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End-stage renal disease is different from chronic kidney disease in upregulating ROS-modulated proinflammatory secretome in PBMCs - A novel multiple-hit model for disease progression

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ABSTRACT

Background: The molecular mechanisms underlying chronic kidney disease (CKD) transition to end-stage renal disease (ESRD) and CKD acceleration of cardiovascular and other tissue inflammations remain poorly determined.

Methods: We conducted a comprehensive data analyses on 7 microarray datasets in peripheral blood mononuclear cells (PBMCs) from patients with CKD and ESRD from NCBI-GEO databases, where we examined the expressions of 2641 secretome genes (**SG**).

Results: *1)* 86.7% middle class (molecular weight >500 Daltons) uremic toxins (UTs) were encoded by SGs; *2)* Upregulation of SGs in PBMCs in patients with ESRD (121 SGs) were significantly higher than that of CKD (44 SGs); *3)* Transcriptomic analyses of PBMC secretome had advantages to identify more comprehensive secretome than conventional secretomic analyses; *4)* ESRD-induced SGs had strong proinflammatory pathways; *5)* Proinflammatory cytokines-based UTs such as IL-1β and IL-18 promoted ESRD modulation of SGs; *6)* ESRD-upregulated co-stimulation receptors CD48 and CD58 increased secretomic upregulation in the PBMCs, which were magnified enormously in tissues; *7)* M1-, and M2-macrophage polarization signals contributed to ESRD- and CKD-upregulated SGs; *8)* ESRD- and CKD-upregulated SGs contained senescence-promoting regulators by upregulating proinflammatory IGFBP7 and downregulating anti-inflammatory TGF-β1 and telomere stabilizer SERPINE1/PAI-1; *9)* ROS pathways played bigger roles in mediating ESRD-upregulated SGs (11.6%) than that in CKD-upregulated SGs (6.8%), and half of ESRD-upregulated SGs were ROS-independent.

Conclusions: Our analysis suggests novel secretomic upregulation in PBMCs of patients with CKD and ESRD, act synergistically with uremic toxins, to promote inflammation and potential disease progression. Our findings have provided novel insights on PBMC secretome upregulation to promote disease progression and may lead to the identification of new therapeutic targets for novel regimens for CKD, ESRD and their accelerated cardiovascular disease, other inflammations and cancers. (Total words: 279).

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1. Introduction

The incidence of chronic kidney disease (**CKD**) is increasing worldwide [1]. A major cause of mortality in patients with CKD has been found to be atherosclerosis-related cardiovascular disease (CVD) [2]. Our and others' recent reports showed that CVD stressors and risk factors such as hyperlipidemia [3,4], hyperglycemia [5], hyperhomocysteinemia [6,7], and chronic kidney disease [8–10], promote atherosclerosis and vascular inflammation via several mechanisms. These mechanisms include endothelial cell (EC) activation [3,11–14] and injury [15]; caspase-1/inflammasome activation [8,10], mitochondrial reactive oxygen species (**ROS**) [4]; Ly6C^{high} mouse monocyte and CD40^{+} human monocyte differentiation [7,16–18]; decreased/trans-differentiated regulatory T cells [19–22] (Treg); impaired vascular repairability of bone marrow-derived progenitor cells [23,24]; down-regulated histone modification enzymes [25] and increased expressions of trained immunity pathway enzymes [26].

CKD is classified into five stages [27] based on glomerular filtration rate (GFR, mL/min. per 1.73 m^2); ≥90 mL/min (stage 1), 60–89 mL/min (stage 2), 30–59 mL/min (stage 3), 15–29 mL/min (stage 4) and <15 mL/min (stage 5). At stage 5, the patient develops end-stage renal disease (**ESRD)** and requires life-long renal replacement therapy (RRT). Clinical evaluations for kidney function include creatinine level, blood urea nitrogen (BUN) assessment and cystatin C level (MedlinePlus, NIH https://medlineplus.gov/kidneytests.html). CVD risk increases significantly according to the stages of CKD, ranging from 1.5-fold in stage 2, to between 20 and 1000-folds with ESRD [28]. Indeed, CVD accounts for 50% of deaths in patients receiving dialysis [29], demonstrating that CKD accelerates atherosclerotic pathology [28], which along with its complications such as myocardial infarction, stroke and peripheral artery disease, are the leading cause of morbidity and mortality in this country, and account for 75% of all CKD deaths from CVD [30]. The molecular and cellular mechanisms underlying CKD-accelerated atherosclerotic pathology, remain unknown.

It has been suggested that CKD-derived uremic toxins (**UTs**) [31], in combination with other risk factors, cause oxidative stress including mitochondrial ROS [4], low-grade inflammation with increased circulating cytokines and endothelial dysfunction [28,32]. Recently, in novel UT metabolomics/gene databases, we analyzed the expression changes of UT receptors and UT synthases in CKD and CVD. We made the following observations: 1) UTs represent only 1/80th of the human serum small-molecule metabolome; 2) Increased in CKD and CVD, some UTs induce or suppress the expression of inflammatory molecules; 3) The expression of UT genes are significantly modulated in CKD patients, and coronary artery disease (CAD) patients; and 4) The expressions of UT genes are upregulated by pathogen/danger associated molecular pattern receptors (PAMPs/DAMPs)/inflammasome-caspase-1 as we reported [8] and tumor necrosis factor-α (TNF-α) pathways but are inhibited in CD4+Foxp3+ regulatory T cells (Treg). These results demonstrate that UTs are selectively increased, and serve as **DAMPs** and homeostasis-associated molecular patterns (HAMPs) that modulate inflammation [33]; and that some UT genes are upregulated in CKD and CAD rather than by purely passive accumulation [10]. One well-characterized UT example is carbamylated low-density lipoprotein (cLDL) [34]. Protein carbamylation has been found in atherosclerotic plaque; and serum level of cLDL is increased significantly in patients with ESRD, which has been shown to have all of the major biological effects relevant to atherosclerosis, including EC injury and dysfunction [35] by binding to oxidized low-density lipoprotein (oxLDL) receptor (LOX-1) [36], increased expression of cell adhesion molecules, monocyte adhesion, and vascular smooth muscle cell (VSMC) proliferation [34]. However, an important question remains whether additional secretory proteins participate in the pathogenesis and inflammatory acceleration of CKD and ESRD.

The secretome, defined as a portion of total proteins secreted by cells to the extracellular space, secures a proper micro-environmental niche, thus maintaining tissue homeostasis [37,38]. Secreted molecules are key mediators in cell-cell interactions and influence the cross-talk with the surrounding tissues in addition to their endocrine functions in long-distance as previously demonstrated by hormones, growth factors, cytokines, adipokines, myokines, cardiokines [39], and chemokines [40]. There is strong evidence supporting that crucial cellular functions such as proliferation, differentiation, communication and migration are strictly regulated from the cell secretome [41]. The major difference between our current study and previous reports on the roles of cytokines and chemokines in CKD pathology is that secretome analyses provide a panoramic view on all the secreted genes in the human genome modulated in CKD and ESRD, as opposed to focusing on only one or a few cytokines/chemokines. Recent reports showed that aberrant endothelial secretome in kidney diseases contribute to fibroblast reprogramming [40]. More importantly, peripheral blood mononuclear cells (PBMCs) are first tier of sensors to uremic toxins and other proinflammatory molecules in serum during kidney dysfunction [42,43]. Gene expression profile, metabolite profile, monocyte counts of PBMCs are identified to provide an access to evaluate and predict the settings of CVD and CKD [7,44–46]. The PBMC morphology, Treg/Th17 disequilibrium and activation of TLRs on membrane of PBMCs promote vascular calcification and endothelial dysfunction, which are closely related to cardiovascular risk in CKD patients [47–49]. Meanwhile, glomerular inflammation is correlated with IL-6 and IL-1β secretion in the peripheral blood [50]. However, an important question remains whether CKD and ESRD upregulate the secretome gene expressions in innate immune cells such as PBMCs, by which chronic systemic and tissue inflammations get accelerated.

In order to broaden our understanding of CKD and ESRD-accelerated inflammation, we hypothesized that CKD and ESRD induce differential secretomic gene (**SG**) expression patterns in PBMCs [51], by which CKD and ESRD accelerate inflammation. We conducted a comprehensive data analyses on a microarray dataset (GEO ID:GSE15072) containing genomic screening of PBMCs from patients with CKD and ESRD from the NIH-NCBI-GEO databases (https://www.ncbi.nlm.nih.gov/gds/), where we examined expressions of 2641 secretome genes (**SG**). We made the following findings: *1)* 86.7% middle class UTs were encoded by SGs; *2)* Upregulations of SGs in PBMCs in patients with ESRD (121 SGs) were significantly higher than that of CKD (44 SGs); *3)* ESRD-induced SGs had strong proinflammatory pathways; *4)* Proinflammatory cytokines-based UTs such as IL-1β and IL-18 promote ESRD modulation of SGs; *5)* ESRD-upregulated co-stimulation receptors CD48 and CD58 increase secretomic upregulation in the PBMCs, which are magnified enormously in tissues; *6)* M1-, and M2-macrophage polarization signals contribute to ESRD- and CKD-upregulated SGs; *7)* ESRD- and CKD-upregulated SGs contain senescence-promoting regulators by upregulating proinflammatory IGFBP7 and downregulating anti-inflammatory TGF-β1 and telomere stabilizer SERPINE1/PAI-1; and *8)* ROS pathways play bigger roles in mediating ESRD-upregulated SGs (11.6%) than that in CKD-upregulated SGs (6.8%), and half of ESRD-upregulated SGs are ROS-independent. Novel PBMC-secretome acts synergistically with uremic toxins, to promote inflammation and potential disease progression. Our findings provided novel insights on secretomic upregulation in PBMCs of patients with CKD and ESRD and identification of new therapeutic targets on CKD, various inflammations and cancers.

2. Materials and methods

2.1. Expression profile of secretomic genes (SGs) and innate immunomic genes (IIGs) in PBMC from patients with CKD and with ESRD

Microarray datasets were collected from National Institutes of Health (NIH)-National Center for Biotechnology Information (NCBI)-Gene Expression Omnibus (GEO) databases (https://www.ncbi.nlm.nih.gov/gds/) and analyzed with an online software GEO2R (https://

www.ncbi.nlm.nih.gov/geo/geo2r/). The numbers of 7 GEO datasets were listed in Table 2A. The detailed information of these GEO datasets was shown in Table 2A and other tables.

2.2. Statistical analysis of microarray data

As we reported [26,52], we applied a statistical method similar to that meta-analysis and analyzed the expressions of 9 house-keeping genes including ACTB, GAPDH, PGK1, PPIA, B2M, YWHAZ, SDHA, HMBS, TBP (Supplement Table of Housekeeping Genes) in all GEO datasets regardless of species that were chosen for this study. The house-keeping gene list was extracted from the list provided by Eisenberg and Levanon [53]. Briefly, the mean log fold change (LogFC) of house-keeping genes between treatment and control groups vary from −1.27 to 1.28. As this variation was very narrow, we concluded that the datasets (Table 2A) are of high quality. The target genes with expression changes more than 2-folds in CKD and ESRD were defined as the upregulated genes, while genes with their expression decreased more than 2-fold in CKD and ESRD were defined as downregulated genes $|\log FC| > 1$).

2.3. Ingenuity Pathway Analysis

We utilized Ingenuity Pathway Analysis (**IPA**, Qiagen, https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/) to characterize clinical relevance and molecular and cellular functions related to the identified genes in our microarray analysis. Differentially expressed genes were identified and uploaded into IPA for analysis. The core and pathways analysis was used to identify molecular and cellular pathways, as we have previously reported [52,54].

3. Results

3.1. 86.7% middle class (molecular weight >500 Daltons) uremic toxins (UTs) were encoded by secretomic genes (SGs)

We recently reported that UTs, classified in three major groups including 1) small solutes, 2) protein-bound uremic toxins, 3) middle molecules, are significantly upregulated and modulated in patients with chronic kidney disease (CKD) [10,55–57]. To improve our understanding of how many uremic toxins are encoded by secretomic genes, we hypothesized that **SGs** encode the majority of middle class uremic toxins (**UTs**). To test this hypothesis, we collected a comprehensive list of human SGs containing 2641 secreted protein genes as predicted by majority decision-based method for secreted proteins (MDSEC) used for protein classification within the Protein Atlas (https://www.proteinatlas.org/search/protein_class:Secreted+proteins+predicted+by+MDSEC, accessed December 4, 2019) [51]. As others reported, the expression levels of mRNAs are strongly correlated to that of proteins when comparing samples of the same cell type/tissue [58], justifying for us to estimate SG changes in the diseases using peripheral blood mononuclear cells (**PBMC**) transcriptomic changes for the SG changes. A list of 30 middle class UTs were collected from the European Uremic Toxins (EUTox) Database (www.uremic-toxins.org, assessed on December 4, 2019) [59–61]. As shown in Table 1A, 26 out of 30 middle molecular class UTs (86.7%) were well-characterized cytokines and encoded by SGs. In addition, as shown in Table 1B, the Ingenuity Pathway Analysis (**IPA**) showed that at least five out of top 10 pathways of middle class UTs were closely related with cytokine-associated signaling functions including apelin (adipokine) liver signaling [62], role of hypercytokinemia and hyperchemokinemia, role of cytokines in mediating communication between immune cells, differential regulation of cytokine production in macrophages and T helper cells by IL-17A and IL-17F, and IL-10 signaling. If we associate the rest of five UTs top pathways with innate immune responses, and tissue inflammation including communication between innate and adaptive immune cells, glucocorticoid receptor signaling, graft-versus-host disease signaling [63], triggering receptor expressed on myeloid cells-1 (TREM1) signaling [64], and cardiac hypertrophy signaling [65], then we can classify all the top 10 pathways of UTs play significant roles in promoting inflammations related to ESRD. Therefore, these results suggest that secretomic changes in the PBMCs contributed to the generation of middle class UTs and therefore may play significant roles in the progression of CKD and end-stage renal disease (**ESRD**); and that secretomic changes detected in transcriptomic approaches reflected secretomic changes in protein levels, demonstrated by UTs, detected on protein levels, as examples, at least partially.

