Chronic Kidney Disease Induces Inflammatory CD40⁺ Monocyte Differentiation via Homocysteine Elevation and DNA Hypomethylation

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**Rationale:** Patients with chronic kidney disease (CKD) develop hyperhomocysteinemia and have a higher cardiovascular mortality than those without hyperhomocysteinemia by 10-fold.

**Objective:** We investigated monocyte differentiation in human CKD and cardiovascular disease (CVD).

**Methods and Results:** We identified CD40 as a CKD-related monocyte activation gene using CKD-monocyte -mRNA array analysis and classified CD40 monocyte (CD40⁺CD14⁺) as a stronger inflammatory subset than the intermediate monocyte (CD14⁺CD16⁻) subset. We recruited 27 patients with CVD/CKD and 14 healthy subjects and found that CD40/CD40 classical/CD40 intermediate monocyte (CD40⁺CD14⁺/CD40⁺CD14⁺CD16⁻/CD40⁺CD14⁺CD16⁻), plasma homocysteine, S-adenosylhomocysteine, and S-adenosylmethionine levels were higher in CVD and further elevated in CVD+CKD. CD40 and CD40 intermediate subsets were positively correlated with plasma/cellular homocysteine levels, S-adenosylhomocysteine and S-adenosylmethionine but negatively correlated with estimated glomerular filtration rate. Hyperhomocysteinemia was established as a likely mediator for CKD-induced CD40 intermediate monocyte, and reduced S-adenosylhomocysteine/S-adenosylmethionine was established for CKD-induced CD40/CD40 intermediate monocyte. Soluble CD40 ligand, tumor necrosis factor (TNF)-α/interleukin (IL)-6/interferon (IFN)-γ levels were elevated in CVD/CKD. CKD serum/homocysteine/CD40L/increased TNF-α/IL-6/IFN-γ-induced CD40/CD40 intermediate monocyte in peripheral blood monocyte. Homocysteine and CKD serum-induced CD40 monocyte were prevented by neutralizing antibodies against CD40L/TNF-α/IL-6. DNA hypomethylation was found on nuclear factor-kB consensus element in CD40 promoter in white blood cells from patients with CKD with lower S-adenosylhomocysteine/S-adenosylmethionine ratios. Finally, homocysteine inhibited DNA methyltransferase-1 activity and promoted CD40 intermediate monocyte differentiation, which was reversed by folic acid in peripheral blood monocyte.

**Conclusions:** CD40 monocyte is a novel inflammatory monocyte subset that appears to be a biomarker for CKD severity. Hyperhomocysteinemia mediates CD40 monocyte differentiation via soluble CD40 ligand induction and CD40 DNA hypomethylation in CKD. (Circ Res. 2016;119:1226-1241. DOI: 10.1161/CIRCRESAHA.116.308750.)

**Key Words:** CD40 ligand ■ DNA methylation ■ homocysteine ■ monocytes ■ renal insufficiency, chronic

Homocysteine and its metabolite, S-adenosylhomocysteine, are uremic toxins that accumulate in the plasma of patients with chronic kidney disease (CKD) because of impaired extrarenal metabolism. Cardiovascular disease (CVD) is prevalent in patients with CKD and accounts for 50% of deaths in the end-stage renal disease. Patients with CKD have a 10 to 30× higher cardiovascular mortality. Hyperhomocysteinemia has been established as an independent risk factor for CVD and a cause of cardiovascular events in CKD and can be used as a biomarker to predict the prognosis of CVD outcome in CKD. Homocysteine-lowering therapy was initially reported to be not beneficial to general vascular outcomes in secondary prevention trials, but VITATOPS (folic acid-based therapy) and HOPE2 (Heart Outcomes Prevention Evaluation 2), but were reported later to have significantly reduced stroke recurrence and death in the post hoc analysis of the same trials. The
The causative effect of hyperhomocysteinemia on CVD is supported by several important trails including a large primary prevention trial, CSPPT (China Stroke Primary Prevention Trial), and a large population-based cohort study. These 2 studies showed that folic acid therapy reduced overall CVD and stroke incidence in hypertensive patients and that folic acid fortification reduced stroke mortality in the US general population, respectively; however, the mechanism underlying the induction of CVD by hyperhomocysteinemia in CKD patient population is unknown.

Monocytes are highly plastic and heterogeneous. Their functional phenotype could change in response to environmental stimuli such as uremic toxins. It is recently recognized that monocytes can be divided into 3 subsets, classical (CD14++CD16–), intermediate (CD14+CD16+), and nonclassical (CD14+CD16++), based on cell surface marker expression. The intermediate monocyte subset is known to be a cellular hallmark of chronic inflammation that is associated with the burden of CVD, CVD in CKD, and death in the end-stage renal disease.

We and others have reported that hyperhomocysteinemia induces systemic and vascular inflammation and cytokine production and that it also potentially accelerates atherosclerosis via inflammatory monocyte differentiation. We demonstrated that hyperhomocysteinemia induces both inflammatory monocyte (Ly6Chigh+middle), the counterpart of human inflammatory monocyte subset in mice, and monocyte-derived inflammatory M1 macrophages (Mφ) in cystathionine β-synthase gene-deficient mice compound with streptozotocin-induced hyperglycemia. We provided evidence showing that hyperhomocysteinemia-induced DNA hypomethylation may be responsible for inflammatory monocyte differentiation.

Homocysteine is a metabolite of methionine, and it can be converted to S-adenosylhomocysteine, a potent inhibitor for methyl transferase. Uremia-associated hyperhomocysteinemia is associated with altered epigenetic regulation and global DNA methylation change. Promoter DNA hypomethylation of p66Shc (SHC1), a stress responsible protein, was identified in blood cells from CKD subjects. Differential DNA methylation patterns were found in genes related to inflammation and oxidation in diseased kidneys. The mechanism of hyperhomocysteinemia-related DNA methylation and its contribution to inflammatory monocyte differentiation in CKD-related CVD have not been studied.

In this study, we identified CD40 monocyte as a biomarker of CKD, found the metabolic connection between homocysteine and methylation metabolites, and discovered their relation to inflammatory monocyte differentiation in patients with CVD with or without CKD.

**Methods**

**Human Subjects**

We analyzed blood samples from 27 patients with CVD (13 CVD and 14 CVD+CKD) from the Temple University Vascular Surgery and Nephrology practice and 14 healthy donors with no history of CKD and CVD from the Thrombosis Center at Temple University. Case subjects had defined clinical and objective investigational evidence of all types of vascular disease in which monocyte counts and hyperhomocysteinemia were identified to be independent risk factors.

Conditions thought to influence homocysteine concentrations and monocyte counts (eg, recent systemic infection or thyroid disease) served as exclusion criteria for both cases and control subjects. CKD stage estimation and demographics/clinical information are described in Online Table I and Online Table II. For subjects with stage 5 CKD, blood samples were collected before using the hemodialysis (more details in Methods in the Online Data Supplement).

**Microarray Analysis**

Microarray data (GSE43484) were analyzed in the R statistical environment using Biobase, GEOquery, and limma Bioconductor projects and interpreted by DAVID bioinformatics resources tools. Genome-wide gene expression profiles were established from freshly isolated peripheral blood monocytes from CKD stage 4 to 5 patients and healthy subjects with a median age of 59 years. (more details in Methods in the Online Data Supplement).

**Metabolite (Homocysteine, S-Adenosylmethionine, and S-Adenosylhomocysteine) Analyses**

White blood cells (WBCs) were isolated after red blood cell lysis. Plasma and cellular metabolites were measured using liquid chromatography-electrospray ionization tandem mass spectrometry. Plasma and cellular metabolites were measured using liquid chromatography-electrospray ionization tandem mass spectrometry. (more details in Methods in the Online Data Supplement).

