Hindawi
Stem Cells International
Volume 2019, Article ID 9545261, 12 pages
https://doi.org/10.1155/2019/9545261

Research Article

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

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Received 22 August 2018; Revised 14 January 2019; Accepted 13 February 2019; Published 2 May 2019

Academic Editor: Tao-Sheng Li

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 (CAC_{iv}). Genes and pathways of interest were further evaluated using qPCR comparing CAC_{iv} versus $CD14^+$ monocytic cells. The CAC_{iv} transcriptome strongly related to tissue macrophages, and more specifically to regulatory M2c macrophages. The cytokine expression profile of CAC_{iv} was predominantly immune modulatory and resembled the cytokine expression of tumor-associated macrophages (TAM). Pathway analysis revealed previously unrecognized biological processes in CAC_{iv}, 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*. CAC_{iv} 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 subtype 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 CAC_{iv} 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_{iv}-specific gene signature, to determine the CAC_{iv} cytokine-cytokine receptor fingerprint and to investigate the biological processes that are important in CAC_{iv} 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_{iv} 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_{iv}, 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_{iv}, we collected blood of healthy volunteers. The CAC_{iv} 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_{iv} 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 CACiv and 3 gene expression profiles of CD14+ 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 ∂-value was selected in such a way that the misclassification error rate was minimal. The global clustering pattern of the CAC_{iv} 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_{iv} and $CD14^+$ monocytes.

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

The following strategy was adopted to evaluate the possible macrophage genotype of cultured CAC_{iv}. 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 ∂-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 shrunken 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_{iv} and monocytes.

To evaluate the degree of resemblance between CAC_{iv} 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_{iv} 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_{iv} and $CD14^+$ 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_{iv} 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, 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® gene expression assays (Applied Biosystems) were used for qPCR analysis on a LightCycler® 480 instrument (Roche). All primers were designed to be intron spanning. qPCR was performed using the LightCycler® Taqman® 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 (Cq) was measured using the baseline-independent second derivative maximum method. Normalized relative gene expression was determined by the $E^{-\Delta\Delta Cq}$ method. Assay efficiency (E) was measured by serial dilution of cDNA of pooled samples based on the slope of the standard dilution curve ($E = 10^{(1/\text{-slope})}$-1).

2.6. Statistical Analysis. Statistical analysis was performed in PASW® statistics 18 (IBM Corp.). Graphs were created in GraphPad Prizm®. 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 $CD14^+$ monocytic cells ($\partial = 2.85$, 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 lineage transcriptome was evident (e.g. *RGS2*, *NR4A2*, *FCN1*, *IL1B* and *SELL*). 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 *SLCO2B1*, 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 *SCLO2B1* 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*