Table 1a
Middle class uremic toxins (UTs) classified into secretomic proteins and non-secretomic toxins from the EUTox Work Group (http://www.uremic-toxins.org/). Middle class UTs defined as molecular weight >500 Daltons. All 2641 Secretomic Genes (SGs) and UTs are listed in Supplement Table 1.

	name	related gene symbol
Secretomic UTs (86.67%)	Adiponectin	ADIPOQ
	Adrenomedullin	ADM
	Atrial Natriuretic Peptide (ANP)	NPPA
	Basic fibroblast growth factor (BFGF)	FGF2
	Calcitonin gene-related peptide (CGRP)	CALCA
	Cholecystokinin	CCK
	Clara cell protein (CC16)	SCGB1A1
	Complement Factor D	CFD
	Cystatin C	CST3
	Endothelin	EDN1, EDN2, EDN3
	Guanylin	GUCA2A,GUCA2B, GUCA2C
	Interleukin-18	IL18
	Interleukin-1β	IL1B
	Interleukin-6	IL6
	Methionine-Enkephalin	PENK
	Motiline	MLN
	Neuropeptide Y	NPY
	Parathyroid hormone	PTH
	Resistin	RETN
	Substance P	TAC1
	Tumor Necrosis Factor Alpha (TNF)	TNF
	Uroguanylin	GUCA2A, GUCA2B
	Vasoactive intestinal peptide (VIP)	VIP
	Vasopressin (ADH)	AVP
	β-2-Microglobulin	B2M
	β-Endorphin	POMC
	Degranulation Inhibiting Protein I	
non-secretomic UTs (13.33%)	Delta-sleep Inducing Peptide	TSC22D3
	Hyaluronic acid (Hyaluronan)	HAS1,HAS2
	λ-Ig Light Chain	IGLC1, IGLC2, IGLC3, IGLC7

3.2. Upregulations of SGs in PBMCs in patients with ESRD (121 SGs) were significantly higher than that of CKD (44 SGs); and among ESRD specifically modulated 975 SGs, ESRD upregulated 116 SGs (11.9%) but downregulated 859 SGs (88.1%), respectively

To improve our understanding of how many secreted molecules are generated in the prototypic innate immune cell types, PBMCs, in patients with CKD and ESRD, we hypothesized that the expressions of secretome in PBMCs from patients with CKD and ESRD are modulated in comparing to that of healthy controls. As shown in Table 2B, we identified significant secretomic mRNA expression changes in PBMCs, from patients with CKD and patients with ESRD (see the references for the information regarding the classification of CKD and ESRD, patients and controls) [66–68]. Total 44 out of 2641 SGs (1.67%) were

Table 1b
Top 10 pathways of middle class UTs classified by Ingenuity Pathway Analysis (IPA) are closely related with cytokine-associated signaling functions. Top 10 pathways of all of 2641 SGs are shown in Supplement Fig. 1 with the full list of pathways of middle class UTs and SGs are shown in Supplement Table 2.

Ingenuity Canonical Pathways	-log (p-value)	Ratio*
Apelin Liver Signaling Pathway	7.13	0.154
Communication between Innate and Adaptive Immune Cells	6.41	0.0521
Role of Hypercytokinemia/hyperchemokinemia in the Pathogenesis of Influenza	6.23	0.093
Glucocorticoid Receptor Signaling	6.12	0.0208
Graft-versus-Host Disease Signaling	6.03	0.0833
Role of Cytokines in Mediating Communication between Immune Cells	5.82	0.0741
Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-17A and IL-17F	5.55	0.167
IL-10 Signaling	5.39	0.058
TREM1 Signaling	5.25	0.0533
Cardiac Hypertrophy Signaling (Enhanced)	5.06	0.0144

* The number of SGs classed/total genes in this pathway.

Table 2a
Seven microarray datasets collected from the NIH-NCBI-GeoData Sets database and were analyzed in this study (https://www.ncbi.nlm.nih.gov/gds/). Nine canonical housekeeping genes (ACTB, GAPDH, PGK1, PPIA, B2M, YWHAZ, SDHA, HMBS, TBP) were used to verify the quality of all the datasets with their expression variation listed in the supplemental "Supplement table of Housekeeping Genes".

No.	GEO ID	Species	cell type/tissue	Disease Comparison/Treatment[a]	PMID
1	GSE15072	Human	Peripheral blood mononuclear cells	ESRD vs. CKD vs. health	24189015/19698090
2	GSE103500	human	human blood leukocytes	WT vs. IL-1b-treated	N/A
3	GSE103500	human	human blood leukocytes	WT vs. IL-18-treated	N/A
4	GSE15215	Human	human plasmacytoid dendritic cells	$CD2^+$ DCs vs. $CD2^-$ DCs	19454677
5	GSE85346	Human	Macrophage	M1 vs. M0, M2a vs. M0, M2b vs. M0	27990286
6	GSE100671	Human	leukemic cells	WT vs. NOX2−/− PLB-985 cells	29967760
7	GSE7810	mouse	type II cells isolated from lungs	WT vs. Nrf2−/− mice Type II cell	17895394

[a] WT: wild type; IL: Interleukin; DC: dendritic cells; M1: M1 Macrophages; M0: M0 Macrophages; NOX2: NADPH Oxidase 2; Nrf2: nuclear factor, erythroid 2 like 2.

upregulated in PBMCs from patients with CKD; in comparison, 121 out of total 2641 SGs (4.58%) were upregulated in the PBMCs from patients with ESRD. In addition, 55 out of 2641 SGs (2.08%) were downregulated in the PBMCs from patients with CKD; and 928 out of 2641 SGs (35.14%) were downregulated in the PBMCs from patients with ESRD. These results suggest that 1) panoramic view of secretomic changes in PBMCs can be generated by analyzing microarray data from patients with CKD and ESRD; 2) secretomic changes in PBMCs may contribute significantly to generation of UTs in patients with chronic kidney disease (CKD) and end-stage renal disease (ESRD) (also see Fig. 3A); and 3) UTs-based secretome in PBMCs in patients with CKD and ESRD may play significant roles in promoting CKD- and ESRD-accelerated systemic and tissue inflammations (also see Table 4D).

Secretomic upregulation and downregulation in the PBMCs from patients with CKD and ESRD can be categorized into four groups by Venn Diagram analysis, Fig. 1. The results showed that: *1)* 25 SGs upregulated in CKD (25/44, 56.8%) shared with that downregulated in ESRD, and shared four SGs with that upregulated in ESRD. The 25 SGs upregulated in CKD but downregulated in ESRD had potential in inhibiting the progression of ESRD. The four SGs downregulated in CKD but upregulated in ESRD suggest: *a)* their regulatory pathways switched from downregulation in CKD to upregulation in ESRD; and *b)* their potential roles in promoting ESRD; *2)* 44 out of 55 SGs (80%) downregulated in CKD shared with that downregulated in ESRD, suggesting their potential roles in suppressing the progression of CKD and ESRD. One SG, endoplasmic reticulum calcium-binding protein reticulocalbin 2 (RCN2) was increased in ESRD but decreased in CKD, suggesting its potential markers for diagnosis and prognosis of ESRD; *3)* 116 out of 121 SGs (95.9%) upregulated in ESRD were ESRD-specific, suggesting their roles in promoting the progression of ESRD; and *4)* 859 out of 928 SGs (92.6%) downregulated in ESRD were ESRD-specific, suggesting their potential roles in inhibiting the ESRD progression.

Therefore, we have demonstrated for the first time that ESRD upregulates 116 SGs that may promote ESRD progression and downregulates 859 SGs that may inhibit ESRD progression in the PBMCs in patients with ESRD. If ESRD-specific upregulated 116 SGs plus ESRD-specific downregulated 859 SGs as 100% (975 SGs), this might suggest that ESRD upregulates specifically only 11.9% SGs to promote disease progression and downregulates 88.1% SGs for disease progression, indicating for the first time that as high as 88.1% SGs in the PBMCs may play homeostatic functions which could potentially contribute to the inhibition of ESRD progression.

Table 2b
The numbers of SGs upregulated in the PBMCs from patients with ESRD (121 SGs, 4.58%) were significantly higher than that in CKD (44 SGs, 1.67%) while more dramatic changes of the numbers of downregulated SGs (928 SGs, 35.14%) were observed in the PBMCs from patients with ESRD than that in CKD (55 SGs, 2.08%). (Gene list for all these up- and downregulated SGs in CKD and ESRD are listed in Supplement Table 3.)

		CKD	ERSD
up-regulated Cutoff: P < 0.05, log FC > 1	number percentage	44 1.67% (44/2641)	121 4.58% (121/2641)
down-regulated Cutoff: P < 0.05, log FC < −1	number percentage	55 2.08% (55/2641)	928 35.14% (928/2641)

3.3. ESRD-upregulated SGs had 2 folds higher percentages of the cytoplasm and nucleus subcellular groups than the controls; and had the higher percentages of five out of 13 SG functional groups including enzyme, kinase, peptide, transcription regulator, and transmembrane in comparison to the controls

We used IPA to map the subcellular locations for CKD- and ESRD-modulated SGs. As shown in Table 3A, CKD-upregulated SGs had higher

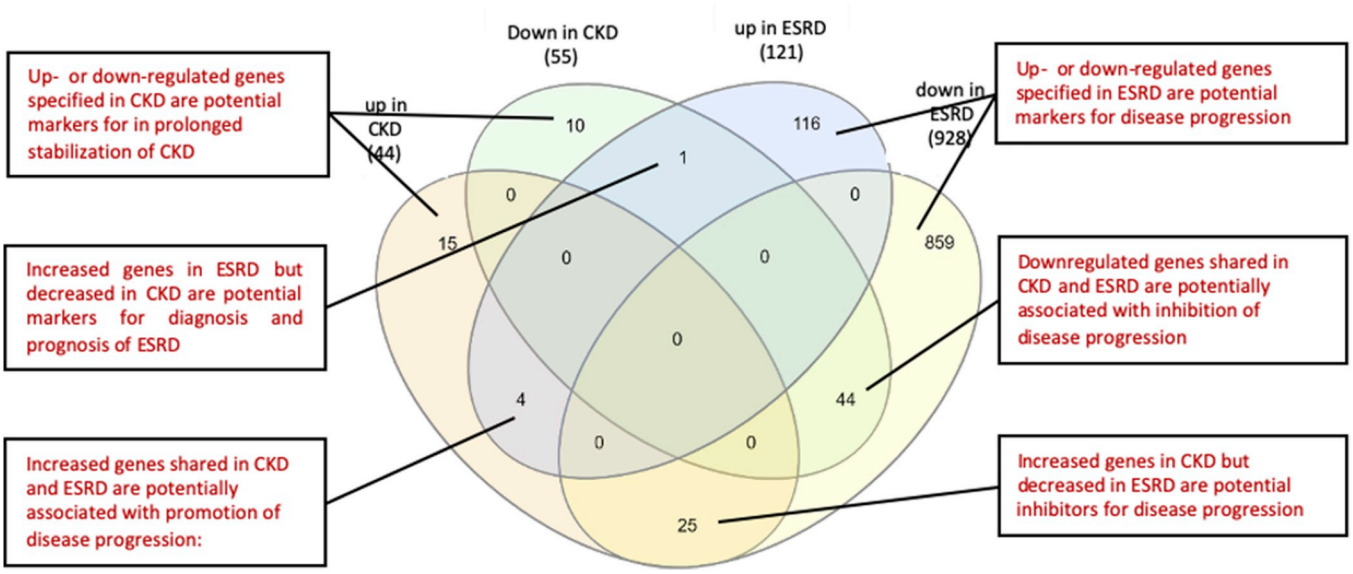

Fig. 1. Venn diagram analysis of the secretomic upregulation and downregulation in the PBMCs from patients with CKD and ESRD. This secretomic regulation can be broken into six categories. (Gene lists for each category are listed in Supplement Table 4.)

percentages of extracellular space SGs (56.82%) and plasma membrane SGs (20.45%) than that of total SGs controls, 49% and 16.19% respectively; but CKD-downregulated SGs had decreased percentages of extracellular space SGs (41.82%) in comparison to that total SGs control (49%). ESRD-upregulated SGs had higher percentages of cytoplasm SGs (47.11%) and nucleus SGs (8.26%) than that of total SGs controls, 22.08% and 4.46%, respectively. In contrast, ESRD-downregulated SGs had decreased percentages of cytoplasm SGs (15.95%) and other group SGs (3.23%) in comparison to that total SGs control (cytoplasm, 22.08%), and other group SGs (8.27%), respectively.

