP2   |    |    |    |    |     |
| NRIH3  |    |    |    |    |     |
| MERTK  |    |    |    |    |     |
| IQGAP2 |    |    |    |    |     |
| ABC5S  |    |    |    |    |     |
| SLC31A1 | * |    |    |    |     |
| CCL18  | *  |    |    |    |     |
| VSI4   |    |    |    |    |     |
| ZFYVE26 | * |    |    |    |     |
| ATP16O1A | * |    |    |    |     |
| MT5S1  |    |    |    |    |     |
| ATP6V1A | * |    |    |    |     |
| SLCT7A8 | * |    |    |    |     |
| CD40   |    |    |    |    |     |
| ADORA3 | *  |    |    |    |     |
| MITF   | *  |    |    |    |     |
| TLR5   | *  |    |    |    |     |
| DAB2   | *  |    |    |    |     |
| TDRD3  | *  |    |    |    |     |
| GLA    | *  |    |    |    |     |
| MARCO  |    |    |    |    |     |
| GYP1B1 |    |    |    |    |     |
| PSEN1  |    |    |    |    |     |
| LHPPL2 | *  |    |    |    |     |
| RIN2   |    |    |    |    |     |
| HIVP2  |    |    |    |    |     |
| SNX1   |    |    |    |    |     |
| IL7R   |    |    |    |    |     |
| PLXNC1 |    |    |    |    |     |
| HSD17B4 | * |    |    |    |     |
| SEC22B |    |    |    |    |     |

Figure 2: Expression of CAC iv signature related genes in myeloid cells. To demonstrate the lineage relationships between CAC iv and other cell types, we highlighted those genes of the CAC iv signature that were found to be significantly upregulated and also specific for a particular cell type. *: very specific for macrophages. Mφ: macrophage; Mo: monocyte; EC: endothelial cell; DC: dendritic cell; Ocl: osteoclast.
CCL17, CCL18 and CCL22, is a cluster that has been associated specifically with the M2 macrophage profile. However, because CCL17 and CCL22 expression did not reach statistical significance, this association in CACiv remains speculative. Since also the M1 macrophage-associated chemokine CCL2 was upregulated in CACiv, this chemokine profile strongly resembles the chemokinetic fingerprint of TAM [17]. Moreover, TAM are characterized by low expression of inflammatory cytokines, such as IL1β, IL12β and TNF, lending further support to the similar genotypic appearance of CACiv and TAM.

Interestingly, CACiv also expressed a number of M1 macrophage-associated pro-inflammatory markers, such as IL1α and IL23α. Stimulation of cardiac myofibroblasts with IL1α has been shown to lead to the production of extracellular matrix metalloproteinases (MMPs), such as MMP2 and MMP9, and inhibits the expression of ADAMTS1, an angiogenesis inhibitor [18]. IL23α has also been shown to upregulate MMP9 activity and enhance angiogenesis [19]. Together, the expression of these M1-associated cytokines could endow CACiv with important tissue remodeling and angiogenic properties in the setting of MI.

Figure 3: Gene expression analysis. Gene expression of (a) M2c macrophage-related, (b) endothelial, (c) cytokine/cytokine receptor and (d) cholesterol transporter pathway genes in CACiv (green) vs. CD14+ monocytes (red) vs. human umbilical vein endothelial cells (HUVEC) (grey). Only the relevant statistical significance between CACiv and CD14+ monocytes is depicted. NRQ: normalized relative quantity. ** p < 0.01; *** p < 0.001.
Figure 4: CAC<sub>iv</sub> differentiate towards a predominantly M2 macrophage genotype. Boxplots comparing the relative expression of the genes in
(a) the monocyte-specific gene signature (MONO), (b) the macrophage-specific gene signature (MACRO), (c) the LPS- and IFN-γ-stimulated
M1 macrophage-specific gene signature (MACRO M1), and (d) the IL4-stimulated M2 macrophage-specific gene signature (MACRO M2),
between CAC<sub>iv</sub> and CD14<sup>+</sup> monocyctic cells (MONO). Positive values signify a stronger degree of similarity of the genetic expression of CAC<sub>iv</sub>
or CD14<sup>+</sup> monocyctic cells to the specified cell type.
Finally, we identified the CCR7 chemokine receptor as the most differentially expressed chemokine receptor in Cd14^+ monocytic cells, although its expression did not reach statistical significance. This chemokine receptor is commonly found on mature DCs and facilitates lymphoid tissue homing. Interestingly, CXCR4, the receptor for the hypoxia-inducible chemokine...
CXCL12, showed higher expression on monocytes than on CACiv, suggesting that differentiated CACiv do not display increased hypoxia-directed tissue homing capacity compared to CD14+ monocytes on. qPCR analysis, the expression of IL10 and CCR7 was confirmed to be significantly upregulated in CACiv (Figure 3(c)).