**Plasma Soluble CD40 ligand Measurement**

Platelets were the predominant source of soluble CD40 ligand (sCD40L) in plasma. Freezing–thawing of the prepared plasma causes lysis of residual platelets. On the day of blood collection, platelets in plasma were removed. Plasma levels of CD40L were measured by ELISA.

**Peripheral Blood Mononuclear Cell Cultivation and Differentiation**

Peripheral blood mononuclear cells (PBMCs) were isolated from 50 mL of whole blood and treated with human recombinant-CD40L, tumor necrosis factor (TNF)-α or interleukin (IL)-6, mouse IgG, mouse anti-human IL-6, mouse anti-human TNF-α, or folic acid (more details in Methods in the Online Data Supplement).

**Blood Monocyte Isolation and Cultivation**

CD14+ monocyte was isolated as described, with modification. Cells were washed and stained with anti-CD14 antibody. Approximately 3 to 5×10^6 cells were isolated with a 95% purity of CD14+ monocyte from 30×10^6 PBMCs.

**Serum Cytokine Array**

Serum cytokine levels were determined by using a commercially available array kit according to manufacturer instructions (human cytokine array Q1; RayBiotech).

**CD40 Promoter DNA Methylation Mapping in WBC**

1×10^6 WBC were used for genomic DNA preparation and CD40 core promoter methylation mapping (more details in Methods in the Online Data Supplement).
DNA Methyltransferase Protein and Activity Analysis
PBMC were cultured as above. Nuclear protein was extracted and assayed for DNA methyltransferase (DNMT) protein levels and DNMTs activity as described29 (more details in Methods in the Online Data Supplement).

CpG Island and Core Promoter Mapping
A promoter CpG island was searched using a CpG Island Search engine (http://cpgislands.usc.edu). Transcription factor binding sites were mapped as identified previously28,31 and predicted by database TESS (more details in Methods in the Online Data Supplement).

Mediation Analysis
We investigated the mediation effects (MEs) of plasma/ cellular homocysteine and S-adenosylmethionine/S-adenosylhomocysteine ratio in the 3 CKD-induced inflammatory monocyte subsets, ie, intermediate monocyte, CD40 monocyte, and CD40 intermediate monocyte. The direct and total residual effects of estimated glomerular filtration rate (eGFR) on monocyte subsets were estimated using the standard mediation method by testing the cellular/plasma homocysteine or S-adenosylmethionine/S-adenosylhomocysteine as a mediator26–34 (more details in Methods in the Online Data Supplement).

Results

CKD Induces CD40 Monocyte That Expresses Stronger Inflammatory Markers
Using a database mining strategy, we analyzed human CKD monocyte-mRNA screening data and compared gene expression profiles between stage 4 and 5 CKD patients and healthy subjects.27 We discovered 109 genes with altered mRNA levels in at least 1 of the 3 CKD susceptible disease categories, and 14 of them were in all 3 disease categories. Thirty-one genes were altered in at least 1 of the 3 monocyte activation gene categories. Interestingly, CD40 is the only gene that belongs to all 3 monocyte activation-related categories (Figure 1A). This implies that CD40 is a potential marker for monocyte activation in the patient population with CKD. We established gating methods for CD40 monocyte (CD40+CD14+) in Figure 1B. CD40 expression was observed in 65% monocyte compared with the paired isotype control. To validate whether CD40 expression is related to inflammatory feature of monocyte, we examined the expression of 9 inflammatory markers in CD40 monocyte by costaining using antibodies against T-cell activation surface markers CD86/CD80/HLA-DR, adhesion receptors CD62L/CD49d/CD11b,38 and chemokine receptors CCR2/CCR5/CX3CR112 (Figure 1C). CD40+CD14+ monocyte expressed higher levels of inflammatory markers CD86/HLA-DR/CD11b/CD49d/CCR2/CCR5/CX3CR1 compared with CD40–CD14+ monocyte. In general, CD40 monocyte is similar to that in intermediate monocyte (CD14+CD16+), which was denoted as inflammatory monocyte subset on the common nomenclature (Figure 1D) with respect to inflammatory markers. Interestingly, T-cell activation marker CD86 and chemokine receptor CCR2 were higher in CD40 monocyte than in intermediate monocyte (Figure 1C).

Macrophage, CD40 Monocyte, and CD40 Intermediate Monocyte Are Increased in CKD and CVD Subjects
We characterized circulating monocyte (CD14+), macrophage (CD14+CD16+), and dendritic cells (HLA-DR+CD16+) in human subjects (Online Figure IIA) and found that macrophage was elevated from 3.5±0.35% in PBMC in healthy subjects to 5.8±0.72% in CKD+CVD patients (1.7-fold induction), whereas monocyte and dendritic cell were not significantly different between the 2 groups. CD40 macrophage and CD40 monocyte were significantly increased in CVD+CKD patients (Online Figure IIB). The intermediate monocyte subset was previously considered to be the inflammatory monocyte and a predictor of cardiovascular event in CKD subjects.13,14 The classical monocyte subset (CD14+CD16+) and nonclassical monocyte subset (CD14+CD16−) were thought to carry out phagocytosis and patrolling functions based on common nomenclature (Figure 1D). We characterized circulating monocyte subsets in all subjects (Figure 2B). Monocyte cloud is 5% to 10% of WBC and contains ≈85% monocyte. Compared with that in healthy subjects, the classical monocyte subset was decreased from 87±1.5% of monocyte to 79±2.5% and 79±2.3% for CVD and CKD+CVD patients, respectively. The intermediate monocyte subset was increased from 8.4±0.7% of monocyte in healthy subjects to 14±1.5% and 13±1.1% in CVD and CKD+CVD patients, respectively, whereas nonclassical monocyte was not significantly different between the 3 groups. CD40+ population was 37% in the intermediate monocyte subset, which was significantly higher than that in the classical (15%) and nonclassical (28%) monocyte subsets (Figure 2C). When compared with the healthy subjects, classical monocyte was lower whereas intermediate and nonclassical monocyte were higher in CVD; however, it was not further increased in CVD+CKD patients (Figure 2D through 2F). Nonetheless, CD40 monocyte, CD40 classical, and CD40 intermediate subsets were elevated in CVD and CVD+CKD patients and seemed to increase linearly with the elevation of CKD severity (Figure 2G, 2H, and 2J).

Plasma Glucose Are Increased in CKD+CVD Patients, but Not Correlated With Inflammatory Monocyte
Plasma glucose levels were increased from 116 mg/dL in healthy subjects to 151 mg/dL in CVD+CKD patients (a 30% induction; Online Figure IIIE). It was weakly correlated with CD40 intermediate monocyte (r=0.35; P=0.03) and not correlated with intermediate monocyte and CD40 monocyte by simple regression analysis (r=0.35; P=0.03 and r=0.35; P=0.03; Online Figure IIIG).