We then used IPA to map the 13 function groups for CKD- and ESRD-modulated SGs (Table 3A). In CKD-upregulated SGs, the percentages of five out of 13 functional groups including ion channel, kinase, peptide, transcription regulator, and transmembrane were increased. In ESRD-upregulated SGs, the percentages of five out of 13 functional groups including enzyme, kinase, peptide, transcription regulator, and transmembrane were increased in comparison to that of total SGs. These results demonstrated that CKD upregulated SGs have upregulated functional groups of SGs similar to that of ESRD except for the functional groups of enzyme and ion channel. Of note, some PBMC secretome proteins identified with a transcriptomic approach localized in the subcellular locations other than the supernatants of cultured cells and plasma that conventional secretomic analyses sampled and examined. Therefore, our data have also demonstrated that

Table 3a
All of the percentages of five subcellular location groups of SGs and 14 functional groups of SGs were significantly changed in CKD and ESRD compared with that of total SG controls according to IPA results. Gene list for all these up- and downregulated SGs in CKD and ESRD as well as all of the SGs are listed in Supplement Table 3. (# - number of SGs, % - percentage in each location).

classification		Total SGs (control)		up in CKD*		down in CKD*		up in ESRD*		down in ESRD*	
	group	#	%	#	%	#	%	#	%	#	%
Location	cytoplasm	574	22.08%	8	18.18%	12	21.82%	57	47.11%	148	15.95%
	extracellular space	1274	49.00%	25	56.82%	23	41.82%	31	25.62%	532	57.33%
	nucleus	116	4.46%	1	2.27%	4	7.27%	10	8.26%	31	3.34%
	other	215	8.27%	1	2.27%	4	7.27%	1	0.83%	30	3.23%
	plasma membrane	421	16.19%	9	20.45%	12	21.82%	22	18.18%	187	20.15%
total		2600	100.00%	44	100.00%	55	100.00%	121	100.00%	928	100%
Functional group	cytokine	156	6.00%	1	2.27%	4	7.27%	6	4.96%	68	7.33%
	enzyme	464	17.85%	5	11.36%	9	16.36%	32	26.45%	151	16.27%
	G-protein coupled receptor	18	0.69%	0	0.00%	0	0.00%	0	0.00%	11	1.19%
	growth factor	120	4.62%	2	4.55%	3	5.45%	6	4.96%	69	7.44%
	ion channel	10	0.38%	1	2.27%	1	1.82%	0	0.00%	8	0.86%
	kinase	47	1.81%	2	4.55%	4	7.27%	4	3.31%	21	2.26%
	other	1279	49.19%	18	40.91%	22	40.00%	39	32.23%	389	41.92%
	peptide	232	8.92%	8	18.18%	2	3.64%	13	10.74%	108	11.64%
	phosphatase	25	0.96%	0	0.00%	0	0.00%	0	0.00%	11	1.19%
	transcription regulator	46	1.77%	2	4.55%	3	5.45%	5	4.13%	12	1.29%
	translation regulator	2	0.08%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
	transmembrane receptor	115	4.42%	4	9.09%	4	7.27%	11	9.09%	45	4.85%
	transporter	86	3.31%	1	2.27%	3	5.45%	5	4.13%	35	3.77%
total		2600	100.00%	44	100.00%	55	100.00%	121	100.00%	928	100.00%

*P < 0.05.

Table 3b
Transcription factor analysis using TRANSFAC database (http://genexplain.com/transfac/) through the access of the GATHER database (https://changlab.uth.tmc.edu/gather/) showed that top 10 transcription factors filtered by P value were involved in each of the four groups of SGs such as CKD upregulated SGs, CKD-downregulated SGs, ESRD-upregulated SGs and ESRD-downregulated SGs. Of note, five out of ten transcription factors that are involved in ESRD downregulated SGs are isoforms of E2F1.

	Annotation	-ln (p value)*	main function/effect	PMID
up SGs in CKD	E2F1(V$E2F1_Q6_01)	5.94	control cell-cycle progression from G1 to S phase	7969176
	fork head box J 2(V$FOXJ2_01)	5.94	suporession of migration and invasion	25873280
	NF-kappaB (p65) (V$NFKAPPAB65_01)	5.13	proinflammation to disease progression	30135182
	E2F1(V$E2F1_Q6)	5.08	control cell-cycle progression from G1 to S phase	7969176
	KROX (V$KROX_Q6)	5.01	tooth development	12489153
	DP-1 heterodimer (V$E2F1DP1_01: E2F-1)	4.31	cell cycle	8405995
	E2A (V$E2A_Q2)	4.31	pathogenesis of lymphocytic leukemia.	26301816
	LEF1(V$LEF1TCF1_Q4)	3.89	cell proliferation	31623618
	upstream stimulating factor (V$USF_Q6)	3.84	lipid metabolism and atherosclerosis	19910639
	activator protein 1(V$AP1_Q4)	3.84	cell growth, differentiation, and apoptosis	15564374
down SGs in CKD	nuclear respiratory factor 2(V$NRF2_01)	5.56	oxidative stress	27646262
	SREBP(V$SREBP_Q3)	5.51	glucose metabolism	28920951
	NFKB(V$NFKB_Q6_01)	5.24	proinflammatory response	31101940
	c-ETS-1 binding site (V$ETS1_B)	4.9	cell differentiation	30566881
	AP-1 binding site (V$AP1_C)	4.84	cell growth, differentiation, and apoptosis	15564374
	c-Ets-2 binding sites (V$ETS2_B)	4.84	osteogenesis	11175361
	ZTA (V$ZTA_Q2)	4.78	Epstein-Barr Virus Reactivation	27708396
	c-Rel (V$CREL_01)	4.61	tumorigenesis	26757421
	DEAF1(V$DEAF1_01)	4.61	intellectual disability	24726472
	TEL2(V$TEL2_Q6)	4.49	hematopoiesis	28693791
up SGs in ESRD	Egr-1(V$EGR1_01)	8.42	inflammation and fibrosis	21511034
	Egr-2(V$EGR2_01)	7.4	maintenance of peripheral nerve myelin	15836632
	CEBPGAMMA (V$CEBPGAMMA_Q6)	6.61	antioxidant regulator	26667036
	PIT1(V$PIT1_Q6)	6	obesity and insulin resistance	27568561
	Ikaros 3(V$IK3_01)	5.94	T helper cell 2 transcription factor	21469117
	Hepatic nuclear factor 1(V$HNF1_C)	5.79	transcription inducer for proinflammatory molecules such as C-reactive protein, IL-6, HNF1a and HNF4a in CKD	29330688
	early growth response gene 3 product (V$EGR3_01)	5.18	IL-1b co-expressed inflammatory gene	31612215
	POU1F1(V$POU1F1_Q6)	5.13	inflammation/ immunity and hormone regulator	27709372

(*continued on next page*)

Table 3b (*continued*)

	Annotation	-ln (p value)*	main function/effect	PMID
	E4BP4(V$E4BP4_01)	4.96	obesity and insulin resistance	27050305
	cell division control protein 5(V $CDC5_01)	4.72	unguarded cellular proliferation	18583928
down SGs in ESRD	E2F1(V$E2F1_Q6)	12.62	control cell-cycle progression from G1 to S phase	7969176
	nuclear respiratory factor 2(V$NRF2_01)	12.62	oxidative stress	27646262
	E2F1(V $E2F1_Q6_01)	12.62	control cell-cycle progression from G1 to S phase	7969176
	E2F1(V $E2F1_Q3_01)	12.62	control cell-cycle progression from G1 to S phase	7969176
	E2F(V$E2F_Q6)	12.62	control cell-cycle progression from G1 to S phase	7969176
	E2F(V$E2F_Q3_01)	12.62	control cell-cycle progression from G1 to S phase	7969176
	KROX (V$KROX_Q6)	12.62	tooth development	12489153
	CREB(V $CREBATF_Q6)	12.62	formation of long-lasting memories	20223527
	CREB(V $CREB_Q4_01)	12.62	formation of long-lasting memories	20223527
	NRF1(V$NRF1_Q6)	12.62	Cholesterol Homeostasis	29149604

*P value were calculated based on the probability of seeing a Bayes factor of a particular magnitude in a query.

transcriptomic analyses of PBMC secretome have advantages to identify more comprehensive secretome than conventional secretomic analyses [69].

3.4. Although CKD- and ESRD-upregulated SGs were highly diversified in signaling, ESRD-induced SGs had strong proinflammatory pathways

To characterize the signaling pathways that CKD- and ESRD-modulated SGs are involved in, we adapted **IPA** to map SGs pathways. As shown in Fig. 2A, IPA indicated that upregulated and downregulated SGs in CKD were highly diversified in signaling pathways and were not classified into any signaling pathways in a statistically significant manner. The results suggest that there are diversified and multi-regulatory factor-based signals involved in controlling SGs modulations in CKD. In Fig. 2B, the IPA results showed that 22 out of total 121 (18.2%) upregulated SGs in the PBMCs from patients with ESRD were classified in five active pathways according to IPA core analysis including dermatan sulfate biosynthesis, neuroprotective role of Thimet oligopeptidase (THOP1) [70] in Alzheimer's disease, IL-8 signaling, cardiac hypertrophy and neuroinflammation signaling pathway. The rest of 99 SGs (81.8%) upregulated SGs in the PBMCs from patients with ESRD were in a diversified manner similar to that observed in CKD. Of note, the chondroitin sulfate/dermatan sulfate (CS/DS)-containing proteoglycans (CS/DSPGs) are extracellular matrix (ECM) molecules, which mediate the accumulation of lipoproteins in the sub-intimal spaces, a key event occurring during the pathobiology of atherosclerosis and the progression of vascular damage [71]. Further Venn Diagram analysis shown in Fig. 2C and, 18 SGs in the four out of five pathways indicated that the SGs upregulated in ESRD have strong proinflammatory roles in PBMCs, especially newly identified chondroitin sulfate/dermatan sulfate (CS/DS)-containing proteoglycans (CS/DSPGs).

A total of 113 active pathways were identified in ESRD downregulated SGs via IPA analysis. Six out of 113 pathways (5.31%) were positively activated by downregulated SGs in ESRD, including SPINK1 Pancreatic Cancer Pathway, Inhibition of Matrix Metalloproteases, PPAR Signaling, Apelin Cardiac Fibroblast Signaling Pathway, Antioxidant Action of Vitamin C, and PTEN Signaling. The rest of the pathways were downregulated, suggesting that a large number of SGs pathways in the PBMCs from patients with ESRD were downregulated for ESRD progression, which potentially drives physiological functions and homeostasis. Of note, the five active pathways induced by upregulated SGs in ESRD (Fig. 2B) were also included in 113 active pathways induced by downregulated SGs in ESRD according to Venn Diagram, which showed that some components in these ESRD-upregulated pathways can be fully functional in the absence of the other components in these pathways downregulated in ESRD.

In addition, to identify the upstream regulating transcription factors (**TFs**) for CKD- and ESRD-modulated SGs, we used the GATHER database (https://gather.genome.duke.edu/) [72] to map the TF binding in the promoters of the modulated SGs. As shown in Table 3B and Fig. 2F, the top 10 TFs bound to the promoters of CKD-upregulated SGs were E2F1, FoxJ2, NFkBp65, E2F1, ROX, DP-1 heterodimer, E2A, LEF1TCF1, upstream stimulating factor and activator protein 1, which were different from that of ESRD-upregulated SGs. The top 10 TFs bound to the promoters of ESRD-upregulated SGs included Egr-1 (inflammation and fibrosis) [73], Egr-2 (maintenance of peripheral nerve myelin), CCAAT/enhancer-binding protein (CEBPgamma) (antioxidant regulator) [74], phosphate inorganic transporter 1 (PIT1) (obesity and insulin resistance) [75], Ikaros 3 (T helper cell 2 transcription factor) [76], hepatic nuclear factor 1 (HNF1) (transcription inducer for proinflammatory molecules such as C-reactive protein, IL-6, HNF1a and HNF4a in CKD) [77], early growth response gene 3 (IL-1β co-expressed inflammatory gene) [78], POU1F1 (inflammation/immunity and hormone regulator) [79], E4BP4 (obesity and insulin resistance) [80], and cell-division control protein 5. In addition, we noticed that nuclear respiratory factor 2 (different from the antioxidant transcription factor nuclear factor-erythroid-derived 2-like 2, Nrf2) [81] was