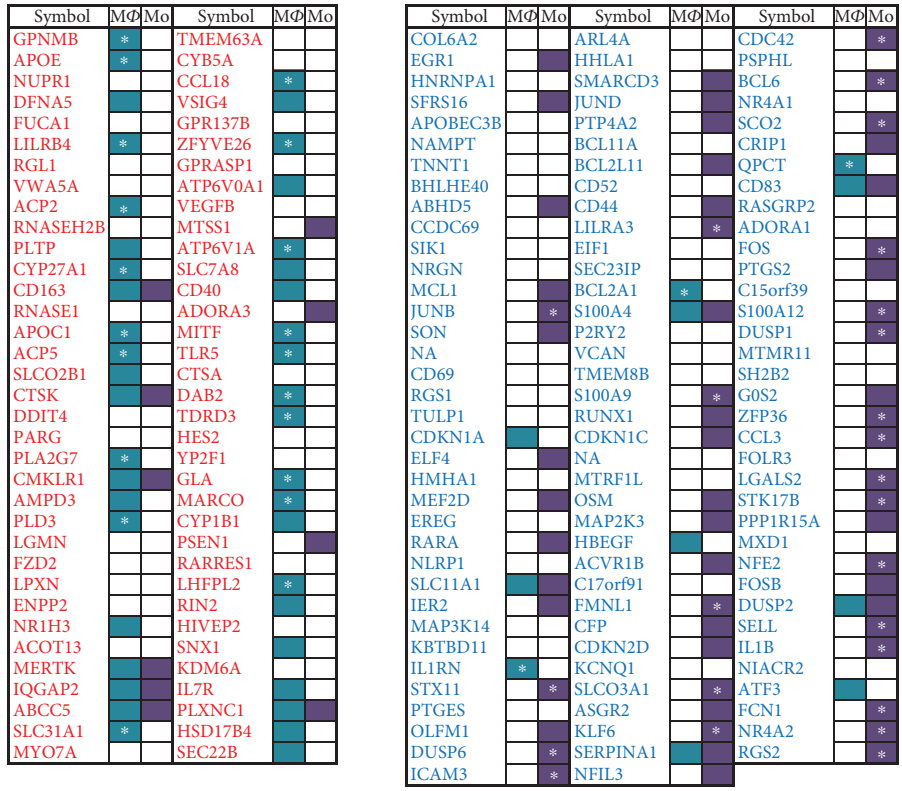

FIGURE 1: CAC_{iv} gene signature. 70 genes were significantly upregulated versus 107 genes were significantly downregulated in CAC_{iv} versus $CD14^+$ monocytic cells. The upregulated (red) and downregulated (blue) genes express a close lineage relationship with macrophages and monocytes, respectively. We highlighted those genes of the CAC_{iv} signature that are specific for either macrophages or monocytes. *: very specific. Mϕ: macrophage; Mo: monocyte.

and VSIG4), and additionally observed repression of several genes regulating transformation of macrophages into foam cells (*RGS2*, *NR4A2 and S100A12*).

To elaborate further on the resemblance of the M2 macrophage genotype and the transcriptome of cultured CAC_{iv}, we used a microarray data set (GSE5099) on the differentiation of monocytes into macrophages, and of macrophages into the M1 or M2 macrophage subtype (macrophages cultured for 18 h with LPS and IFN-γ or IL4, 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 differentation, 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 investigate 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-

Symbol	MΦ	Mo	EC	DC	Ocl	Full name
GPNMB	*					Glycoprotein (transmembrane) nmb
APOE	*					Apolipoprotein E
DFNA5						Deafness, autosomal domnant 5
LILRB4	*					Leukocyte immunoglobulin-like receptor
ACP2	*					Acid phosphatase 2, lysosomal
PLTP						phospholipid transfer protein
CYP27A1						cytochrome P450, famikly 27, subfamily A, polypeptide 1
CD163						CD163 molecule
RNASE1						Ribonuclease pancreatic
APOC1	*					Apolipoprotein C-1
ACP5	*					Acid phosphatase 5, tartrate-resistant
SLCO2B1						Solute carrier organic anion transporter family, member 2B1
CTSK						Cathepsin K
PLA2G7	*					Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)