Homocysteine Levels Are Increased in the Plasma and WBC in CKD+CVD and Positively Correlated With Inflammatory Monocyte Subsets and Hypomethylation Status
We examined relevant traditional risk factors for CVD/CKD and methylation metabolites and found that plasma homocysteine levels were increased from 9.1 μmol/L in healthy subjects to 12.7 and 20.3 μmol/L in CVD and CVD+CKD patients, respectively (Figure 3A; Online Figure IIIA). It was positively correlated with plasma S-adenosylhomocysteine and S-adenosylmethionine (Figure 3G) and all 3 inflammatory monocyte subsets (intermediate, CD40, and CD40 intermediate monocyte; Figure 3F), whereas being negatively correlated with eGFR (Figure 3I). Cellular homocysteine
Figure 1. Chronic kidney disease (CKD) induces CD40 expressing in monocyte (MC) and CD40+CD14+ MC inflammatory classification. A. Disease and MC activation-related genes are altered in human CKD MC. Data mining analysis was performed by DAVID bioinformatics resources tool (as described in Materials and Methods) using human CKD MC mRNA screening database. (Continued)
Figure 1 Continued. Fourteen genes with altered expression were identified and jointly associated with 3 CKD susceptible diseases as shown in the first Venn diagram and are listed in the table. CD40 is the sole gene that was induced in CKD MC and jointly associated with 3 MC activation–related categories, as shown in the second Venn diagram. B, MC subset gating. C, CD40 MC classification and MC activation marker analysis. White blood cells (WBCs) from healthy subjects were isolated and stained with anti-CD14, anti-CD16, and anti-CD40 antibodies, and costained with surface markers for T-cell activation (CD80, CD86, and HLA-DR, an MHC class II cell surface receptor), adhesion receptors (CD62L, CD11b, and CD49d), and chemokine receptors (CCR5, CX3CR1, and CCR2). MCs are defined as CD14+ cells from MCC. Representative dot plots and histograms depict CD40– cells. MC activation markers were quantified in CD40− vs CD40+ MC or common MC subsets (CD14−CD16+, CD14−CD16+, and CD14−CD16+; CD40 MC) and analyzed by flow cytometry. C, Common subsets by nomenclature. Schematic description summarizes the commonly recognized MC classification and function (common nomenclature). D, Working model. Values represent mean±SEM (n=4); *P<0.05 vs CD40− MC; †P<0.05 vs CD40+ MC; ‡P<0.05 vs CD14−CD16+ MC. CVD indicates cardiovascular disease; and MCC, monocyte cloud.

level was increased from 3.7 nmol/L/g in healthy subjects to 8 nmol/L/g in CVD+CKD patients (Figure 3B), positively correlated with plasma homocysteine (Figure 3C) and cellular S-adenosylhomocysteine and S-adenosylmethionine (Figure 3H), and negatively correlated with eGFR (Figure 3I). We considered the homocysteine metabolic cycle as the methylation cycle because of the fact that its metabolites (S-adenosylhomocysteine and S-adenosylmethionine) determine methylation status (Figure 3 schematic) and examined S-adenosylhomocysteine and S-adenosylmethionine in the plasma and WBC. Compared with the healthy subjects, CVD and CKD+CVD patients’ plasma S-adenosylhomocysteine levels were increased from 16.8±1.67 nmol/L to 25±2.6 nmol/L and 128±34 nmol/L (1.48- and 5.12-fold induction; Figure 3D; Online Figure IIIB), and plasma S-adenosylmethionine levels were increased from 86.6±5.5 nmol/L to 95±6.2 nmol/L and 148.7±18.4 nmol/L (1.7- and 1.56-fold induction), respectively (Figure 3D; Online Figure IIIC). Plasma S-adenosylmethionine/S-adenosylhomocysteine ratio, an indicator of methylation status, was decreased from 5.8 to 3.9 and 2 in CVD and CKD+CVD patients (1.48- and 2-fold reduction; Figure 3D; Online Figure IIIID), respectively. We divided human subjects into normal (plasma homocysteine level <15 μmol/L) and hyperhomocysteinemia (plasma homocysteine level ≥15 μmol/L) groups* and found that intermediate, CD40, and CD40 intermediate monocyte were increased from 10.1±0.8%, 13.1±2.6%, and 5.7±1.3% of monocyte in normal group to 13.5±1.3%, 26.1±3.9%, and 10.7±2% in hyperhomocysteinemia group, respectively (Figure 3E); they were positively correlated with plasma homocysteine levels (Figure 3F). Plasma/cellular homocysteine levels were positively correlated with plasma/cellular homocysteine and plasma S-adenosylmethionine and S-adenosylmethionine levels, respectively (Figure 3G and 3H). eGFR level was negatively correlated with plasma/cellular homocysteine and plasma methylation metabolites S-adenosylmethionine and S-adenosylhomocysteine (Figure 3I and 3J), and CD40 and CD40 intermediate monocyte (Online Figure IIIE, respectively). Plasma total cholesterol levels were not significantly different, but plasma glucose levels were increased from 116 mg/dL in healthy subjects to 151 mg/dL in CKD+CVD patients (Online Figure IVE and IVF).

Plasma Homocysteine Level Is Likely a Mediator for CKD-Induced CD40 Intermediate Monocyte Differentiation (Mediation Analysis With Adjustment)

We performed mediation analysis to further determine the role of hyperhomocysteinemia in CKD-induced inflammatory monocyte (Figure 4). After having adjusted for, age, sex, hypertension, and hypercholesterolemia whenever necessary, we observed a significant direct reduction effect of eGFR on plasma homocysteine (α=–0.081*; P<0.05; 95% confidence interval [CI], 0.156 to –0.006) (Figure 4A through 4C), but not on cellular homocysteine (α=–0.030; P>0.10; 95% CI, –0.065 to 0.006; Figure 4D through 4F). eGFR also demonstrated a significant total negative effect on CD40 monocyte (β=–0.192*; P<0.05; 95% CI, –0.353 to –0.031) and CD40 intermediate monocyte (β=–0.079*; P<0.05; 95% CI, –0.150 to –0.007), but not on intermediate monocyte (Figure 4A through 4F). Plasma homocysteine elevation exhibited a marginally significant ME on CD40 intermediate monocyte differentiation (β=0.279*; P<0.10; 95% CI, –0.057 to 0.615), demonstrating a 29% ME on the eGFR deficiency-induced CD40 intermediate monocyte (ME=29%), the residual effect of eGFR became insignificant (β=–0.064; P>0.10; 95% CI, –0.136 to 0.007) in the presence of the ME of plasma homocysteine (Figure 4C). No significant MEs were found for plasma homocysteine on the other 2 CD40 monocyte subsets or for cellular homocysteine on the 3 CD40 monocyte subsets under consideration (Figure 4A and 4B and 4D through 4F); however, eGFR showed a consistent residual effect on CD40 monocyte (β=–0.205*; P<0.05; 95% CI, –0.408 to –0.003) and CD40 intermediate monocyte (β=–0.118*; P<0.05; 95% CI, –0.218 to –0.118) even in the presence of the possible ME of cellular homocysteine (Figure 4E and 4F).

Reduced Plasma/Cellular S-Adenosylmethionine/S-Adenosylhomocysteine Ratio Is a Stronger Mediator for CKD-Induced CD40 Monocyte Differentiation (Mediation Analysis With Adjustment)