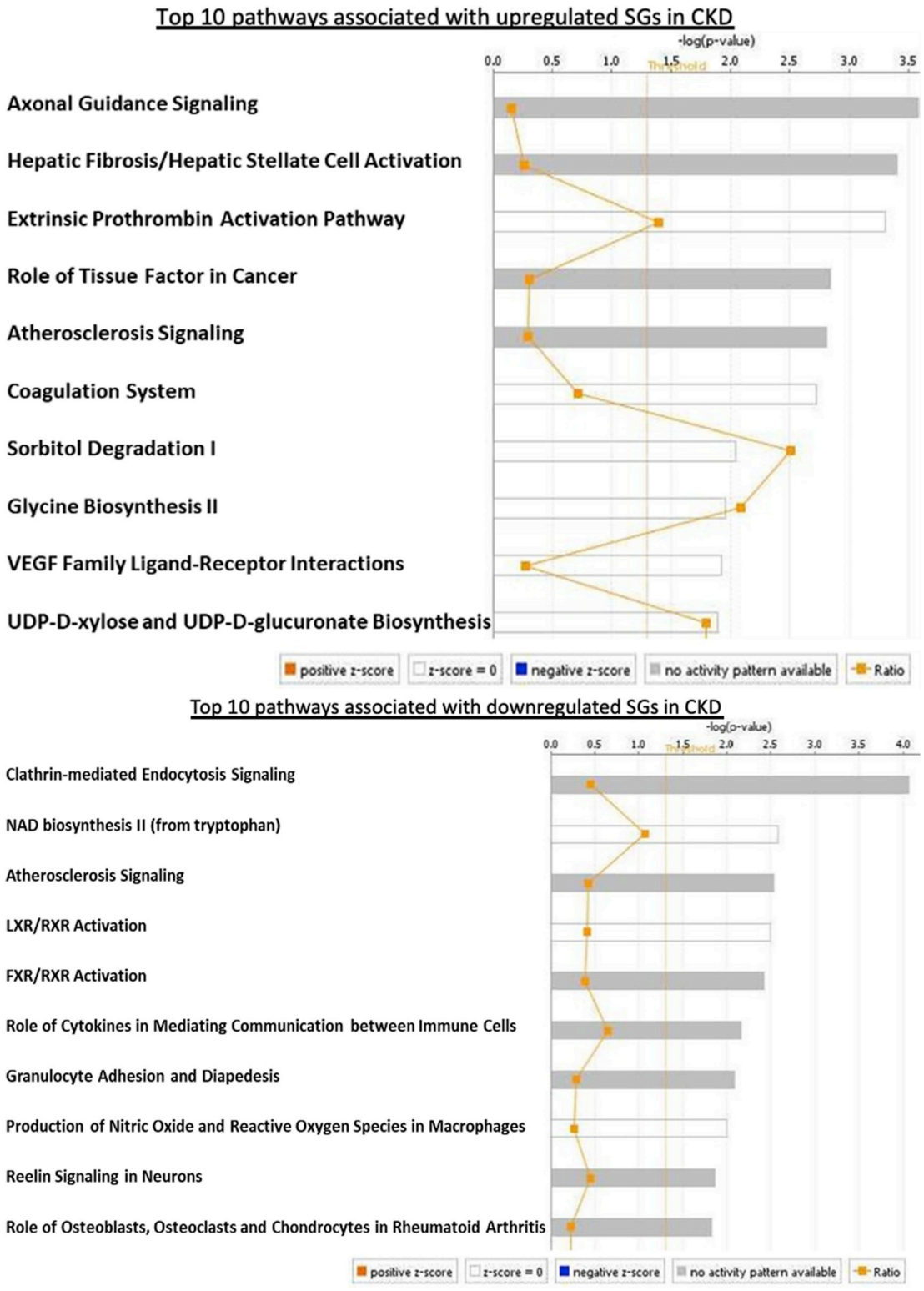

Fig. 2a. Top 10 pathways of upregulated and downregulated SGs in CKD from IPA. These pathways were highly diversified in signaling pathways and were not classified into any signaling pathways in a statistically significant manner (cutoff: P value < 0.05, $|z\text{-score}| > 2$). (Lists of all pathways associated with these up- and downregulated SGs in CKD via IPA are listed in Supplement Table 5.)

downregulated in ESRD-downregulated SGs (Table 3B and Fig. 2F). In summary, the seven TFs out of ten TFs identified with the Gather database indicated that ESRD-upregulated SGs promote inflammation, obesity and insulin resistance and fibrosis.

The Venn Diagram Analysis results (Fig. 3A) on the three secretory gene groups such as 35 UT genes (encoded for 30 UTs in Table 1A), 44 CKD upregulated SGs and 121 ESRD-upregulated SGs showed that: 1) UTs have no overlaps with CKD-upregulated SGs; 2) UTs have two toxins (CFD, and RETN) overlapped with ESRD-upregulated SGs; 3) ESRD-upregulated SGs have four SGs (ADAM Metallopeptidase Domain 9 (ADAM9), complement C3 (C3), Heat Shock Protein 90 Beta Family Member 1 (HSP90B1), and S100 Calcium Binding Protein A12

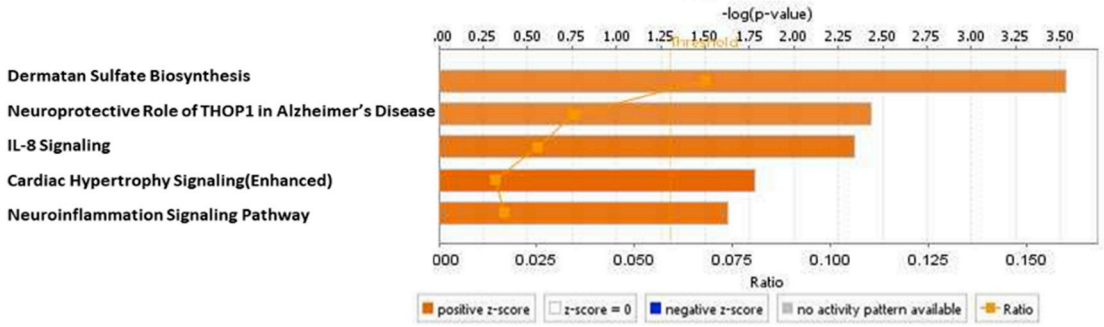

Fig. 2b. 22 SGs out of total 121 (18.2%) upregulated SGs in the PBMCs from patients with ESRD were classified in five active pathways according to IPA core analysis (cutoff: |z-score| > 2). The other 99 SGs (81.8%) were in a diversified manner similar to that in CKD. (Lists for all pathways associated with these up- and down-regulated SGs in ESRD via IPA are listed in Supplement Table 5.)

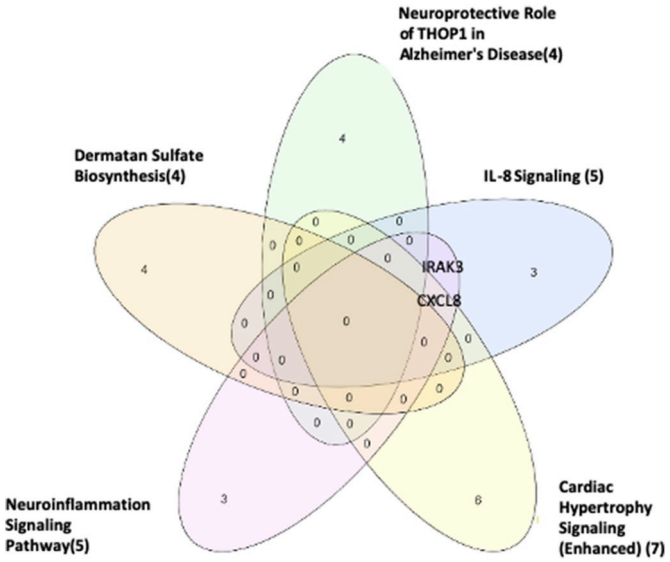

Fig. 2c. The Venn Diagram Analysis of the five signaling pathways specified in Fig. 2B. Interleukin 1 receptor-associated kinase 3 (IRAK3) is involved in two pathways (IL-8 signaling and Neuroinflammation Signaling Pathway). C-X-C Motif Chemokine Ligand 8 (CXCL8) is involved in three pathways (IL-8 Signaling, Cardiac Hypertrophy and Neuroinflammation Signaling Pathway). (Gene list for these five pathways associated with these up- and downregulated SGs in ESRD via IPA are listed in Supplement Table 6.)

(S100A12)) overlapped with CKD-upregulated SGs. In addition, one signaling pathway "Role of Cytokines in Mediating Communication between Immune Cells" was shared by the top 10 pathways associated with UTs and the five active pathways upregulated by SGs in ESRD. These results suggest that the signaling pathway "Role of Cytokines in Mediating Communication between Immune Cells" may be significant in ESRD progression.

ClueGo (v2.5.4) from Cytoscape (v3.7.2) was also used to verify a close functional relationship between UT genes further and up-regulated SGs in ESRD (Fig. 3B) [82–84]. Of note, the small dots were the genes that connected UT gene group and upregulated SGs in ESRD. ClueGo identified four ESRD-upregulated SGs pathways (big balls in blue color), neuropeptide receptor finding, regulation of endocrine process, lung fibrosis and hyaluronan metabolic process. In addition, ClueGo identified five UT genes pathways, such as prostaglandin biosynthetic process (big balls in red color). Moreover, ClueGo found 20 shared (big balls in grey color, connected) regulatory pathways (Supplemental Table 8).

In summary (Table 4D), based on the four aspects above including *1)* IPA analyses on proinflammatory signaling pathways of ESRD-upregulated SGs (Fig. 2B) and *)* IPA analyses on activating profibrotic pathways in ESRD-downregulated SGs (Fig. 2D); *3)* Gather/TRANSFAC-identified TFs involved in proinflammatory and profibrotic pathways (Table 3B); *4)* ClueGo (Cytoscape)-identified ESRD-upregulated SGs shared with that of UTs (Fig. 3B), our results demonstrated that UT genes and ESRD-upregulated SGs share many signaling pathways, especially in some pro-inflammatory/profibrotic pathways. These results were correlated well with our recent report on our new model of inflammation-driven upregulation for uremic toxin generation rather than the traditional model of passive accumulation of metabolites fully due to kidney dysfunction to generate uremic toxins [10].

3.5. Proinflammatory cytokines-based middle class UTs such as interleukin-1β (IL-1β) and IL-18 promote ESRD modulation of SGs

To identify the molecular mechanisms underlying ESRD modulation of SGs, we selected proinflammatory cytokine-based UTs such as IL-1β and IL-18 stimuli to demonstrate proof of principle. As shown in Table 4A, IL-1β stimulation of PBMCs resulted in upregulation of five out of 121 ESRD-upregulated SGs such as C-X-C Motif Chemokine Ligand 2 (CXCL2), Interleukin 1 Receptor Associated Kinase 3 (IRAK3), Phospholipase A2 Group VII (PLA2G7), Sphingomyelin Phosphodiesterase Acid Like 3A (SMPDL3A) and Thrombospondin 1 (THBS1). Of note, the Geo dataset (microarray experiments) used as many as 17 innate immune stimuli, IL-1β stimulated data were chosen for our analysis since the rest of innate immune stimuli were not the reported UTs. In addition, IL-1β stimulation downregulated 29 out of 928 ESRD-downregulated SGs (Table 2B) (3.13%). Moreover, IL-18 stimulation upregulated five out of 121 ESRD-upregulated SGs (4.13%) such as complement factor D (CFD), Interleukin-6 Receptor Subunit Beta (IL6st), IRAK3, Platelet-Derived Growth Factor C (PDGFC), and SIL1 Nucleotide Exchange Factor (SIL1); and downregulated 29 out of 928 ESRD-downregulated SGs (Table 2B) (3.13%). Furthermore, IL-18 downregulated 105 out of 928 ESRD-downregulated SGs (11.31%). Our results have demonstrated for the first time that as a novel mechanism underlying the phenotype of ESRD-induced SG changes in the PBMCs from patients with ESRD, proinflammatory cytokines-based middle class UTs such as IL-1β and IL-18 promote ESRD modulation of SGs; and that the ESRD-downregulated SGs contribute to physiological functions. Their downregulation by proinflammatory cytokines IL-1β and IL-18 further strengthen this conclusion.