CMKLR1						Chemokine-like receptor 1
AMPD3						Adenosine monophosphate deaminase 3
PLD3	*					Phospholipase D family, member 3
LGMN						Legumain
FZD2						Frizzled homolog 2 (Drosoophila)
LPXN						Leupaxin
ENPP2						Ectonucleotide pyrophosphatase/phosphodiesterase 2
NR1H3						Nuclear receptor subfamily 1, group H, member 3
MERTK						C-mer proto-oncogene tyrosine kinase
IQGAP2						IQ motif caontaining GTPase-activating protein 2
ABCC5						Multidrug resistance-associated protein 5
SLC31A1	*					Solute carrier family 31 (copper transporters), member 1
CCL18	*					Chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)
VSIG4						V-set and immunoglobulin domain-containing 4
ZFYVE26	*					Zinc finger, FYVE domain-containing 26
ATP6V0A1						ATPase, H+ transporting, lysosomal V0 subunit a1
MTSS1						Metastasis suppressor 1
ATP6V1A	*					ATPase, H+ transporting, lysosomal 70 kDa, V1 subunit A
SLC7A8						Solute carrier family 7 (amino acid transporter, L-type), member 8
CD40						CD40 molecule, TNF receptor superfamily member 5
ADORA3						adenosine A3 receptor
MITF	*					Microphthalmia-associated transcription factor
TLR5	*					Toll-like receptor 5
DAB2	*					Disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)
TDRD3	*					Tudor domain containing 3
GLA	*					Galactosidase, alpha
MARCO	*					Macrophage receptor with collagenous structure
CYP1B1						Cytochrome P450, family 1, subfamily B, polypeptide 1
PSEN1						Presenilin 1
LHFPL2	*					Lipoma HMGIC fusion parner-like 2
RIN2						Ras and Rab interactor 2
HIVEP2						Human immunodeficiency virus type I enhancer-binding protein 2
SNX1						Sorting nexin 1
IL7R						Interleikin 7 receptor
PLXNC1						Plexin C1
HSD17B4						Hydroxysteroid (17-beta) dehydrogenase 4
SEC22B						SEC22 vesicle traffiking protein homolog B (S. cerevisiae) (gene/pseudogene)