We also performed mediation analysis to determine the role of methylation status (S-adenosylhomocysteine/S-adenosylmethionine ratio) on CKD-induced inflammatory monocyte differentiation (Figure 4). We observed a significant positive direct effect of eGFR on plasma S-adenosylmethionine/S-adenosylhomocysteine (α=0.048*; P<0.05; 95% CI, 0.026 to 0.070; Figure 4G through 4I) but not on cellular S-adenosylmethionine/S-adenosylhomocysteine (α=0.027; P>0.10; 95% CI, 0.024 to 0.078; Figure 4J through 4L). Reduced plasma S-adenosylmethionine/S-adenosylhomocysteine exhibited a significant ME on CD40 monocyte and CD40 intermediate monocyte (β=–3.146*; P<0.05; 95% CI, –5.531 to –0.791 and β=–1.266*; P<0.05; 95% CI, –2.298 to –0.233), with 79% and 77% MEs on the eGFR deficiency–induced CD40 monocyte and CD40 intermediate monocyte (ME=79% and 77%). The residual effect of eGFR became insignificant (β=–0.029; P>0.10; 95% CI, –0.216 to 0.158 and c′=–0.021; P>0.10; 95% CI, –0.103 to 0.060) in the presence of the ME.
Figure 2. CD40 monocyte (MC) subsets markers chronic kidney disease (CKD) stages in cardiovascular disease (CVD) subjects. Peripheral blood was collected from human subjects for MC subset and blood chemistry analyses. White blood cells were isolated after red blood cell lysis and stained with antibodies against CD14, CD16, and CD40 for flow cytometric analysis. A. Characteristics of subjects. Diabetes mellitus were determined based on whether the patient was currently receiving hypoglycemic therapy or whether their fasting glucose level was ≥126 mg/dL. Hypertension, hyperlipidemia, and CKD stage were determined as described in Materials and Methods in the Online Data Supplement. Representative dot plots depict 3 commonly recognized MC subsets; CD14++CD16– classical, CD14++CD16+ intermediate, and CD14+CD16++ nonclassical MC. B. CD40 MC gating in MC subsets. CD40 MC and common MC subsets are gated as described in Figure 1B. The percentage of CD40+ cells in 3 MC subsets was quantified. D-F, MC subsets. Common MC subsets were quantified and profiled in all subject groups. G-I, CD40 MC subsets. J, Total CD40 MC. CD40+ cells in common MC subsets and total CD40 MC were quantified and profiled in all subject groups. Arrows indicate the direction of significant changes. The schematic description summarizes the working model. Values represent mean±SEM; *P<0.05 vs healthy; #P<0.05 vs CVD. MCC indicates monocyte cloud; and PBMC, peripheral blood mononuclear cells.
Figure 3. Homocysteine (Hcy) levels are increased in patients with chronic kidney disease (CKD), and positively correlated with intermediate, CD40, and CD40 intermediate monocyte (MC) subsets. Peripheral blood was collected from human subjects for MC subset analysis, as described in Figure 2, and for Hcy/methylation metabolite analysis as described in the Materials and Methods in the Online Data Supplement. Correlation was determined by linear regression analysis. A–C, Plasma Hcy, cellular Hcy, and correlation of plasma Hcy with cellular Hcy. D, Plasma methylation metabolites. E and F, MC subset change in hyperhomocysteinemia (HHcy) condition and its correlation with plasma Hcy levels. Subjects were divided into 2 groups, plasma Hcy <15 μmol/L (normal Hcy, n=25) and >15 μmol/L (HHcy, n=16). MC populations for intermediate MC, CD40, and CD40 intermediate MC subsets were quantified and analyzed for correlation with plasma Hcy. G and H, Correlation of plasma and cellular Hcy with methylation metabolites and MC subsets. I and J, Correlation of estimated glomerular filtration rate (eGFR) with Hcy and methylation metabolites. Hcy and methylation metabolites are described in the framed methylation cycle. The schematic description in the frame summarizes the working model. Each dot represents one subject. Values represent mean±SEM; *P<0.05 vs healthy; #P<0.05 vs CVD. Arrows indicate the direction of significant changes. SAH indicates S-adenosylhomocysteine; and SAM, S-adenosylmethionine.
of plasma S-adenosylmethionine/S-adenosylhomocysteine (Figure 4H and 4I).

Cellular S-adenosylmethionine/S-adenosylhomocysteine ratio reduction exhibited a possible ME on CD40 monocyte and CD40 intermediate monocyte differentiation \( (b=-2.320^*; P<0.05; 95\% CI, -3.654 to -0.986 and b=-0.816^*; P<0.10; 95\% CI, -1.642 to 0.011) \) with a likely 33\% and 28\% ME on the eGFR deficiency–induced CD40 monocyte and CD40 intermediate monocyte (ME=33\% and 28\%), respectively. Moreover, eGFR presented a consistent residual effect on CD40 monocyte and CD40 intermediate monocyte \( (c'=-0.160^*; P<0.05; 95\% CI, -0.295 to 0.1025 and c'=-0.885^*; P<0.05; 95\% CI, -0.171 to 0.001) \) in the presence of the possible ME of cellular S-adenosylmethionine/S-adenosylhomocysteine ratio reduction (Figure 4J through 4L); however, such findings were very weak because the direct effect of eGFR on cellular S-adenosylmethionine/S-adenosylhomocysteine was not statistically significant \( (a=0.027; P>0.10; 95\% CI, -0.024 to 0.078; Figure 4J through 4L). \)

**CDK Serum and Homocysteine Treatment Induce Intermediate, CD40 Monocyte, and CD40 Intermediate Monocyte Differentiation in PBMC**

To confirm the ME of hyperhomocysteinemia on CDK-induced CD40 monocyte differentiation, we cultured human PBMC and treated it with patients’ serum and exogenous homocysteine (100 μmol/L). CVD serum and homocysteine treatment increased intermediate monocyte by 1.4- and 1.34-folds (Figure 5B), respectively. CD40 monocyte was increased by 1.2- and 1.4-folds in CVD and CDK+CVD serum-treated groups and by 1.4-folds in homocysteine-treated group, respectively (Figure 5C). CD40 intermediate monocyte was increased by 1.4-folds in CDK+CVD serum-treated...
group but was not significantly different in CVD serum- and homocysteine-treated PBMC (Figure 5D).

**Plasma sCD40L Is Increased in CKD and CVD.** CD40L Induces Intermediate and CD40 Intermediate Monocyte Differentiation in PBMC

We examined plasma levels of sCD40L in all subjects and found that it was elevated from 171±17 in healthy subjects to 267±30 pg/mL in patients with CVD and 247±27 pg/mL in patients with CKD+CVD (156% and 144% induction, respectively; Figure 6A). In addition, plasma sCD40L levels were positively correlated with plasma homocysteine levels and intermediate monocyte (Figure 6B). This may imply that elevated CD40L in CVD patients’ serum induces inflammatory monocyte; hence, we investigated the effect of CD40L on the early stage monocyte differentiation in IFN-γ–primed (72 hours) PBMC (Figure 6C). CD40L induced monocyte differentiation in a dose-sensitive manner and elevated monocyte by 1.3-, 1.8-, and 1.5-folds at 24, 48, and 72 hours, respectively (Figure 6D), and intermediate monocyte by 1.9-folds at 72 hours (Figure 6E). It is known that CD40–CD40L signaling induces CD40 and CD86 expression in monocyte. We confirmed that CD40L increased CD86 and CD40 monocyte by 1.6- and 1.4-fold, respectively (Figure 6F and 6G) and induced CD40 intermediate monocyte differentiation by 1.7-folds (Figure 6G). CD40L-neutralizing antibody partially reversed CKD serum-induced CD40 intermediate monocyte from 1.73- to 1.32-folds (Figure 6H).