In Tables 3A and 4C, IPA classified the parts of the SGs modulated in the PBMCs from patients with CKD and patients with ESRD as the cytokine group. The results in Table 4C showed that 4 out of 55 SGs (7.2%) downregulated in CKD (also shown in Table 2B) were cytokines such as IL-24, interleukin 36 receptor antagonist (IL36RN), platelet factor 4 (PF4), and ectodysplasin A (EDA); one Dickkopf WNT signaling pathway inhibitor 3 (DKK3, a tumor suppressor) out of 44 SGs (2.27%) upregulated in CKD was cytokine; 68 out of 928 SGs (7.3%) downregulated in ESRD were cytokines; and 6 out of 121 SGs (4.96%)

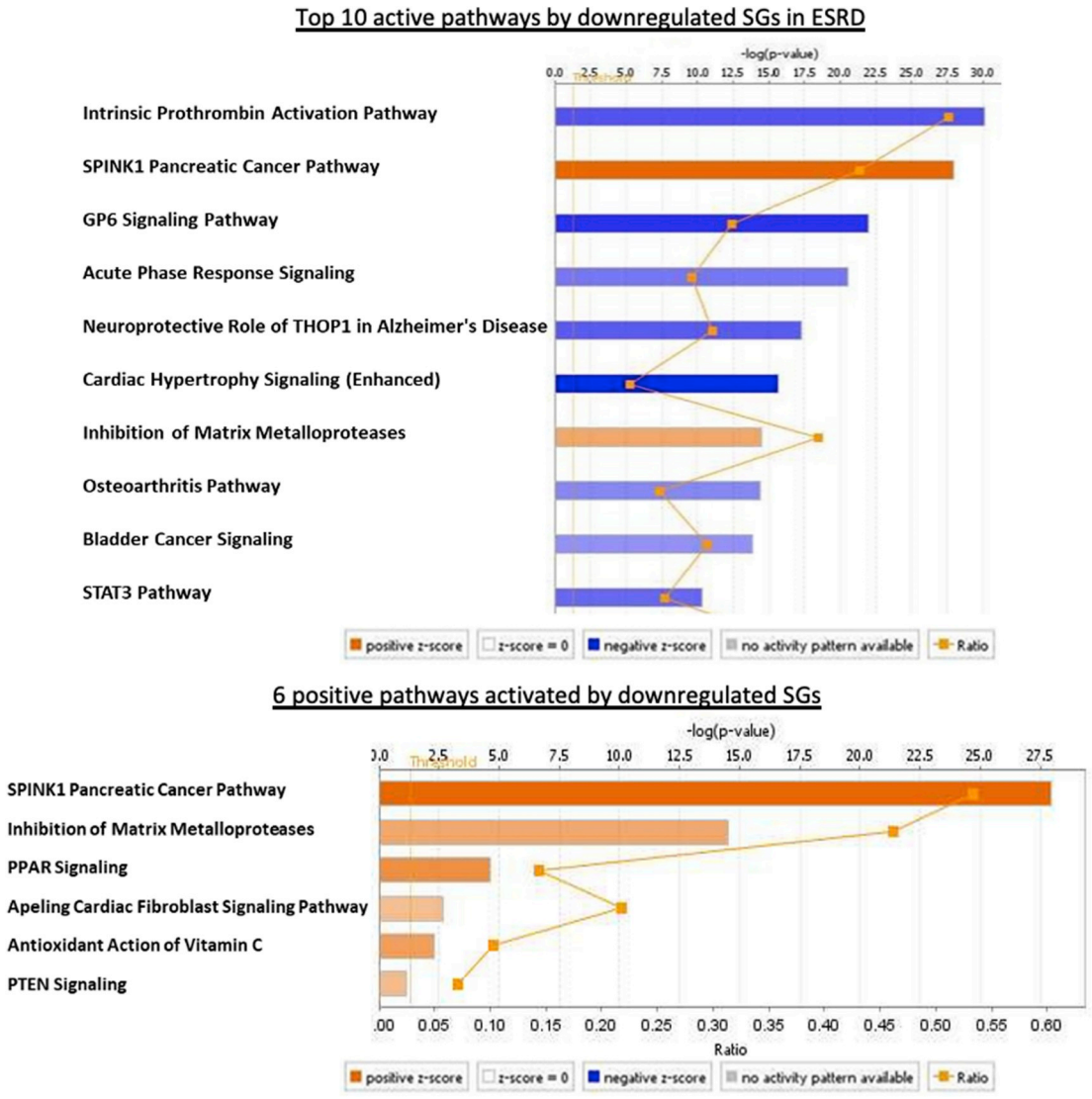

Fig. 2d. A total of 113 active pathways were identified in ESRD down-regulated SGs according to IPA (cutoff: |z-score| > 2). The top 10 active pathways are shown (top). Only six active pathways (5.31%) were positively activated by downregulated SGs in ESRD (bottom), including SPINK1 Pancreatic Cancer Pathway, Inhibition of Matrix Metalloproteases, PPAR Signaling, Apelin Cardiac Fibroblast Signaling Pathway, Antioxidant Action of Vitamin C, and PTEN Signaling. The rest of the pathways were downregulated. (Gene list for these 113 pathways associated with these up- and downregulated SGs in ESRD via IPA are listed in Supplement Table 7.)

upregulated in ESRD were cytokines including inflammation-modulating aminoacyl tRNA synthetase complex interacting multifunctional protein 1 (AIMP1), inflammatory C-X-C motif chemokine ligand 2 (CXCL2), inflammatory CXCL8, T cell and B cell promoting IL7, T cell and natural killer cell-activating IL-15 and inflammatory X–C motif chemokine ligand 1 (XCL1).

In Fig. 3C, the Venn Diagram Analysis results showed that four cytokines downregulated in CKD were shared with that downregulated in ESRD. The expanded list of 68 cytokines downregulated in ESRD can be split into two groups. First are the chemokine subset of cytokines. Chemokines are involved in cell migration, activation and tissue injury and thus key mediators of inflammation, especially in cardiovascular disease [85]. According to previous studies, the majority of these proteins were involved in the homeostatic function of immune cells due to their ligand promiscuity [86]. The profile of chemokines downregulated in ERSD were mostly “homeostatic” compartments rather than “inflammatory” ones. Typically, CCL2, 3, 4, 5, 11, CXCL1, 2, 8, and 10 played proinflammatory roles in kidney disease; and these chemokines were not included in this list [87–89]. Secondly, pro-inflammatory cytokine drivers were “common” in kidney disease including IL6, IL8, IL10, IL17 and IL18 and were not included in our downregulated cytokine list [88]. These cytokines may be contributed by cell types other than PBMCs during kidney diseases. Interestingly, both proinflammatory and anti-inflammatory members (IL-1Ra and IL-36G) were all downregulated, indicating that a compensatory balance weighed by PBMCs was associated with disease progression [90]. Of note, IL10 family members, including IL-19, IL-22, IL-24, IL-26 and IL-28 were downregulated [90]. Based on the overall proinflammatory phenotype associated with secretomic changes during ESRD, these results suggested that in addition to the modulation by cytokines and chemokines, additional secretomic changes modulated by other mechanisms may play significant roles in disease progression.

One cytokine upregulated in CKD, DKK3, was shared with that downregulated in ESRD. The six cytokines upregulated in ESRD were ESRD-specific. The results suggest that *first*, the numbers of SGs in the cytokine groups upregulated in ESRD are significantly higher than that of CKD; and *second*, highly focused six cytokines and chemokines upregulated in ESRD may play significant roles in promoting ESRD progression and systemic inflammations. To integrate all the findings on proinflammatory cytokines from UTs and from SGs upregulated in

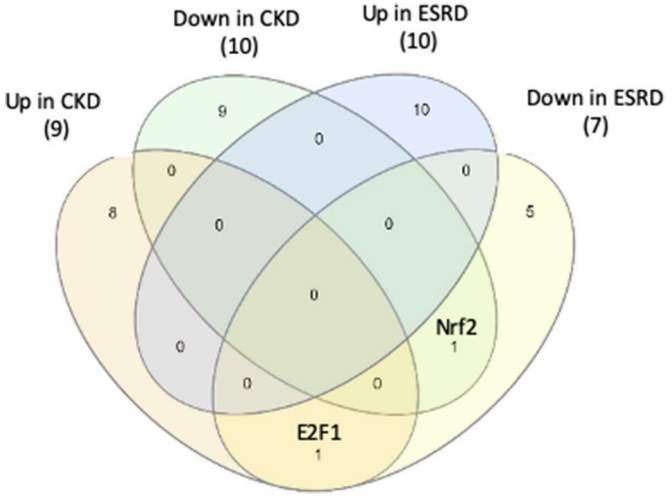

Fig. 2f. The Venn Diagram of transcript factors analysis in Table 3B. E2F1 was shared by CKD-upregulated SGs and ESRD-downregulated SGs in ESRD. In addition, nuclear respiratory factor 2 (Nrf2), a key transcription factor in Redox Oxygen Species (ROS), was shared in two groups of SGs, CKD-downregulated SGs and ESRD-downregulated SGs, indicating these two transcription factors may serve as an important inhibitor of disease progression.

ESRD, we proposed a new mechanism in Fig. 3D. Proinflammatory cytokines (primary, upstream) play significant roles in combination with other uremic toxins and other mechanisms in upregulating SGs (secondary, downstream), promoting the pathogenesis of ESRD and inflammations. Of note, the classification of primary/upstream cytokines and secondary/downstream cytokines is conceptual to demonstrate the cytokine interaction as the proof of principle. We used the proinflammatory cytokines IL-1β and IL-18 from UTs as prototypic secretomic proteins to demonstrate the mutual promotion and modulation among the secretomic proteins as the role-switching of "primary" or "secondary" cytokines during ESRD. Future time course experiments will be needed to characterize chronological upregulation of cytokines in upregulated UTs and SGs in ESRD.

3.6. ESRD-upregulated co-stimulation receptors CD48 and CD58 increase secretomic upregulation in the PBMCs; may not be even limited in the PBMCs, CD48/CD58-CD2 signaling can be actually magnified enormously in tissues

We hypothesized that CKD- and ESRD-upregulated T cell activation co-stimulation receptors (**CSRs**) and co-inhibition receptors (immune checkpoint receptors, **ICRs**) [91], as prototypic cell membrane receptors in mediating cell-cell interactions, upregulate SGs in PBMCs (antigen-presenting cells, **APC**) via reverse signaling as we reported [22,92]. As shown in Table 5A, the expression of 14 CSRs, 4 dual-function receptors, and 10 ICRs were examined in the microarrays of the PBMCs from patients with ESRD and CKD (Table 2A) as we reported [92]. The results show that *1)* ESRD upregulates CSRs CD48 and CD58 but downregulates seven out of 14 CSRs including Inducible T Cell Costimulator Ligand (ICOSLG), CD70, TNF Superfamily Member 14 (TNFSF14), CD40, TNFSF15, TNFSF18, and Signaling Lymphocytic Activation Molecule Family Member 1 (SLAMF1); *2)* ESRD downregulated one out of four dual function receptors poliovirus receptor (PVR) (con-stimulation at naïve T cells but co-inhibitory at activated T cells); *3)* ESRD downregulated four out of 10 immune checkpoint receptors (co-inhibition receptors) such as nectin cell adhesion molecule 3 (NECTIN3), programmed cell death 1 ligand 2 (PDCD1LG2), human endogenous retrovirus-H long terminal repeat-associating protein 2 (HHLA2) and butyrophilin like 2 (BTNL2); and *4)* CKD upregulated one immune checkpoint receptor HHLA2 but downregulated one co-stimulation receptor TNFSF8.

To determine whether ESRD upregulated co-stimulation receptors [92], CD48 and CD58, play any causative roles in regulating ESRD-modulated SG expressions, we tried to find available microarray or RNA-sequencing datasets associated with overexpression or deficiency of CD48 and CD58. As no such datasets are available at the time of this writing, we used the GEO datasets (GSE15215) related to CD2, a membrane protein acting as the ligand for both CD48 and CD58 on dendritic cells (DCs, $CD2^+$ DCs versus $CD2^-$ DCs), to determine whether the forward signaling of CD48 and CD58 (from antigen-presenting cells toward T cells) can modulate the expression of SGs modulated in ESRD. These results showed that CD2 upregulates 14 out of 121 SGs (11.6%) upregulated in ESRD; and CD2 downregulates 25 out of 928

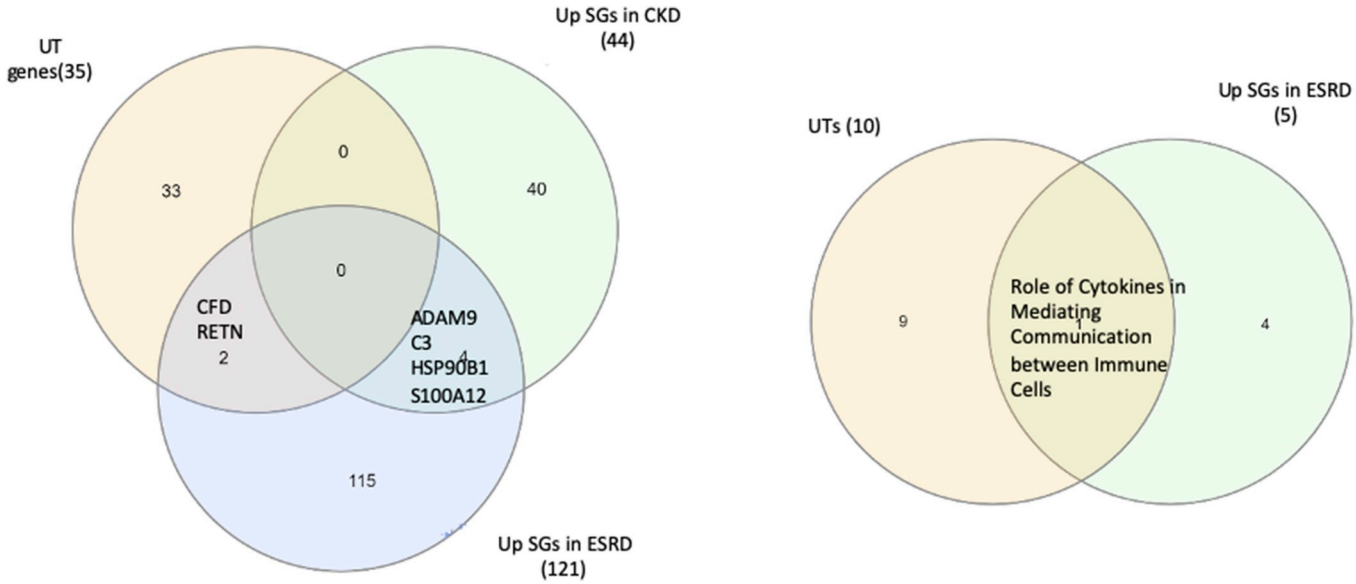

Fig. 3a. The Venn Diagram results on the three groups such as 35 UT genes (encode total 30 UTs), 44 CKD upregulated SGs and 121 ESRD-upregulated SGs showed that 1) UTs have no overlaps with CKD-upregulated SGs; 2) UTs have two toxins (CFD, and RETN) overlapped with ESRD-upregulated SGs; 3) ESRD-upregulated SGs have four SGs (ADAM9, C3, HSP90B1, and S100A12) overlapped with CKD-upregulated SGs. In addition, one signaling pathway "Role of Cytokines in Mediating Communication between Immune Cells" was shared by the top 10 pathways associated with UTs and the five active pathways upregulated by SGs in ESRD.