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. $M\phi$: macrophage; Mo: monocyte; EC: endothelial cell; DC: dendritic cell; Ocl: osteoclast.

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

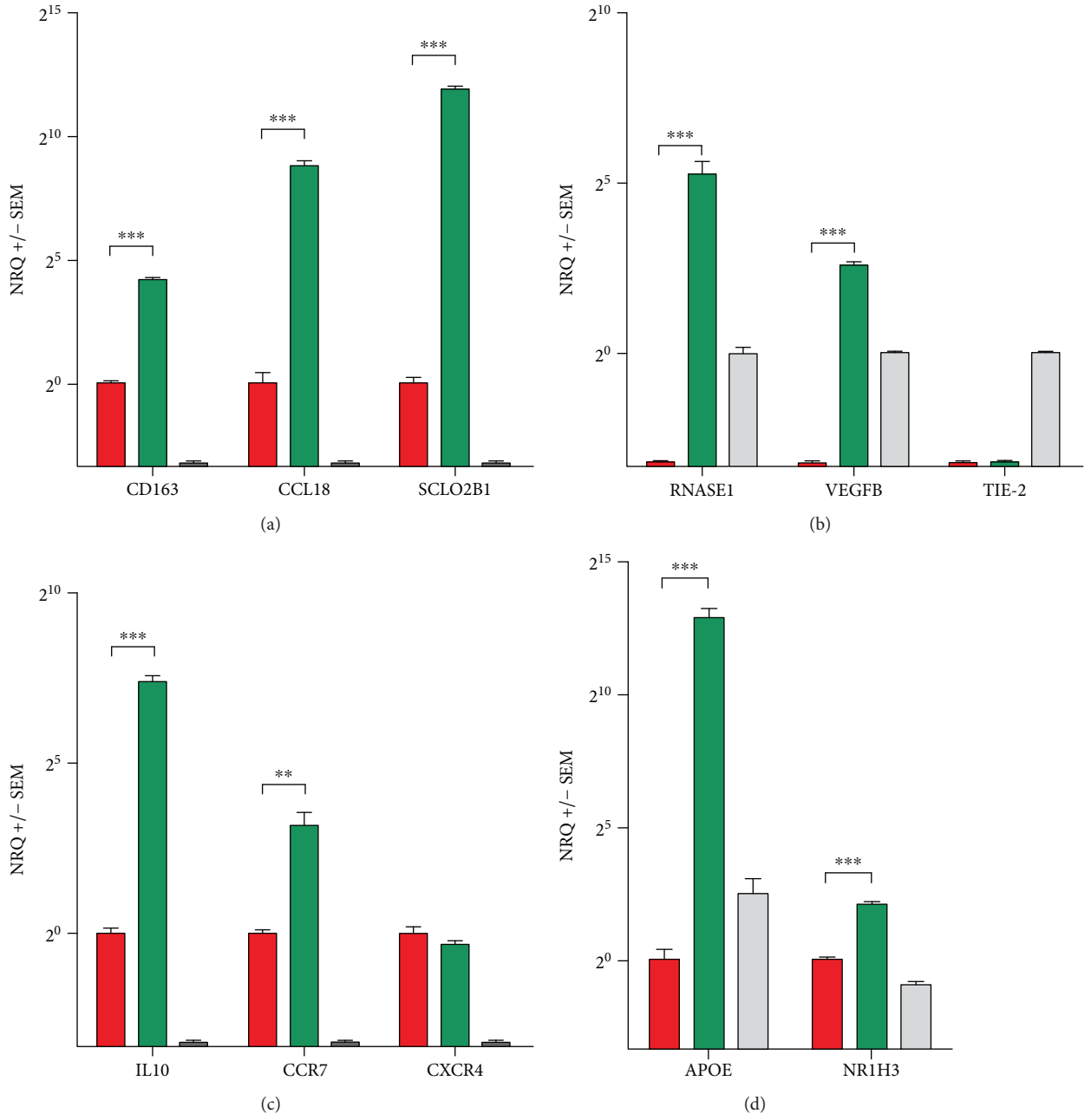

Figure 3: Gene expression analysis. Gene expression of (a) M2c macrophage-related, (b) endothelial, (c) cytokine/cytokine receptor and (d) cholesterol transporter pathway genes in CAC_{iv} (green) vs. $CD14^+$ monocytes (red) vs. human umbilical vein endothelial cells (HUVEC) (grey). Only the relevant statistical significance between CACiv and $CD14^+$ monocytes is depicted. NRQ: normalized relative quantity. ** $p < 0.01$; *** $p < 0.001$.

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 CAC_{iv} remains speculative. Since also the M1 macrophage-associated chemokine CCL2 was upregulated in CAC_{iv}, 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 CAC_{iv} and TAM.

Interestingly, CAC_{iv} 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 CAC_{iv} with important tissue remodeling and angiogenic properties in the setting of MI.