**Monocyte and T-Cell Origin Inflammatory Cytokines Were Induced in Patients With CKD and Synergistically Induced CD40 Monocyte With Homocysteine**

We screened 20 cytokines in the serum of human subjects using ELISA and found that 4 cytokines of monocyte origin (IL-12p70/GM-CSF/IL-6/TNF-α) were elevated in patients with CKD and CVD when compared with those of healthy subjects. Similarly, TNF-α level was increased from 132±12 to 190.6±17 pg/mL and 232.6±26 pg/mL, and IL-6 levels were increased from 13.2±1.9 to 20.2±3 pg/mL and 33.58±1.7 pg/mL in patients with CVD and CKD+CVD, respectively. The absolute levels of CD4+ T-cell–dominant cytokines (IL-4/IFN-γ/IL-5/IL-12p70) were relatively low in the healthy subjects but were elevated in patients with CVD+CKD. The IFN-γ level was elevated from 4.6±0.4 to 4.9±0.4 pg/mL and 7.5±0.4 pg/mL in patients with CVD and CKD+CVD, respectively (Figure 7A). We further examined the synergistic effect of homocysteine and inflammatory cytokines on monocyte differentiation in PBMC. Intermediate inflammatory monocyte was induced by IL-6 by 1.4-fold. CD40 monocyte was induced by homocysteine, TNF-α, IL-6, and IFN-γ by 1.6-, 1.6-, 1.4-, and 1.7-folds, respectively. The combination of homocysteine with TNF-α/IL-6/IFN-γ resulted in further elevation of CD40 monocyte by 2-, 1.9-, and 2.3-folds, respectively. CD40 intermediate monocyte was induced by 1.5-folds in homocysteine group, by 1.7-, 1.4-, and 1.6-folds with TNF-α, IL-6, and IFN-γ treatment. The combination of homocysteine with TNF-α/IL-6/IFN-γ resulted in further
elevation of CD40 intermediate monocyte by 2- to 2.3-folds (Figure 7B), respectively. Neutralizing antibodies against TNF-α and IL-6 completely reversed homocysteine-induced CD40 monocyte and CD40 intermediate MC subsets were examined as described in Figures 1 through 3. Each dot represents 1 subject. C, Gating method for CD40L-induced MC differentiation. D and E, Dose response and time course of CD40L-induced MC differentiation. PBMC was primed with interferon (IFN)-γ (100U/mL) for MC differentiation and treated with human recombinant CD40L (0.4 μg/mL) for indicated times. MC (CD14⁺) and intermediate MC populations are expressed as the fold change to that in control (CT; n=5). F, Co-stimulation response. G, CD40L-induced CD40 MC subsets differentiation. H, CD40L-antibody rescue. PBMC was treated with CD40L (0.4 μg/mL), in the presence of IFN-γ (100U/L), and anti-CD40L (1 μg/mL), polyclonal mouse IgG antibody (1 μg/mL), or 10% pooled chronic kidney disease (CKD) serum as a source of CD40L. CD86-CD14⁺ SMC was determined as the standard CD40L costimulation response. MC subsets were quantified as the fold increase to average value of these in CT (n=4). Schematic description summarizes the working model. Values represent mean±SEM; *P<0.05 vs healthy and CT. Arrows indicate the direction of significant changes. CVD indicates cardiovascular disease; MFI, median fluorescence intensity.

DNA Hypomethylation in CD40 Promoter and DNMT1 Suppression Contribute to Homocysteine-Induced Inflammatory Monocyte Differentiation

Plasma homocysteine levels were negatively correlated with plasma S-adenosylmethionine/S-adenosylhomocysteine ratio, which was also negatively correlated with eGFR. Cellular homocysteine levels were associated with, but not significantly
correlated with cellular S-adenosylmethionine/S-adenosylhomocysteine. Plasma/cellular S-adenosylmethionine/S-adenosylhomocysteine ratios were negatively correlated with intermediate and CD40 intermediate monocyte in patients with CKD and CVD (Figure 8A). Because decreased S-adenosylmethionine/S-adenosylhomocysteine ratios indicate hypomethylation status, we mapped DNA methylation in CD40 promoter in selected subjects with high or low plasma S-adenosylmethionine/S-adenosylhomocysteine ratios (7.2±1.3 versus 2.2±0.7; Figure 8A and 8B). We identified a CpG island (–892/–291) on the CD40 promoter (Figure 8C), which has an IFN-γ/TNF-α responsible core promoter (–561/–396) containing 5 cis-transactive consensus elements (2x nuclear factor [NF]-κB, 2xPU.1, and a Stat1) and 9 potential methylation sites (CpG; Figure 8D). We mapped DNA methylation in the CD40 core promoter region using bisulfite converting and pyrosequencing. DNA methylation at site 1 CpG dinucleotide, an NFκB binding site, was reduced from 11.8% in high S-adenosylmethionine/S-adenosylhomocysteine subjects (Figure 8E). Furthermore, protein levels and activity of DNMT 1, but not 3a, were reduced to 70% in monocyte treated with homocysteine (Figure 8F and 8G). 5-Azacytidine was used as a specific DNMT inhibitor control. Finally, homocysteine-induced CD40 intermediate monocyte was significantly reduced by folic acid, which provides methylation power to remethylate homocysteine and converts it back to methionine (Figure 8H).

Discussion

In this comprehensive clinically driven mechanistic study, we investigated inflammatory monocyte differentiation in human CVD patients, with or without CKD, and in cultured PBMC. We identified CD40 monocyte as an inflammatory monocyte subset that is elevated in patients with CVD+CKD (Figure 8F and 8G). 5-Azacytidine was used as a specific DNMT inhibitor control. Finally, homocysteine-induced CD40 intermediate monocyte was significantly reduced by folic acid, which provides methylation power to remethylate homocysteine and converts it back to methionine (Figure 8H).

Figure 7. Inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-6, and interferon (IFN)-γ are induced in chronic kidney disease (CKD)+cardiovascular disease (CVD) subjects and synergized in CD40 intermediate monocyte (MC) differentiation with homocysteine (Hcy). Peripheral blood was collected from human subjects and screened for serum cytokine levels. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors and cultured for MC differentiation study as described in Figure 5. A, Serum cytokine levels. Serum from 5 human subjects was pooled as 1 assay sample and analyzed for cytokines in quadruplicates using Quantibody multiplex ELISA cytokine array (n=2–3). B, Cytokine potentiated Hcy-induced CD40 MC differentiation. PBMC was treated with α-Hcy (100 μmol/L) and inflammatory cytokines: IFN-γ (100 U/mL), TNF-α (10 ng/mL), IL-6 (100 ng/mL; n=6). C, TNF-α and IL-6 antibodies blocked Hcy-induced CD40 MC in CKD. PBMC was treated with anti-IL-6, anti-TNF-α, polyclonal mlgG antibody, and α-Hcy (100 μmol/L) in the presence of pooled 10% CKD serum as a source of inflammatory cytokines (C) (n=4). Quantification of MC subsets are expressed as the fold change to average value of these in control (CT). Schematic description summarizes the working hypothesis. Values represent mean±SEM; *P<0.05 vs healthy, #P<0.05 vs CVD; *P<0.05 vs CT, #P<0.05 vs Hcy. Arrows indicate the direction of significant changes. MFI indicates median fluorescence intensity.

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Figure 8. CD40 promoter DNA hypomethylation and DNA methyltransferase (DNMT) 1 suppression contribute to homocysteine (Hcy)-induced inflammatory monocyte (MC) differentiation. Peripheral blood was collected from human subjects for metabolite analysis for MC population characterization, and for DNA methylation mapping as described in Figures 1 through 3. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors and cultured as described in Figure 5. A, Plasma S-adenosylmethionine (SAM)/S-adenosylhomocysteine (SAH) correlation with Hcy, estimated glomerular filtration rate (eGFR), and MC subsets. Plasma SAM/SAH was used as an indicator of methylation status. Correlation was determined by linear regression analysis. Subjects with high or low SAM/SAH were selected as representatives for DNA methylation mapping (shown in E) and indicated by empty and black circles to distinguish from other subjects (gray circle). Each dot represents 1 subject.

B, Metabolic parameters selected for DNA methylation mapping.

C, CpG island in CD40 Promoter.

D, Nine CpG pairs in CD40 core promoter (-561/-396).

E, DNA Methylation Mapping (-561/-396) (Pyrosequencing).

F, DNMT protein levels.

G, DNMT activities.