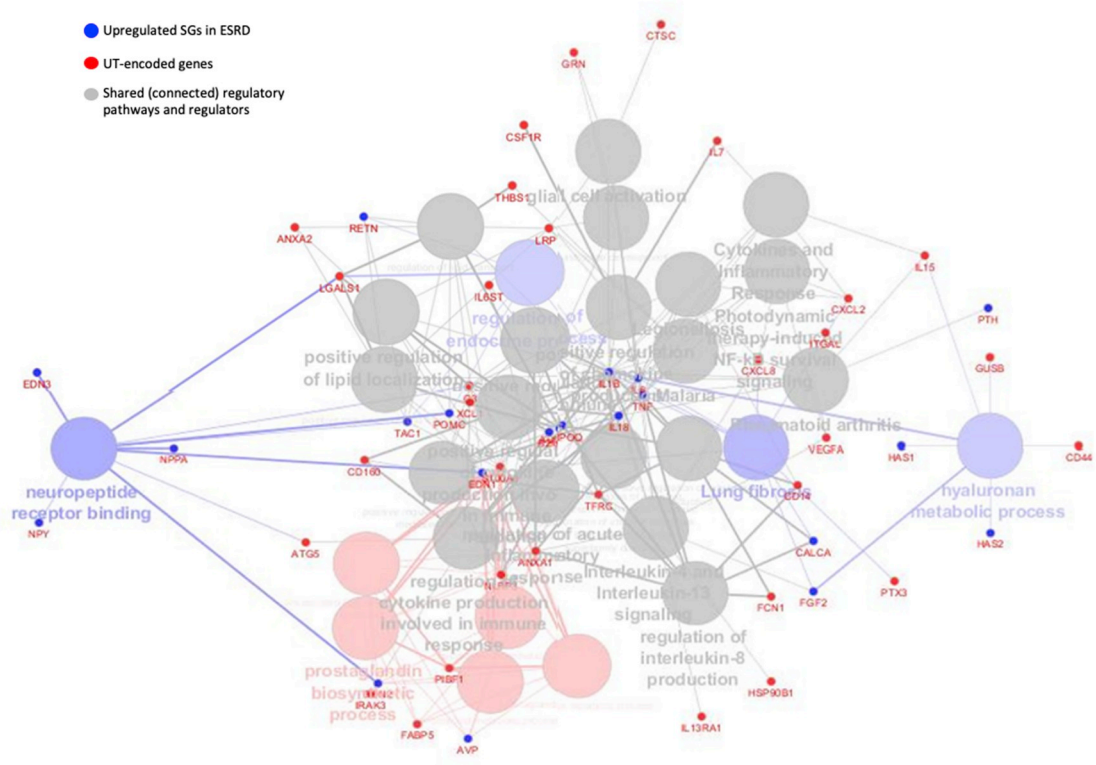

Fig. 3b. The ClueGo v2.5.4 from Cytoscape v3.7.2 used as a secondary software to confirm a close functional relationship between UT-encoded genes and up-regulated SGs in ESRD. (Group-specific and connective function are listed in supplement Table 8.)

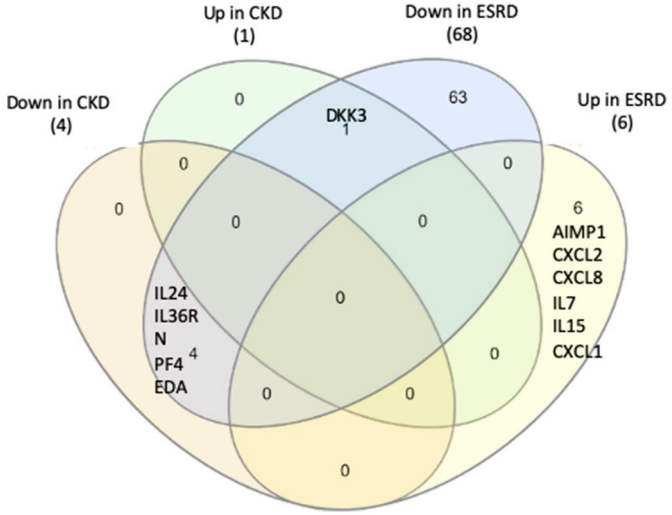

Fig. 3c. The Venn Diagram Analysis results showed that 1) all of the four cytokines (IL24, IL36RN, PF4, EDA) downregulated in CKD are overlapped that downregulated in ESRD; 2) the one cytokine upregulated in CKD is overlapped with that downregulated in ESRD; and 3) six cytokines upregulated in ESRD are not overlapped with the other three groups.

SGs (2.7%) downregulated in ESRD (see Table 5b). We found that CD48/CD58-CD2 signaling may amplify the SGs alteration in ESRD. These results suggest that CD48/CD58-CD2 signaling promotes SGs upregulation in ESRD. Of note, the justifications for this analysis are: *1)* DCs can be the parts of PBMCs in patients with ESRD [93]; *2)* CD2 protein is also expressed in monocytes, B lymphocytes, $CD4^+$ T cells, $CD8^+$ T cells NK cells, platelets, bone marrow stromal cells, which were the CD2 expression data collected from the GeneCards database (https://www.genecards.org/cgi-bin/carddisp.pl?gene=CD2#protein_expression); *3)* new RNA-sequencing data from Human Protein Atlas Database (https://www.proteinatlas.org) indicated that CD48 and CD58 are expressed in every one of 27 tissue examined; and that their ligand (CD2) is also expressed in every one of 27 tissues examined, which are correlated with the CD2 protein expression data collected in the GeneCards database shown above (see Fig. 4a); and *4)* as shown in Fig. 4bB, the protein expression data from the Proteomics Database (https://www.proteomicsdb.org/) showed that CD2 protein can be highly enriched in cytotoxic T-lymphocyte, natural killer cell, bone marrow stromal cell, helper T-lymphocyte, B lymphocyte and monocyte, which indicated the possibility of the signal amplification induced by co-stimulation of CD48/CD58-CD2 pathway. Of note, we reported previously that $CD40^+$ proinflammatory monocytes accelerate inflammation in CKD [7]. Taken together, these data suggest that *first*, ESRD-upregulated CD48 and CD58 increase secretomic upregulation in the PBMCs, whose signals may not be even limited in the PBMCs from patients with ESRD examined in this study; *second*, CD48/CD58-CD2 pathway can be actually magnified enormously in tissue levels so that the CD48/CD58-CD2 pathway-activated PBMCs in blood circulation accelerate vascular and other inflammations; and *third*, reverse signaling from $CD2^+$ T cells to $CD48^+$/$CD58^+$ PBMCs play significant roles in modulating PBMC secretomic changes in ESRD (see Fig. 4c).

3.7. Classically activated macrophages (M1)-, and alternatively activated macrophages (M2)- macrophage polarization signals contribute to ESRD- and CKD-upregulated SGs

We recently identified 20 new disease group-specific and 12 new shared pathways in macrophages in eight groups of 34 diseases including 24 inflammatory organ diseases and 10 types of cancers [94,95]. It has also been reported that M1 proinflammatory macrophages contribute to infection clearance, inflammation and renal injury, and M2 anti-inflammatory macrophages can contribute to the resolution phase of the response to injury [96,97]. We hypothesized

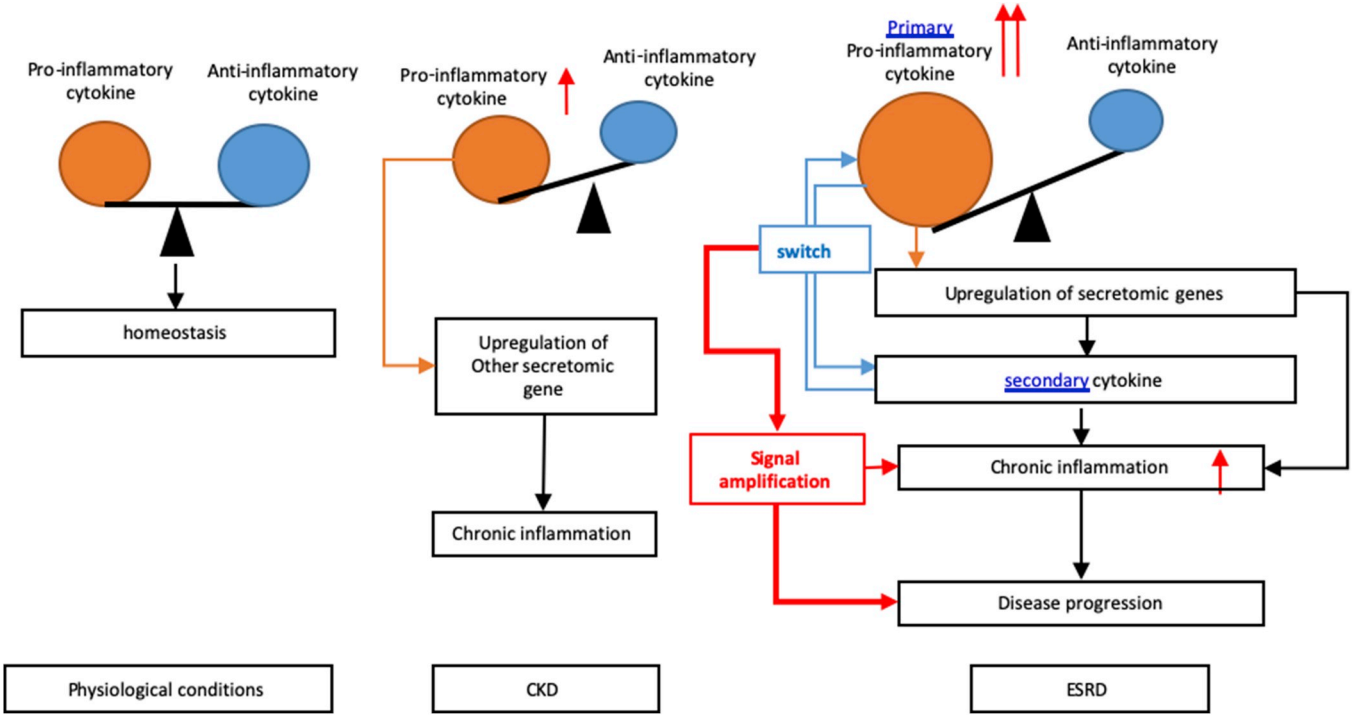

Fig. 3d. Novel mechanism I. Proinflammatory cytokines (primary) play significant roles in combination with uremic toxins and other mechanisms in upregulating SGs (secondary), promoting the pathogenesis of ESRD and inflammations. We used the proinflammatory cytokines as prototypic secretomic proteins to demonstrate the mutual promotion and modulation among the secretomic proteins as the role-switching of "primary" and "secondary" cytokines during ESRD.

that M1 and M2 macrophage polarization signals contribute to CKD- and ESRD-upregulated PBMC SG expressions. As shown in Table 6, eight out of 44 (18.18%) CKD-upregulated SGs were found in M1 macrophage polarization dataset; six out of 44 (13.64%), including three out of 44 (6.8%, M2a), four out of 44 (9.1%, M2b), one out of 44 (2.3%, M2c), CKD-upregulated SGs, were found in M2a, M2b, and M2c macrophage subset polarization, respectively. In addition, 15 out of 121 (12.4%) ESRD-upregulated SGs were found in M1 macrophage polarization dataset; 16 out of 121 (13.2%), including one out of 121 (0.83%, M2a), 11 out of 121 (9.1%, M2b), and 9 out of 121 (7.4%) ESRD-upregulated SGs were found in M2a, M2b, and M2c macrophage polarization, respectively. When examining those modulated SGs with IPA, no significant pathways were found. As shown in Fig. 5, these results suggest that: *1)* macrophage polarization pathways participate CKD-, and ESRD-upregulated secretomic changes in PBMCs in patients with CKD and ESRD; *2)* M1 proinflammatory macrophage polarization signal may play more important roles in facilitating PBMC secretomic upregulations in CKD and ESRD than the signals mediating three M2 macrophage subset polarizations and *3)* M1-, and M2-polarization signaling pathways involving in upregulating SGs are diversified.