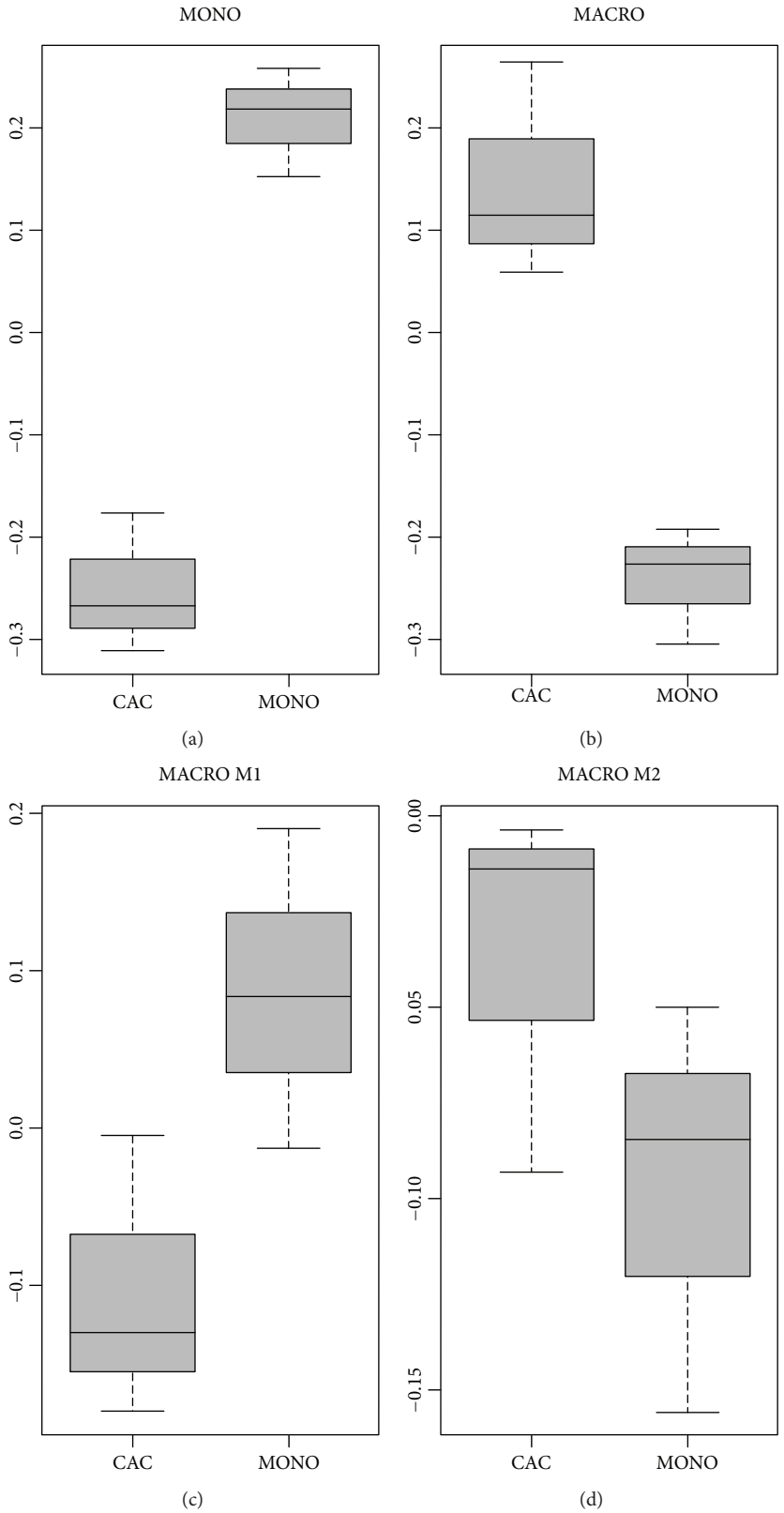

Figure 4: CAC_{iv} differentiate towards a predominanty M2 macrophage genotype. Boxplots comparing the relative expression of the genes in (a) the monocyte-specific gene signature (MONO), (b) the macrophage-specific gene signature (MACRO), (c) the LPS- and IFN-γ-stimulated M1 macrophage-specific gene signature (MACRO M1), and (d) the IL4-stimulated M2 macrophage-specific gene signature (MACRO M2), between CAC_{iv} and $CD14^+$ monocytic cells (MONO). Positive values signify a stronger degree of similarity of the genetic expression of CAC_{iv} or $CD14^+$ monocytic cells to the specified cell type.