H, Hcy-induced CD40 Intermediate MC.
maturation. \textsuperscript{38} We found that CD40+ monocyte expressed similar or higher levels of inflammatory markers (Figure 1) compared with the currently recognized inflammatory intermediate monocyte. Similar to the patrolling nonclassical monocyte, the CD40+ monocyte exhibited anti-inflammatory features with reduced inflammatory markers. We conclude that CD40+ monocyte is a novel and stronger inflammatory subset compared with the currently recognized inflammatory intermediate monocyte.

We found that unlike the intermediate monocyte, CD40 classical/intermediate monocyte and CD40 monocyte are linearly increased with the elevation of CKD severity (Figure 2G, 2H, and 2I). We propose that CD40 monocyte can be used as a diagnosis and prognosis marker for CKD and inflammatory disease, more accurately than the intermediate monocyte, to predict and represent CKD severity.

In the efforts to identify the metabolic mediator responsible for inflammatory monocyte differentiation in CKD, we found that plasma homocysteine and its metabolite, S-adenosylhomocysteine, were increased in CKD and positively correlated with 3 inflammatory monocyte subsets (Figure 3). The negative correlation between eGFR and homocysteine metabolites supported a strong metabolic link between kidney function and homocysteine metabolites. This is not influenced by the clearance of metabolites because blood samples were collected from patients with end-stage renal disease before dialysis.

Our data suggest that CKD is a stronger inducer for CD40 monocyte differentiation than is an increased glucose level. eGFR has a significant direct effect on CD40 monocyte and CD40 intermediate monocyte differentiation (Figure 4B, 4C, 4E, and 4F). Plasma glucose levels are significantly increased in patients with CVD+CKD (Online Figure IIIE), but its correlation with CD40 monocyte and CD40 intermediate monocyte is very weak (Online Figure IIIG).

The ME of plasma homocysteine elevation was further supported by mediation analysis (Figure 4). Hyperhomocysteinemia has a direct effect on CD40 intermediate monocyte differentiation and a 29% ME on eGFR deficiency–induced CD40 intermediate monocyte differentiation. The direct and mediating effect of homocysteine on CD40 monocyte differentiation was further validated in PBMC by using exogenous homocysteine treatment. We found that both DL-homocysteine (100 μmol/L) and CKD serum induced CD40 monocyte by 140% and 139% (Figure 5C), respectively. This is consistent with our previous findings in mouse models of hyperhomocysteinemia.\textsuperscript{15,17,18} This study suggests that hyperhomocysteinemia mediates CKD-induced CD40 monocyte differentiation and can be a potential therapeutic target for CKD. The role of CD40 in inflammatory monocyte differentiation and vascular disease is supported by a previous report showing that CD40 deficiency reduced blood inflammatory monocyte and atherosclerosis in apoE\textsuperscript{−/−} mice.\textsuperscript{39}

We discovered that reduced plasma/cellular S-adenosylmethionine/S-adenosylhomocysteine ratio has a stronger direct effect on CD40 monocyte differentiation than that of increased plasma homocysteine levels. Reduced plasma S-adenosylmethionine/S-adenosylhomocysteine has a 77% and 79% ME on eGFR deficiency–induced CD40 intermediate monocyte and CD40 monocyte differentiation. These data suggest that S-adenosylmethionine/S-adenosylhomocysteine ratios may be influenced by other metabolic pathway, independent from homocysteine metabolism, and may be a more potent mechanism for CD40 monocyte differentiation.

We demonstrated that plasma CD40L levels are positively correlated with plasma hyperhomocysteinemia and circulating inflammatory monocyte in CVD and CKD subjects and that CD40L induces CD40 monocyte differentiation in PBMC (Figure 6). Plasma sCD40L is considered to be an essential inflammatory biomarker for CVD. It is mostly generated by activated platelets and elevated in stage-5 CKD patients with CVD.\textsuperscript{40} Consistent with our findings, sCD40L levels were found to be elevated in hyperhomocysteinemia patients and positively correlated with homocysteine.\textsuperscript{35} Our study is the first to connect CD40L elevation with monocyte differentiation and to validate this connection in experimental models. We discovered that CD40 system blocking, such as neutralizing antibody against CD40L, reverses CKD patient serum–induced CD40 monocyte differentiation and could be a potential therapeutic strategy to reduce inflammatory response in patients with CKD and CVD.

In addition to sCD40L, previous reports suggested that IFN-γ induces CD40 transcription in microglia/macrophages.\textsuperscript{30} We found that circulating inflammatory cytokines TNF-α, IL-6, and IFN-γ are increased in patients with CKD and CVD. TNF-α antibody reversed CKD serum– and CVD serum+homocysteine-induced CD40 monocyte, whereas IL-6 antibody only blocks such effect induced by CKD serum+homocysteine (Figure 7). We propose a model that hyperhomocysteinemia and TNF-α directly induce CD40 monocyte differentiation and that IL-6 promotes hyperhomocysteinemia-induced monocyte in CKD (Figure 7 working model). Taken together, our data indicate that sCD40L and inflammatory cytokines in combination with homocysteine synergize inflammatory monocyte differentiation in CKD.

The hypomethylation status in CKD was likely because of S-adenosylhomocysteine elevation as it dictated S-adenosylmethionine/S-adenosylhomocysteine ratio reduction regardless the increased plasma S-adenosylmethylonine levels observed (Figure 3D). In some homocysteine-lowering trials, unchanged plasma S-adenosylhomocysteine levels and S-adenosylmethionine/S-adenosylhomocysteine ratios

Figure 8 Continued. CD40 core promoter region (~561/~396). E, DNA methylation mapping. white blood cells from high and low SAM/SAH subjects (empty and black dots in A and B) were used for genomic DNA extraction and DNA methylation mapping by bisulfite converting and pyrosequencing. The percentage of methylated cytosine of 1 to 9 CpG pairs was quantified. F and G, DNMT protein levels and activity. MC was enriched by replating from PBMC and treated with DL-Hcy. DNMT1 and 3a protein levels and activities were examined (n=6). H, Hcy-induced CD40 intermediate MC, PBMCs were isolated, primed as described in Figure 5 and treated with folic acid before DL-Hcy (n=6). Azc, DNMT1 inhibitor, was used as negative control. Schematic description summarizes the working model. Values represent mean±SEM; *P<0.05 vs control (CT) and high SAM/SAH, #P<0.05 vs Hcy. Arrows indicate the direction of significant changes. Azc, 5-azacytidine; FACS, fluorescence-activated cell sorter; and Met, methionine.
have been suggested to be the explanation for the absence of benefits in CVD prevention. Our findings provided a metabolic basis for future clinical trial design. We found that S-adenosylhomocysteine levels are exponentially increased in stage-5 CKD subjects (Figure 3G; Online Figure IIIB), indicating that S-adenosylhomocysteine is a pathogenic factor. We propose that future clinical trials should consider S-adenosylhomocysteine and methylation targeted therapy, especially for stage-5 CKD, to improve the benefit of homocysteine-lowering therapy. The pathogenic effect of S-adenosylhomocysteine is also supported by its biochemical feature, as the Km for S-adenosylhomocysteine, the Michaelis constant for substrate concentration for 50% of maximal reaction rate, is smaller than the Km for S-adenosylmethionine. Lower S-adenosylhomocysteine concentration is needed to compete with S-adenosylmethionine for binding to methyltransferases. Therefore, S-adenosylhomocysteine has a higher affinity for methyltransferase. Lower S-adenosylhomocysteine concentration is needed to compete with S-adenosylmethionine for binding to methyltransferase. In this study, we demonstrated that hyperhomocysteinemia and S-adenosylmethionine/S-adenosylhomocysteine correlate with inflammatory monocyte subsets in patients with CVD and CKD (Figures 3, 4, and 8). We propose that hyperhomocysteinemia and S-adenosylhomocysteine are metabolic sensors that determine hypomethylation status and are responsible for inflammatory monocyte differentiation and the progression of CVD and CKD. These data are consistent with our previous findings, showing that hypomethylation induces inflammatory monocyte and macrophage in mice, and lead us to hypothesize that hyperhomocysteinemia induces both sCD40L and CD40 expression via hypomethylation-related mechanism in CKD.