3.8. ESRD- and CKD-upregulated SGs in PBMCs contain senescence-promoting regulators by upregulating proinflammatory growth factor IGFBP7 and downregulating anti-inflammatory cytokine TGF-β1 and leukocyte telomere length stabilizer SERPINE1/PAI-1

The senescence program is implicated in diverse biological processes, including embryogenesis, tissue regeneration and repair, tumorigenesis, aging and inflammation. Two main classes of senescent cells have been identified: acute and chronic senescent cells. Acute senescent cells are generated during coordinated, beneficial biological processes characterized by a defined senescence trigger, transient senescent-cell signaling functions, and eventual senescent-cell clearance. In contrast, chronic senescent cells arise more slowly from cumulative, diverse stresses and are inefficiently eliminated, leading to their accumulation and deleterious effects through a secretory phenotype [98]. Senescent cells secrete a variety of proteins collectively known as the senescence-associated secretory phenotype (SASP) [99]. Recent murine studies have shown that depletion of chronically senescent cells extends healthy lifespan and delays age-associated disease, implicating senescence and the senescence-associated secretory phenotype as drivers of organ (kidney) dysfunction [100]. Previous reports suggest that secretomic changes in CKD and ESRD modulate cellular senescence and disease-modulated aging process, renal fibrosis and cancers. In addition to senescence in the kidney, senescent vascular cells, both endothelial and smooth muscle cells, participate in atherosclerosis; senescent preadipocytes and adipocytes have been shown to lead to insulin resistance [101]. Thus, we hypothesize that CKD- and ESRD-related senescence signaling contributes to upregulated SGs in PBMCs. To examine this hypothesis, we compared 71 senescence regulators [102] with CKD-regulated SGs and ESRD-upregulated SGs, respectively. As shown in Table 7, *first*, no senescence regulators matched with CKD-upregulated SGs; *second*, two out of 71 senescence regulatory genes were decreased in CKD downregulated SGs [102], specifically ID1 (a member of ID family of helix-loop-helix transcriptional regulatory proteins, a kidney damage inhibitor and target of bone morphogenetic proteins [103]) and secreted protein acidic and rich in cysteine (SPARC). It has been reported that SPARC accelerates disease progression in experimental crescentic glomerulonephritis [104]; and SPARC leads to a progressive reduction in podocyte number, thus fueling the future development of glomerulosclerosis [105]. In addition, two out of 71 senescence regulatory genes including cellular repressor of E1A stimulated genes 1 (CREG1) and insulin-like growth factor-binding protein 7 (IGFBP7) were increased in ESRD-upregulated SGs. Of note, CREG1 haploinsufficiency confers increased susceptibility of adipose tissue to inflammation, leading to aggravated obesity and insulin resistance when challenged with a high fat diet [106]. IGFBP7 is one of the growth factors upregulated in patients with inflammatory breast cancer [107]. Moreover, five out of 71 senescence regulators such as serpin family E member 1, (SERPINE1, plasminogen activator inhibitor 1, PAI-1),

Table 4a
As a novel mechanism, cytokine-based m.w. UTs can amplify the ESRD signals in inducing secretomic changes in the PBMCs from patients with ESRD. The first example is that 4.13% of upregulated SGs in ESRD were upregulated in Interleukine-1 beta (IL-1B)-treated human blood leukocytes (GEO ID: GSE103500) and 3.13% were downregulated.

Uremic Toxin	Primary Change	Gene	P.Value	logFC
IL-1B GSE103500	upregulated ESRD SGs 5 (4.13%)	CXCL2	6.53E-05	4.959039
		IRAK3	0.002333	1.783057
		PLA2G7	0.033483	1.751108
		SMPDL3A	0.038285	1.081
		THBS1	0.02777	1.307772
	downregulated ESRD SGs 29 (3.13%)	ADAM12	0.003898	−2.62067
		B3GNT3	0.02258	−3.46046
		BRINP2	0.044496	−5.65328
		CAMP	0.024428	−1.31643
		CLPS	0.049925	−4.62095
		EGFL7	0.042206	−2.06849
		FGF6	0.015567	−4.70715
		FN1	0.018602	−2.11926
		GREM1	0.031547	−1.73607
		INSL6	0.012816	−3.0913
		IZUMO4	0.029209	−5.18373
		KLK10	0.017674	−2.11833
		KLK11	0.037963	−2.50173
		LIPF	0.028216	−1.33852
		LRRC17	0.015359	−7.35399
		MIA	0.013393	−1.3311
		MMP11	0.021633	−1.8703
		MMP28	0.004459	−3.13363
		MUC2	0.034662	−2.09434
		NRG1	0.038783	−1.98528
		OPRPN	0.049001	−3.26088
		PGC	0.034397	−1.69163
		POFUT1	0.011287	−1.93524
		PON1	0.036631	−4.57456
		PRL	0.015419	−4.23378
		PRLR	0.044216	−1.79944
		PVR	0.034772	−4.70854
		SERPINE1	0.012631	−1.57299
		SPARCL1	0.023002	−2.49064

Table 4b
As a novel mechanism, cytokine-based m.w. UTs can amplify secretomic changes in ESRD. The second example is that 4.13% upregulated SGs in ESRD were upregulated in IL-18-treated human blood leukocytes (GEO ID: GSE103500) and 11.31% were downregulated (the full gene list is attached in supplement Table 9).

Uremic Toxin	upregulated ESRD SGs	Gene	P.Value	logFC
IL-18	5 (4.13%)	CFD	0.007418	1.755116
		IL6ST	0.007849	3.737921
		IRAK3	0.005272	1.357006
		PDGFC	0.01745	1.366625
		SIL1	0.024563	1.612347
	downregulated ESRD SGs 105 (11.31%)	ADAM12	0.047778	−3.15133
		ALPPL2	0.036972	−1.34452
		AMBN	0.048698	−1.73612
		AMELX	0.010632	−2.25732
		ANTXR1	0.032292	−2.91483
		ARHGAP6	0.017996	−3.93008

SPARC, transforming growth factor β1 (TGFβ1), insulin-like growth factor 1 (IGF1), and insulin-like growth factor-binding protein 5 (IGFBP5) were downregulated in ESRD-downregulated SGs. Of note, TGF-β1 is a key reactive oxygen species (ROS) promoting cytokine in renal fibrosis [108]. TGF-β1 promotes the cell cycle G2/M arrest based senescence-associated secretory phenotype (SASP) rather than DNA-damage based G1/S arrest [109]. TGFβ1-Smads form an anti-proliferation pathway [110]. Anti-aging gene Klotho deficiency exacerbates early diabetic nephropathy via enhancing TGFβ1 signaling in kidneys, which is a strong inducer of cellular senescence in a mouse model of chronic kidney injury [111]. Insulin-like grow factor 1 (IGF1) were increased in secretomic genes upregulated in ESRD. Circulating IGF-1 forms a complex with two other proteins – the IGF binding protein (IGFBP) and the acid labile subunit (ALS). High level concentrations of circulating IGF-1 are related to a higher risk of prostate, colorectal and breast cancers [112]. The results suggest that secretomic genes modulated in PBMCs from patients with ESRD modulate senescence via the following mechanisms: *first*, increasing expressions of proinflammatory growth factor IGFBP7; *second*, promoting inflammation and inhibiting fibrosis by decreasing TGF-β1 [113]; *third*, on the other hand, as potential negative feedback mechanisms, inhibiting inflammation and kidney injury by upregulating anti-inflammatory CREG1 and downregulating proinflammatory SPARC, respectively; *fourth*, inhibiting higher risk of prostate, colorectal and breast cancers by decreasing IGF1 and IGFBP5; and *fifth*, promoting senescence by decreasing leukocyte telomere length (LTL) [114] via inhibiting SERPINE1/PAI-1 expression [115]. Therefore, our data suggest that modulating secretomic gene expressions in PBMCs may have beneficial therapeutic effects in the treatment of ESRD-related cancer and aging-related diseases [116] (see Fig. 6).

3.9. Reactive oxygen species (ROS) pathways play much bigger roles in ESRD-upregulated SGs (11.6%) than that in CKD-upregulated SGs (6.8%), and half of ESRD-upregulated SGs are ROS-independent

It has been well documented that reactive oxygen species (ROS) plays a key role in regulating pathophysiological signaling in endothelial cell activation [117], cardiovascular diseases [118] and chronic kidney disease/end-stage renal disease [119]. We also reported that mitochondrial ROS plays a significant role in mediating EC activation [4,120,121]. Overproduction of ROS by impaired mitochondria can lead to positive feedback to enhance the cellular damage and generate uremic toxins, especially those produced by oxidation or peroxidation, such as creatine, urea and Melatonin [122,123]. This process aggravated by the accumulation of uremic toxins was hallmarked by mitochondria dysfunction defined as increased proton leaks as we reported [4,120,121,124,125], impaired mitochondria dynamics, alteration of mitochondria morphology and remodeling, which lead to dysfunction of podocytes and endothelial cells in the kidney. Mitochondria is both a source and a target for uremic toxins. In addition, it has been reported that uremia is associated with a reduction in the numbers and functions of lymphoid cells, whereas numbers of myeloid cells in uremic patients are either normal or increased with increased production of inflammatory cytokines and ROS [119]. Moreover, to find the evidence that ROS pathway genes are modulated by CKD and ESRD, the 84 oxidative and anti-oxidative regulatory genes [126] were examined. As shown in Table 8A, ESRD upregulated an antioxidant enzyme peroxiredoxin 4 (PRDX4) and a potential neuron development regulator prion protein (PRNP), and downregulated eight oxidative/anti-oxidative genes including antioxidant glutathione peroxidase 3 (GPX3), anti-oxidant glutathione peroxidase 5 (GPX5), antimicrobial lactoperoxidase (LPO), microbicidal myeloperoxidase (MPO), anti-oxidant superoxide dismutase 3 (SOD3), anti-inflammatory cytokine IL19, proinflammatory cytokine IL-22, anti-inflammatory apolipoprotein E (APOE) in PBMCs, respectively. These results suggest that ESRD downregulates more antioxidant enzymes/proteins than upregulate them, whereby promoting ROX generation. In addition, CKD downregulated one anti-oxidative gene SOD3. These results suggest that ESRD and CKD modulate ROS regulatome (oxidative and anti-oxidative regulatory genes).

However, an important question remains whether ROS signaling and antioxidant signaling mediate CKD and ESRD-modulation of SGs. Thus, we examined a novel hypothesis that ROS signaling and anti-oxidant signaling mediate CKD-, and ESRD-, modulation of SGs. By

Table 4c
The IPA classified the parts of the SGs modulated in the PBMCs from patients with CKD and patients with ESRD as the cytokine group. The results showed that 4 out of 55 SGs (7.2%) downregulated in CKD (also shown in Table 2B) were cytokines; one out of 44 SGs (2.27%) upregulated in CKD was cytokine; 68 out of 928 SGs (7.3%) downregulated in ESRD were cytokines; and 6 out of 121 SGs (4.96%) upregulated in ESRD were cytokines.