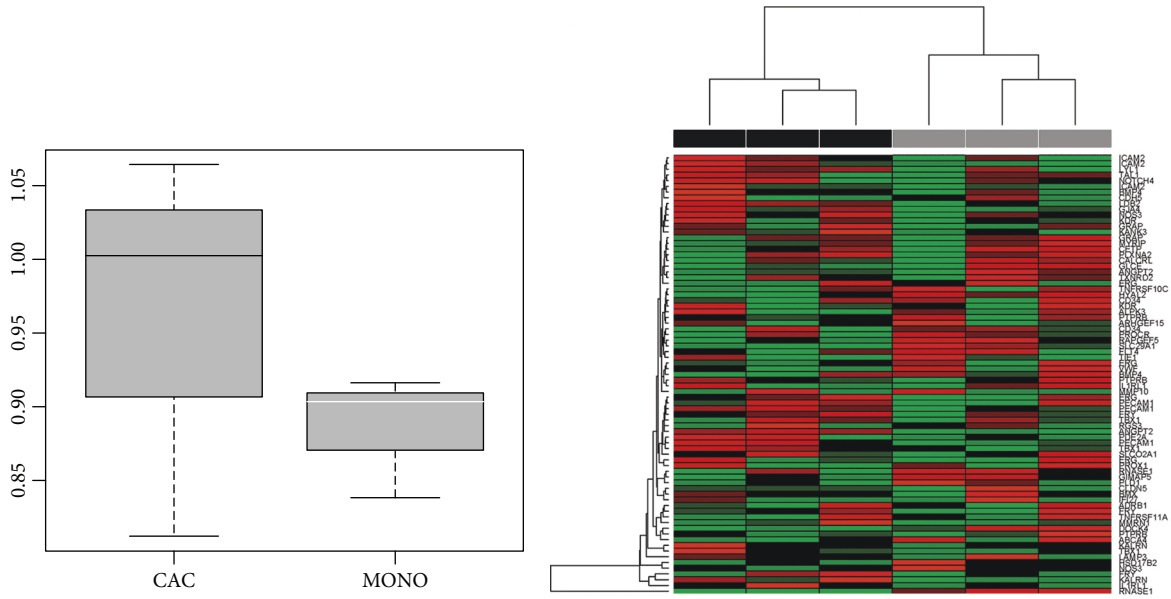

Figure 5: Analysis of EC-associated genes in the CAC_{iv} profile. (a) Boxplot comparing the relative expression of an endothelial gene set between CAC_{iv} and $CD14^+$ monocytic cells. There is no significant difference in similarity of the genetic expression of CAC_{iv} or $CD14^+$ monocytic cells to an EC-specific gene signature. (b) UHC analysis highlighting the relative expression of EC-associated genes in CAC_{iv} (grey) and monocytes (black). *RNASE1* is indicated as most differentially expressed in CAC_{iv} compared to $CD14^+$ monocytic cells. We observed a lack of consistency in EC-associated gene expression between different CAC_{iv} culture samples. Red: upregulation; green: downregulation.

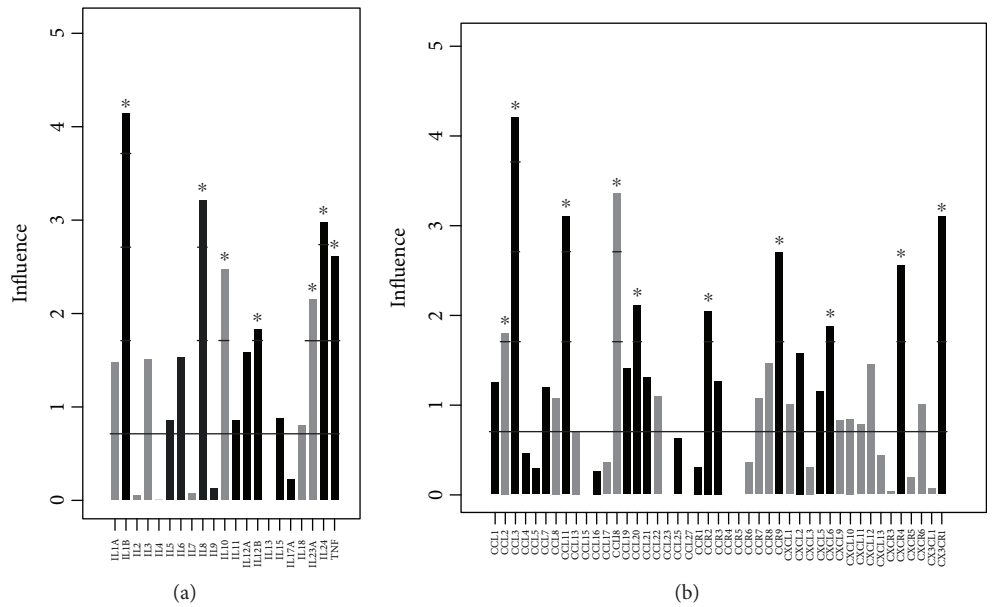

Figure 6: CAC_{iv} -related cytokine and chemokine/chemokine receptor expression profile. Gene expression plot depicting (a) cytokines or (b) chemokines and chemokine receptors that were differentially overexpressed in CAC_{iv} (grey) vs. monocytes (black). The influence (*y*-axis) represents the number of standard deviations (SD) the gene expression of each gene exceeds the null hypothesis that there would be no difference between both groups (z-score). Genes with an influence of ≥1.96 show a statistically significant differential gene expression ($p < 0.05$). * $p < 0.05$.

Finally, we identified the CCR7 chemokine receptor as the most differentially expressed chemokine receptor in Calthough its expression did not reach statistical significance. This chemokine receptor is commonly found on mature DCs [20] and facilitates lymphoid tissue homing. Interestingly, CXCR4, the receptor for the hypoxia-inducible chemokine