We tested the DNA hypomethylation hypothesis in hyperhomocysteinemia- and CKC-induced CD40 monocyte differentiation. We discovered that CpG methylation in CD40 core promoter at NFκB binding site is reduced in patients with low S-adenosylmethionine/S-adenosylhomocysteine ratios and that homocysteine-induced CD40 intermediate monocyte differentiation can be rescued using a remethylation reagent folic acid in PBMC (Figure 8). These findings are consistent with the notions that NFκB is essential for monocyte differentiation and that altered DNA methylation at the NFκB binding site influences intestinal metaplasia, epithelial dedifferentiation, and carcinogenesis.

In summary, we have found that CD40 monocyte, CD40 classical monocyte, and CD40 intermediate monocyte are induced in CVD and further elevated with the progress of CKD, which is likely mediated by elevated plasma homocysteine levels and reduced S-adenosylmethionine/S-adenosylhomocysteine ratios. Hyperhomocysteinemia induces CD40 monocyte via sCD40L induction and CD40 promoter DNA hypomethylation caused by S-adenosylhomocysteine induction and DNMT1 suppression (Online Figure I, working model). It is known that CD40–CD40L interaction leads to enhanced T-cell costimulation, myeloid cell maturation, and B-cell proliferation. Clinical trials targeting CD40 using monoclonal antibodies, Dacuzumab, are ongoing for B-cell lymphoma to inhibit B-cell proliferation and 4D11 for immunosuppressant after organ transplantation to inhibit antigen-presenting monocyte maturation. Our study presents a novel model for CD40–CD40L interaction in monocyte differentiation.

Our study is a typical clinically driven mechanistic research that used multilayers of sophisticated discovery tools and approaches. It incorporated comprehensive genetic, immunologic, molecular and cellular, biological, metabolic, and statistical strategies. We provided substantial evidence ranging from gene and novel monocyte subset identification to metabolic profiling, mediator discovery, and DNA methylation characterization in patients with CKD. Importantly, we used multiple rescue strategies that reversed CKD serum and homocysteine-induced inflammatory monocyte differentiation and presented novel therapeutic strategies for treating CKD and CVD, including α-CD40L, α-TNF-α, α-IL-6 and folic acid therapies. Our work established a solid foundation for further research into inflammatory response and CVD in CKD.

Future studies will validate the key mechanistic findings in a larger sample size and in different disease populations; such studies should identify the regulatory mechanism underlying CD40 monocyte differentiation and the cross talk between metabolic, cellular, and molecular changes in CKD. Continued research on CD40 monocyte differentiation could lead to the discovery of novel therapeutic targets for inflammatory disease, especially for CKD-related CVD.

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Disclosures

None.

References


What Is Known?

- CD14⁺CD16⁻, an inflammatory monocyte subset, is increased in human chronic kidney disease (CKD) and cardiovascular disease (CVD).
- CD40 is expressed in antigen presenting cells and CD40–CD40L signaling activates T cells.
- Plasma homocysteine levels are increased in human CKD and CVD.

What New Information Does This Article Contribute?

- Classified CD40⁺ monocytes as a stronger inflammatory monocyte subset, which can predict and represent CKD severity.
- Hyperhomocysteinemia and reduced S-adenosylmethionine/S-adenosylhomocysteine ratio mediate CKD-induced CD40⁺ monocyte differentiation.
- CD40 ligand is elevated in CVD/CKD and can induce inflammatory monocyte differentiation. Homocysteine mediated CKD-induced CD40⁺ monocyte differentiation via S-adenosylhomocysteine elevation determined hypomethylation status (reduced S-adenosylmethionine/S-adenosylhomocysteine ratio) and CD40 DNA hypomethylation on nuclear factor-κB consensus element.
- Homocysteine suppressed DNA methyltransferase 1 activity and induced CD40 intermediate monocyte differentiation in peripheral blood mononuclear cell.
- Folic acid prevented homocysteine-induced CD40 intermediate MC differentiation in peripheral blood mononuclear cell.

In this study, we investigated monocyte differentiation in human CKD and CVD. We identified CD40 as a CKD-related monocyte activation gene and classified CD40⁺CD14⁻ monocytes as a stronger inflammatory subset than the currently recognized inflammatory intermediate CD14⁺CD16⁻ monocytes. We found that CD40 and CD40 intermediate monocytes, homocysteine, S-adenosylhomocysteine, and S-adenosylmethionine levels were increased in CVD and further elevated in CVD+CKD patients. We established hyperhomocysteinemia as a mediator for CKD-induced CD40 intermediate monocyte differentiation. We propose CD40L as a mediator for CKD/homocysteine-induced CD40 monocyte differentiation because it was increased in CVD/CKD and the antibody against CD40 prevented CD40 patient serum/homocysteine-induced CD40 monocyte differentiation. We characterized DNA methylation and identified hypomethylation on the nuclear factor-κB site in CD40 promoter in white blood cells from CKD subjects with lower S-adenosylmethionine/S-adenosylhomocysteine ratios. Finally, homocysteine inhibited DNA methyltransferase-1 activity and promoted CD40 intermediate MC differentiation, which was reversed by folic acid in peripheral blood mononuclear cells.
Chronic Kidney Disease Induces Inflammatory CD40⁺ Monocyte Differentiation via Homocysteine Elevation and DNA Hypomethylation

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Supplemental Material

Chronic kidney disease induces inflammatory CD40 monocyte differentiation via homocysteine elevation and DNA hypomethylation

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**Supplement Materials and Methods:**

**Human Subjects** — This study was approved by the Temple University School of Medicine IRB. We analyzed blood samples from 27 patients with CVD (13 CVD and 14 CVD+CKD) from the Temple University Vascular Surgery and Nephrology practice and 14 healthy donors with no history of CKD and CVD from the Thrombosis Center at Temple University. Diabetes were determined based on if the patient was currently receiving hypoglycemic therapy or if their fasting glucose level was ≥ 126 mg/dL. Hypertension was determined as a systolic blood pressure ≥ 140 mmHg and a diastolic blood pressure ≥ 90 mmHg. Hyperlipidemia was determined by a total cholesterol level ≥ 240 mg/dl. According to clinical practice guidelines of CKD evaluation, classification, and risk stratification, CKD stage was estimated by glomerular filtration rate (GFR) using the CKD-Epidemiology Collaboration equation as described in Online Table 1. The demographics and clinical information, lipid-lowering therapies and modifiable risk factors that co-occur with CKD such as diabetes, hypertension, and hyperlipidemia were listed in Online Table 2. Plasma was collected from the EDTA tube within 2 hours after blood collection by spinning at 230g for 15 minutes at room temperature and stored at -80°C. The remainder of the fresh blood cell pellet in the EDTA tube was processed to white blood cell (WBC) or peripheral blood mononuclear cell (PBMC) analysis.

