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 it 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.[1] 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 x10^6 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 IFNm (hrIFNF, 100U/ml) and treated with hrCD40L, hrTNFα, hrIL-6 (R&D system), mouse IgG (Jackon 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[3] 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 (50ºC, 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, 42ºC 30 m) and used for bisulfite modification to convert all unmethylated cytosines to uracils. DNA was then used forPCR 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 [^{3}H]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 previously[5, 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 mediator[7-9].

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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