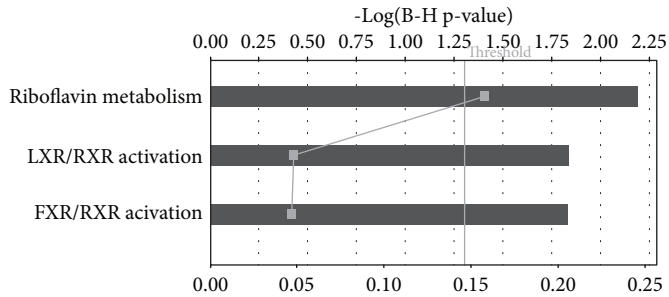

Figure 7: Summary of IPA analysis. The main upregulated canonical pathways are shown. Riboflavin metabolism and LXR/RXR and FXR/RXR activation remain statistically significant even after correction for multiple comparisons (Threshold indicates false discovery rate corrected p-value of 0.05). Bottom axis depicts the ratio of the number of genes upregulated in de CAC_{iv} signature divided by the total number of genes involved in a given pathway (squares).

CXCL12, showed higher expression on monocytes than on CAC_{iv}, suggesting that differentiated CAC_{iv}, do not display increased hypoxia-directed tissue homing capacity compared to $CD14^+$ monocytic cells. On qPCR analysis, the expression of *IL10* and *CCR7* was comfirmed to be significantly upregulated in CAC_{iv} (Figure 3(c)).

3.3. Pathway Analysis. Using IPA software, we determined the biological networks, functions and canonical pathways important to CAC_{iv} biology. The main CAC_{iv}-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 CAC_{iv} 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 CAC_{iv}. 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 CAC_{iv}. This finding is in accordance with the cytokine expression profile of CAC_{iv} and is indicative of a shift towards immunemodulatory IL10 signaling pathways. The upregulation of the FXR/RXR and LXR/RXR associated genes *APOE* and *NR1H3* in CAC_{iv} 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 CAC_{iv} and were able to identify a gene expression profile characteristic of CAC_{iv}. The expression of several key genes was further substantiated with qPCR analysis. We compared the CAC_{iv} transcriptome with microarray data sets dealing with monocyte-macrophage and endothelial cell differentiation and found considerable evidence for macrophage lineage differentiation in the CAC_{iv} genotype but, surprisingly, little evidence for endothelial transdifferentiation. Further analysis of the nature of CAC_{iv} showed high correlation of the CAC_{iv} 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 CAC_{iv} signature, CAC_{iv} 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 monocytic markers, such as CD14, CD11b, CD11c and CD168, as such confining them to the monocyte/macrophage lineage. Considering the lack of evidence for endothelial cel differentation, 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 metallopeptidases 7 and 9, lysosomal acid lipase and APOE. Medina et al. [22], revealed a clear distinction in gene signature between CAC_{iv} and (late) outgrowth endothelial cells (OEC), also known as endothelial colony-forming cells (ECFC). ECFCs were closely related to endothelial cells, whereas the CAC_{iv} 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 CAC_{iv} and ECFC with various other cell types of hematopoietic and mesenchymal origin and concluded that CAC_{iv} 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 CAC_{iv} signature is evident and, together with the evidence from previous studies, almost excludes direct transdifferentiation of CAC_{iv}

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 CAC_{iv} profile probably reflects a macrophage subset with M2 predominance, closely resembling regulatory M2c macrophages. Still, CAC_{iv} express some M1-associated pro-inflammatory markers, such as IL1α and IL23α, which could aid CAC_{iv} to engage in important biological processes, such as tissue remodeling and angiogenesis. Additional studies, comparing the CAC_{iv} 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 CAC_{iv} transcriptome.

LXRs are a family of cholesterol-sensing nuclear receptors regulating lipid homeostasis and cholesterol transport [27]. In macrophages, LXRa 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 CAC_{iv} 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 CAC_{iv} 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 CAC_{iv} 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 CAC_{iv} 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 CAC_{iv} 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 CAC_{iv} 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 CAC_{iv} are genotypically related to regulatory M2c macrophages. CAC_{iv}, however, show little evidence of endothelial cell transdifferentiation. We propose new mechanisms by which CAC_{iv} 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.

Funding

This work was supported by the Research Foundation - Flanders (FWO), grant No. G014906. B. Everaert was supported by a PH.D. fellowship of the Reseach Foundation – Flanders (FWO).

Acknowledgments

The authors would like to acknowledge the efforts of D. Vindevogel and D. De Rijck in editing this text.

Supplementary Materials

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

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