**Microarray Analysis** — Microarray data were downloaded from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE43484 and analyzed in the R statistical environment (http://www.r-project.org) using “Biobase”, “GEOquery”, and “limma” Bioconductor projects (http://www.bioconductor.org/). Data were interpreted by DAVID tools. To identify target genes for MC activation relevant to CVD and CKD, we selected genome-wide gene expression profiles. These were observed from freshly isolated peripheral blood monocytes from CKD stage 4-5 vs Healthy subjects with a median age of 59 years. A total of 109 probe sets had significant differential expression (p<0.05, <1.5 fold change). CKD patients have increased susceptibility to CVD, infections, and poor vaccine response due to impaired cellular and humoral immune response. Because of this, three CKD susceptible disease categories (CVD, immune disease and infection) and three MC activation categories (leukocyte activation, inflammation, and cytokine production) were classified as shown in Venn diagram in Fig. 1A.

**Metabolite analysis (Hcy, SAM, SAH)** — WBCs were isolated after RBC lysis (1:15 volume of hypertonic solution containing 4.2g ammonium chloride in 0.5M Tris-HCl, 10 min at room
temperature) and divided to 2 aliquots—3 x 10^6 WBC in 100µl of 0.4M pericholic acid subjected for SAM/SAH measurement and 1x10^6 WBC for Hcy measurement. Plasma and cellular metabolites were measured by liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS).^2

**Plasma sCD40L measurement** — Platelets are the predominant source of sCD40L in plasma. Freeze-thawing of the prepared plasma causes lysis of residual platelet during the assay steps. On the day of blood collection, platelets in plasma were removed after additional centrifugation at 10,000 rpm for 10 minutes at room temperature and supernatant was transferred as an aliquot. Plasma levels of CD40L were measured by ELISA as manufacturer recommended (Human CD40 ligand/TNFSF5 Quantikine ELISA kit, R&D Systems).

**PBMC cultivation and differentiation** — 50ml of whole blood was collected into a 50 ml tube containing 7.5ml anti-coagulant buffer (85mM sodium citrate, 71.4mM citric acid, and 111mM glucose). PBMCs were isolated by 1077-histopaque gradient density centrifugation (Sigma) to remove granulocyte, seeded in 24 or 48-well tissue culture plate at the density of 1x10^6 cells/ml in DMEM, and then rested for 1 hour. Cells were then primed with human recombinant IFNα (hrIFNF, 100U/ml) and treated with hrCD40L, hrTNFα, hrIL-6 (R&D system), mouse IgG (Jackson immune research), mouse anti-human IL-6 (1936, R&D system), mouse anti-human TNFα (28401, R&D system), or folic acid (Sigma-Aldrich). The attached cells were detached with DMEM, containing 5mM EDTA and 10% FBS, for 10 min at 37°C and pooled with suspending cells. Cells were washed and stained with anti-CD14, -CD16, -CD40 antibodies.

**Blood MC Isolation, Cultivation** — CD14^+ MC was isolated as described with modification. In brief, T75 flasks were coated with 10ml of 2% gelatin for 4 hours at 37°C prior to MC isolation. PBMCs were seeded in gelatin-coated T75 flask at a density of 30x10^6 in 10ml DMEM media rested for 1 hour at 37°C. Suspending cells were aspirated. Attached cells were washed twice with 10ml PBS, detached with DMEM, containing 5mM EDTA and 10% FBS, for 10 min at 37°C and suspended by tapping the plate. Cells were washed and stained with anti-CD14 antibody. Approximately 3~5x10^6 cells were isolated with 95% purity of CD14^+ MC from 30x10^6 PBMCs.

**Serum Cytokine Array** — Peripheral venous blood was collected in a serum plus blood collection tube (BD vacutainer). Blood was put in ice for 2 hours and spun at 500g for 15 minutes at 4°C. Serum aliquots were stored at -80°C. Serum cytokine levels were determined by
using a commercially available array kit according to manufacturer instruction (Human cytokine array Q1, RayBiotech).

**CD40 promoter DNA methylation mapping in WBC** — 1x10^6 WBC were suspended in 40μl proteinase K buffer (50°C, 30 m) and spanned to remove cell debris (14,000 x g for 10 m). Genomic DNA (20μl, 500ng in 45μl) was denatured (5μl of 3N NaOH, 42°C 30 m) and used for bisulfite modification to convert all unmethylated cytosines to uracils. DNA was then used for PCR analysis with specific primers for CD40 core promoter and pyrosequencing (EpigenDx, Inc, Hopkinton, MA). The methylation status was analyzed using QCpG software.

**DNMT protein and activity analysis** — Above prepared MCs were replated in 24 well plates at the density of 1x10^6 MC/ml/well. DNMT protein levels were assayed by Western blot (30μg extract per lane) using antibodies against DNMT1 (1:2000) (Imgenex) and DNMT3a (1:350) (Imgenex). For DNMTs activity assessment, nuclear proteins were extracted from 5x10^6 MC and used for enzymatic reaction (20μg per sample). Nuclear extracts were incubated with double-stranded unmethylated (for DNMT3) or hemimethylated (for DNMT1) DNA substrates in the presence of [^3H]SAM. DNMT1 and DNMT3a activities were examined as described 4.

**CpG island and core promoter mapping** — The promoter CpG island was searched using a CpG Island Search engine (http://cpgislands.usc.edu). A CpG island (-5000/100) is identified as a DNA region with >56.5% CG content and >0.6 CpG ratio. Transcription factor binding sites were mapped as identified previously5, 6 and predicted by the database TESS.

**Mediation analysis** — We investigated the mediation effects of plasma/cellular Hcy and the SAM/SAH ratio in the three CKD-induced inflammatory MC subsets, intermediate MC, CD40 MC, and CD40 intermediate MC differentiation in healthy, CVD, and CVD+CKD subjects. Mediation analysis can test for a causal chain in which one variable affects a second variable that, in turn, affects a third variable. The mediation analysis can identify the intervening variable, the mediator that “mediates” the relationship. This analysis is a multiple step analysis involving both simple and multiple regression analysis. In contrast, the purpose of multiple regression is to determine the relationship of several independent variables with a dependent variable, which is a relatively simple method that only uses the multiple regression analysis procedure.

The direct and total residual effects of eGFR and mediation effects of cellular/plasma Hcy and the SAM/SAH ratio on MC subsets were estimated using the standard mediation regression method by testing the cellular/plasma Hcy or SAM/SAH as a mediator7,9.
The direct (a) and total effect sizes (c) of eGFR on plasma/cellular Hcy or SAM/SAH and CD40 MC subsets were the slopes of eGFR in the corresponding regression models. The mediation effect (b) of Hcy or SAM/SAH were estimated in a regression model with the presence of the residual effect (c’) of eGFR on MC subsets, i.e., both eGFR and Hcy or SAM/SAH were included as independent variables in the regression model for the CD40 MC subsets. All effect sizes and their 95% confidence intervals were calculated using the corresponding linear regression models with adjustment for sex, age, hypercholesterolemia (HC), and hypertension (HTN) whenever necessary. Mediation effect (ME) of Hcy or SAM/SAH, defined as (a*b)/c in percentage, reflected the proportion of the mediator effect in the total direct effect (c) of the eGFR-induced MC differentiation. A $p$-value < 0.05 was considered statistically significant, while a $p$-value < 0.1, marginally statistically significant.

**Statistical Analysis** — Baseline clinical characteristics were adjusted using SPSS software. Statistical analyses were performed with Prism 3.03 software (GraphPad Software). For statistical comparison of independent samples from subjects, an unpaired $t$ test was used for two groups. Unless otherwise stated, a result is claimed to be not statistically significant. Correlation and mediation analysis between the variables were performed using corresponding regression analysis models with adjustment for sex, age, medication, hypercholesterolemia (HC), hypertension (HTN), diabetes, and smoking whenever necessary. No differences were identified before and after adjustment for medication and diabetes conditions.
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