3.3. Pathway Analysis. Using IPA software, we determined the biological networks, functions and canonical pathways important to CACiv biology. The main CACiv-associated biological networks and molecular functions consisted of genes implicated in lipid metabolism, molecular transport, biochemistry of small molecules, inflammatory responses and cardiovascular disease. LXR/RXR (APOC1, APOE, NR1H3, PLTP; \( p = 2.9 \times 10^{-4} \)) and FXR/RXR (APOE, CYP27A1, NR1H3, PLTP; \( p = 4.46 \times 10^{-4} \)) signaling pathways and riboflavin metabolism (ACP5, ACP2, ENPP2; \( p = 6.54 \times 10^{-5} \)) were significantly upregulated in CACiv compared to CD14+ monocytes (Figure 7), whereas genes involved in inflammatory pathways and the acute phase response were repressed, highlighting the anti-inflammatory properties of CACiv. Interestingly, a gene cluster consisting of IL10 antagonistic factors (MAP3K14, FOS, DUSP1, IL1RN, CDKN1A, IL1B, PTGS2, CCL3, CDKN1C; \( p = 3.24 \times 10^{-5} \)), was found to be associated with the monocyte-like profile and appeared to be repressed during differentiation towards CACiv. This finding is in accordance with the cytokine expression profile of CACiv and is indicative of a shift towards immune-modulatory IL10 signaling pathways. The upregulation of the FXR/RXR and LXR/RXR associated genes APOE and NR1H3 in CACiv compared to CD14+ monocytes was confirmed by qPCR analysis (Figure 3(d)).

4. Discussion

In this article we used an in silico microarray analysis approach to explore the genotype of CACiv and were able to identify a gene expression profile characteristic of CACiv. The expression of several key genes was further substantiated with qPCR analysis. We compared the CACiv transcriptome with microarray data sets dealing with monocyte-macrophage and endothelial cell differentiation and found considerable evidence for macrophage lineage differentiation in the CACiv genotype but, surprisingly, little evidence for endothelial transdifferentiation. Further analysis of the nature of CACiv showed high correlation of the CACiv gene signature with the M2 macrophage subtype. Because macrophage M1 and M2 subtypes merely represent the extremes on a wide spectrum of possibilities of macrophage polarization and since we also found some conserved M1 lineage characteristics in the CACiv signature, CACiv most probably constitute a specific intermediate macrophage subtype, with predominant traits of regulatory M2c macrophages [16].

Several studies have dealt with the lineage commitment of EPC. A first study questioning the true progenitor cell nature of EPC was published by Rehman et al. [2], who revealed that mononuclear cells cultured for only a short period under EPC culture conditions mainly yielded cells that expressed monocyte markers, such as CD14, CD11b, CD11c and CD168, as such confining them to the monocyte/macrophage lineage. Considering the lack of evidence for endothelial cell differentiation, Rehman renamed these cells as CAC. Furuhata et al. [21] compared the characteristics of cultured CD34+ mononuclear cells with mature endothelial cells. Using hierarchical clustering, this group reported the absence of endothelial-specific marker expression, such as Tie2, angiopoietin-2, VE-cadherin, endoglin or KDR, even after 14 days in culture and found a high expression of macrophage-specific markers, such as GPNMB, matrix metalloproteinases 7 and 9, lysosomal acid lipase and APOE. Medina et al. [22], revealed a clear distinction in gene signature between CACiv and (late) outgrowth endothelial cells (OEC), also known as endothelial colony-forming cells (ECFC). ECFCs were closely related to endothelial cells, whereas the CACiv genotype clustered with monocytic cells and evidenced an alternative activated M2 macrophage genotype [23]. Finally, a UHC analysis study of Gremmels et al. [24] provided data on the relation of CACiv and ECFC with various other cell types of hematopoietic and mesenchymal origin and concluded that CACiv display a genotype that is restricted to the hematopoietic lineage, whereas ECFC, together with endothelial cell subtypes, belong to a large mesenchymal cell cluster.

The absence of endothelial markers in the CACiv signature is evident and, together with the evidence from previous studies, almost excludes direct transdifferentiation of CACiv.
into EC. Most likely, as demonstrated by Prokopi et al. [25], this hypothesis of transdifferentiation is the result of assay misinterpretation due to the contamination with platelet-derived microparticles of conventional mononuclear cell isolation procedures.

Macrophages show a remarkable degree of plasticity in response to specific environmental stimuli and many distinct macrophage subsets have been described [26]. Broadly speaking, macrophages are polarized along a spectrum of two extremes, M1 and M2 macrophages, which have different genotypes and function. M1 macrophages produce inflammatory cytokines, such as IL1β and TNF, play a role in Th1 responses and in the killing of pathogens and tumor cells. M2 macrophages, on the other hand, display anti-inflammatory properties, facilitate Th2 responses and engage in active tissue remodeling and tumor promotion. Recently, this dichotomized view on macrophage differentiation was challenged by a new paradigm [16] according to which macrophages are classified based on physiological activities, such as host defence, wound healing and immune regulation. Moreover, macrophage subtypes are thought to reflect 'blends' of these basic macrophage 'flavors', resulting in a tissue or disease-specific macrophage genotype. Because we also found expression of markers specific for osteoclasts and DCs, the CACiv profile probably reflects a macrophage subset with M2 predominance, closely resembling regulatory M2c macrophages. Still, CACiv express some M1-associated pro-inflammatory markers, such as IL1α and IL23α, which could aid CACiv to engage in important biological processes, such as tissue remodeling and angiogenesis. Additional studies, comparing the CACiv transcriptome with these and other macrophage-related cell types, are warranted.

Using IPA analysis, we identified riboflavin metabolism and the LXR/RXR and FXR/RXR pathways as the most significantly upregulated biological processes in the CACiv transcriptome.

LXRs are a family of cholesterol-sensing nuclear receptors regulating lipid homeostasis and cholesterol transport [27]. In macrophages, LXRα gene expression has been shown to be upregulated during monocyte to macrophage differentiation [28]. Treatment of ApoE-deficient atherosclerosis-prone mice with LXR agonists significantly reduced atherosclerotic lesion formation [29], highlighting the atheroprotective effects of LXR pathway induction. Interestingly, upregulation of the LXR pathways together with the DC chemokine receptor CCR7 has been reported in CD68+CD14+ macrophages in normal intima devoid of atherosclerotic disease [30]. The CACiv expression profile resembled that described by Trogan et al. [31], who showed in laser-capture microdissection-isolated foam cells that induction of the LXR pathway genes combined with increased CCR7 expression was a hallmark of atherosclerotic plaque regression and that atherosclerotic regression could be inhibited by targeting the CCR7 ligands CCL19 and CCL21. Our findings that LXR pathways and CCR7 are concomitantly upregulated in CACiv biology, could hint a possible role for CAC in reverse plaque remodeling.

We also demonstrated upregulation of FXR/RXR cholesterol-sensing nuclear receptors in the CACiv profile. Activation of FXR pathways in mouse models of atherosclerosis could almost completely inhibit aortic atherosclerotic lesion formation [32], attenuated the pro-inflammatory expression of IL1β, IL6 and TNF [33] and negatively modulated NFκB-mediated inflammation [34]. Furthermore, upregulation of the ABCA1 cholesterol transporter by FXR agonists in ApoE-deficient macrophages, led to the induction of an anti-atherogenic cholesterol 'unloading' mechanism in vivo [33].

Finally, IPA analysis indicated riboflavin metabolism as significantly upregulated in CACiv biology. Riboflavin or vitamin B2 is the central element of the cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). These cofactors are essential for mitochondrial oxidoreductase reactions, cellular oxidative stress resistance and endothelial nitric oxide synthase (eNOS) coupling and eNOS activity [35].

Together, the biological pathways observed in our CACiv profile, seem to relate to reverse cholesterol transport, immunomodulation, energy metabolism, oxidative stress resistance and NO bioavailability. These findings hint at a possible beneficial effect of CACiv therapy in the treatment of atherosclerotic and cardiovascular disease. Alternatively, pathophysiological conditions leading to impaired in vivo CAC function might induce endothelial dysfunction and atherosclerotic plaque formation and progression. However, future experimental studies are required to confirm these hypotheses.

In conclusion, our data indicate that CACiv are genotypically related to regulatory M2c macrophages. CACiv, however, show little evidence of endothelial cell transdifferentiation. We propose new mechanisms by which CACiv could be efficiently applied in the broad field of cardiovascular pathophysiology, more specifically by immunomodulation, tissue remodeling, enhancement of cholesterol efflux and vasculoprotection.

Data Availability

The microarray data used to support the findings of this study have been deposited and are publicly available in the Gene Expression Omnibus (GEO) repository (GSE2040 and GSE5099).

Disclosure

None.

Conflicts of Interest

The author(s) declare(s) that they have no conflicts of interest.

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Supplementary Materials

Basic statistical analysis of microarray data integrity. (Supplementary Materials)

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