	Symbol	Entrez Gene Name	Expr p-value	Expr Log Ratio	Location
down in CKD [4]	IL24	interleukin 24	0.0327	−1.174	Extracellular Space
	IL36RN	interleukin 36 receptor antagonist	0.0319	−1.2	Extracellular Space
	PF4	platelet factor 4	0.0149	−1.11	Extracellular Space
	EDA	ectodysplasin A	0.00949	−1.069	Plasma Membrane
up in CKD (1)	DKK3	dickkopf WNT signaling pathway inhibitor 3	0.0094	1.827	Extracellular Space
down in ESRD [68]	BMP8A	bone morphogenetic protein 8a	0.000409	−2.355	Extracellular Space
	CCL1	C–C motif chemokine ligand 1	0.0184	−1.28	Extracellular Space
	CCL8	C–C motif chemokine ligand 8	0.0221	−1.682	Extracellular Space
	CCL13	C–C motif chemokine ligand 13	5.57E-05	−1.797	Extracellular Space
	CCL17	C–C motif chemokine ligand 17	0.0196	−1.465	Extracellular Space
	CCL18	C–C motif chemokine ligand 18	0.00866	−1.367	Extracellular Space
	CCL19	C–C motif chemokine ligand 19	0.0044	−2.113	Extracellular Space
	CCL21	C–C motif chemokine ligand 21	0.00242	−2.011	Extracellular Space
	CCL22	C–C motif chemokine ligand 22	3.47E-05	−1.656	Extracellular Space
	CCL23	C–C motif chemokine ligand 23	0.00434	−2.54	Extracellular Space
	CCL24	C–C motif chemokine ligand 24	0.00354	−1.942	Extracellular Space
	CCL25	C–C motif chemokine ligand 25	0.0037	−1.042	Extracellular Space
	CRH	corticotropin releasing hormone	0.00196	−1.518	Extracellular Space
	CSF1	colony stimulating factor 1	7.44E-05	−1.84	Extracellular Space
	CSF2	colony stimulating factor 2	0.00115	−1.158	Extracellular Space
	CX3CL1	C-X3-C motif chemokine ligand 1	0.00352	−1.231	Extracellular Space
	CXCL5	C-X-C motif chemokine ligand 5	0.0264	−1.939	Extracellular Space
	CXCL9	C-X-C motif chemokine ligand 9	0.014	−1.063	Extracellular Space
	CXCL11	C-X-C motif chemokine ligand 11	0.0439	−1.101	Extracellular Space
	CXCL12	C-X-C motif chemokine ligand 12	0.000564	−1.422	Extracellular Space
	CXCL14	C-X-C motif chemokine ligand 14	0.00645	−1.535	Extracellular Space
	DKK3	dickkopf WNT signaling pathway inhibitor 3	0.00168	−1.867	Extracellular Space
	EDN1	endothelin 1	0.0271	−1.424	Extracellular Space
	EPO	erythropoietin	0.00154	−2.113	Extracellular Space
	FASLG	Fas ligand	0.000484	−1.074	Extracellular Space
	IFNA5	interferon alpha 5	0.0351	−1.309	Extracellular Space
	IFNA7	interferon alpha 7	0.0259	−1.167	Extracellular Space
	IFNA16	interferon alpha 16	0.0243	−1.327	Extracellular Space
	IFNB1	interferon beta 1	0.0248	−1.679	Extracellular Space
	IFNW1	interferon omega 1	0.000648	−1.794	Extracellular Space
	IL2	interleukin 2	0.00237	−1.245	Extracellular Space
	IL3	interleukin 3	0.00158	−1.82	Extracellular Space
	IL4	interleukin 4	0.000549	−2.658	Extracellular Space
	IL5	interleukin 5	0.00255	−1.791	Extracellular Space
	IL9	interleukin 9	0.00207	−1.852	Extracellular Space
	IL11	interleukin 11	0.00046	−1.87	Extracellular Space
	IL16	interleukin 16	0.000374	−1.476	Extracellular Space
	IL19	interleukin 19	0.00647	−1.564	Extracellular Space
	IL21	interleukin 21	0.000431	−2.258	Extracellular Space
	IL22	interleukin 22	0.0124	−1.315	Extracellular Space
	IL24	interleukin 24	0.000213	−2.527	Extracellular Space
	IL25	interleukin 25	0.00486	−1.597	Extracellular Space
	IL26	interleukin 26	0.00285	−1.789	Extracellular Space
	IL37	interleukin 37	0.031	−1.296	Extracellular Space
	IL17A	interleukin 17A	0.0004	−1.944	Extracellular Space
	IL1A	interleukin 1 alpha	1.52E-07	−2.584	Extracellular Space
	IL1RN	interleukin 1 receptor antagonist	4.04E-05	−1.628	Extracellular Space
	IL36A	interleukin 36 alpha	0.00185	−1.492	Extracellular Space
	IL36G	interleukin 36 gamma	0.0143	−1.615	Extracellular Space
	IL36RN	interleukin 36 receptor antagonist	0.0281	−1.175	Extracellular Space
	LIF	LIF interleukin 6 family cytokine	0.0323	−1.402	Extracellular Space
	LTA	lymphotoxin alpha	2.73E-05	−2.497	Extracellular Space
	OSM	oncostatin M	0.00294	−2.002	Extracellular Space
	PF4	platelet factor 4	0.0432	−1.106	Extracellular Space
	PRL	prolactin	0.00443	−1.693	Extracellular Space
	PRLH	prolactin releasing hormone	0.00346	−1.191	Extracellular Space
	SCG2	secretogranin II	0.00633	−2.162	Extracellular Space
	SCGB1A1	secretoglobin family 1A member 1	0.0166	−1.372	Extracellular Space
	SLURP1	secreted LY6/PLAUR domain containing 1	0.000616	−1.435	Extracellular Space
	SPP1	secreted phosphoprotein 1	0.000139	−2.907	Extracellular Space
	THPO	thrombopoietin	0.00506	−1.895	Extracellular Space
	TNFSF11	TNF superfamily member 11	0.000298	−1.936	Extracellular Space
	TNFSF14	TNF superfamily member 14	1.85E-05	−3.778	Extracellular Space
	WNT1	Wnt family member 1	0.00424	−1.889	Extracellular Space
	WNT2	Wnt family member 2	0.0082	−1.681	Extracellular Space

(*continued on next page*)

Table 4c (*continued*)

	Symbol	Entrez Gene Name	Expr p-value	Expr Log Ratio	Location
	WNT4	Wnt family member 4	0.00799	−1.371	Extracellular Space
	WNT5A	Wnt family member 5A	0.00932	−2.049	Extracellular Space
	EDA	ectodysplasin A	3.23E-06	−1.747	Plasma Membrane
up in ESRD [6]	AIMP1	aminoacyl tRNA synthetase complex interacting multifunctional protein 1	8.15E-09	1.927	Extracellular Space
	CXCL2	C-X-C motif chemokine ligand 2	0.000396	3.051	Extracellular Space
	CXCL8	C-X-C motif chemokine ligand 8	1.78E-06	4.136	Extracellular Space
	IL7	interleukin 7	0.0188	1.258	Extracellular Space
	IL15	interleukin 15	5.13E-06	1.645	Extracellular Space
	XCL1	X–C motif chemokine ligand 1	0.00893	1.131	Extracellular Space

Table 4d
Proinflammatory and profibrotic molecules, pathways and transcription factors played important roles in pathophysiological process of ESRD.

Method	Table/Figure	Result
IPA	Fig. 2B	upregulated SGs in ESRD can active proinflammatory pathways such as IL8 signaling, neuroinflammation signaling pathway.
IPA	Fig. 2D	downregulated SGs in ESRD can active profibrotic pathways such as inhibition of matrix metalloporteases
TRANSFAC	Table 3C	upregulated SGs in ESRD may be modulated by transcription factors involved in inflammation and fibrosis (Egr-1, POU1F1)
ClueGo	Fig. 3B	UT-encoded genes and up-regulated SGs in ESRD are shared in some pro-inflammatory pathways。
GEO2R	Tables 4A and 4B	Proinflammatory uremic cytokines IL1b and IL18 in UTs can amplify the upregulation of SGs in ESRD
IPA	Table4C	Cytokines can be modulated during CKD progression to induce imbalance of anti-inflammatory and proinflammatory function.

mining the microarray datasets in the NIH-NCBI-GeoDataset database, we found several microarray datasets with the inhibition of nicotinamide adenine dinucleotide phosphate oxidase 2 (NOX2) [118] and the deficiency of antioxidant transcription factor nuclear factor erythroid 2–related factor 2 (Nrf2) [118]. Nrf2 was chosen since Nrf2 was considered as a novel therapeutic target for mitochondria dysfunction in chronic kidney disease by animal experimental researches [121,127–132]. In Fig. 7A, we classified all the SGs into four groups: *first*, the SGs increased in NOX2 inhibited cells and decreased in Nrf2 deficient cells are ROS-suppressed genes; *second*, the SGs expressions decreased in NOX2 inhibited cells and increased in Nrf2 deficient cells are ROS-promoted genes; *third*, the SGs either promoted by ROS pathways in one group of microarray datasets or suppressed by ROS pathways in another groups of microarray datasets are ROS pathways-unsettled genes; and *fourth*, the SGs that were not significantly modulated in NOX2 inhibited and Nrf2 deficient are ROS-independent genes (Fig. 7A). Of note, only 1030 out of 2641 SGs were found in the NOX2-, Nrf2-deficient datasets and were focused in our analyses. As shown in Table 8B, 144 out of 1030 SGs (14.0%) were ROS-suppressed SGs; 191 out of 1030 SGs (18.5%) were ROS-promoted SGs; 3 out of 1030 SGs (0.29%) were ROS-unsettled SGs; and 692 out of 1030 SGs (67.2%) were ROS-independent. In addition, 17 out of CKD-upregulated 44 SGs (38.6%) were found to be modulated in ROS-related manners including 5 as ROS-suppressed SGs (29.41%), 3 as ROS-promoted SGs (17.65%), 0 as ROS-unsettled SGs, and 9 as ROS-independent SGs (52.94%); moreover, 26 out of CKD-downregulated 55 SGs (47.3%) were found to be modulated in ROS-related manners including 2 as ROS-suppressed SGs (7.69%), 8 as ROS-promoted SGs (30.77%), 0 as ROS-unsettled SGs, and 16 as ROS-independent SGs (61.54%); furthermore, 84 out of ESRD-upregulated 121 SGs (69.4%) were found to be modulated in ROS-related manners including 6 as ROS-suppressed SGs (7.14%), 14 as ROS-promoted SGs (16.67%), 0 as ROS-unsettled SGs, and 64 as ROS-independent SGs (76.19%); and finally, 341 out of ESRD-downregulated 928 SGs (36.7%) were found to be modulated in ROS related manners including 59 as ROS-suppressed SGs (17.30%), 74 as ROS-promoted SGs (21.70%), 2 as ROS-unsettled SGs (0.59%), and 206 as ROS-independent SGs (60.41%). These results suggest that *first*, ROS play much bigger roles in ESRD-upregulated SGs (69.4%) than that in CKD-upregulated SGs (38.6%); *second*, ROS-independent ESRD-upregulated SGs (76.19%) are much more than ROS-independent CKD-upregulated SGs (52.94%), indicating that ESRD uses more ROS-independent mechanisms than CKD in upregulating SGs in the PBMCs; and *third*, ROS-suppressed CKD-upregulated SGs (29.41%) are much higher than that of ESRD-upregulated SGs (7.14%) (see Fig. 7b).

4. Discussion

Since UTs have been first identified via low throughput mass spectrometry, 130 UTs are documented in the European Uremic Solutes (EUTox) Database (http://www.uremic-toxins.org/DataBase.html). It remains unknown whether secreted proteins generated in innate immune cells in response to UTs stimulation can contribute to the pathogenesis of CKD and ESRD. We recently reported that uremic toxins (UTs) are selectively increased and serve as danger signal-associated molecular patterns (DAMPs) and homeostasis-associated molecular patterns (HAMPs) that modulate inflammation. These results also show that some UT genes are upregulated in CKD and CAD via caspase-1/inflammatory cytokine pathways, rather than by purely passive accumulation. Our findings raised the possibility that UTs-stimulated innate immune cells and other types of cells increase their secretome, which contributes to the CKD/ESRD progression. As reported, the secretory proteins are important for maintaining cell-cell communication and proliferation. Examples of secretory proteins include hormones, digestive enzymes, cytokines, chemokines, interferons (IFNs), colony-stimulating factors (CSFs), growth factors, and tumor necrosis factors (TNFs) [133]. However, secretomic studies in CKD and other metabolic diseases have been at a low pace due to the low throughput technologies [134]. Our previous reports demonstrated that innate immune cells, PBMCs containing such as $Ly6C^{high}$ (mice) [7,17,18,135–137] and $CD40^{+}$ (human) [7] monocytes, contribute significantly to the pathogenesis of metabolic vascular diseases including CKD [7]. However, three important questions remained, *first*, whether UTs are the only soluble molecular drivers for the progression of CKD to ESRD; *second*, whether secretomic changes in innate immune cells, PBMCs, upregulated in CKD and ESRD contribute to the pathogenesis and progression

Table 5a
Most of 28 co-stimulation receptors and immune checkpoint (co-inhibition) receptors (expressed in the antigen presenting cell (APC) surface) are modulated in the PBMCs from patients with CKD and ESRD. The results showed that: 1) seven out of 14 co-stimulation receptors, one out of four dual receptors (functional as co-stimulation for naïve T cells and co-inhibition receptors for activated T cells), and four out of 10 immune checkpoint receptors were downregulated in ESRD, respectively; 2) two co-stimulation receptors CD48 and CD58 were upregulated in ESRD; 3) one immune checkpoint receptor HHLA2 was upregulated in CKD; and 4) one co-stimulation receptor TNFSF8 was downregulated in CKD.

Effect		up in CKD	down in CKD	up in ESRD	down in ESRD
	Gene symbol	logFC	logFC	logFC	logFC
co-stimulation	ICOSLG				−1.9552
	CD70				−1.5467
	TNFSF14				−3.77804
	CD40				−2.93849
	TNFSF9				
	TNFSF4				
	TNFSF15				−1.11762
	TNFSF18				−1.25532
	TNFSF8		−1.36894		
	TIMD4				
	SLAMF1				−1.51147
	CD48			1.089174	
	SEMA4A				
	CD58			1.68807	
co-stimulation at naïve TC and co-inhibitory at activated TC	CD80				
	CD96				
	PVR				−1.21184
	IL2RB				
co-inhibition	LGALS9				
	NECTIN3				−1.6273
	TNFRSF14				
	PDCD1LG2				−1.4391
	CD274				
	CD276				
	VTCN1				
	VSIR				
	HHLA2	1.191813			−1.41072
	BTNL2				−2.12092

Table 5b
Since there are no datasets of CD48 and CD58 deficient/overexpressed microarray datasets available in the NIH-NCBI-GEO database, we use the GEO datasets (GSE15215) related to CD2, a membrane protein acting as CD48/CD58 ligand on dendritic cells (DCs), $CD2^+$ DCs versus $CD2^-$ DCs to determine whether the forward signaling of CD48 and CD58 can modulate the expression of SGs modulated in ESRD. These results showed that CD2 upregulates 14 out of 121 SGs (11.6%) upregulated in ESRD; and CD2 downregulates 25 out of 928 SGs (2.7%) downregulated in ESRD.

upregulated in ESRD (14/121, 11.6%)			downregulated in ESRD (25/928, 2.7%)		
Gene	p value	log FC	Gene	p value	log FC
ANXA1	0.018331	2.18431	ADM2	0.004031	−1.64776
ANXA2	0.006552	1.797703	BMP1	0.027839	−1.26577
ASGR1	0.022441	2.235951	C2CD2	0.028997	−1.03495
CTSH	0.004221	1.910232	COL4A6	0.007275	−1.47307
EREG	0.020802	1.079933	COL8A1	0.035575	−1.64511
HS2ST1	0.001885	2.614528	CRISP1	0.003	−2.27406
IGFBP7	0.001049	2.731112	CRLF2	0.003679	−2.21456
IRAK3	0.006719	2.871895	CXCL5	0.00911	−2.13001
MTHFD2	0.01361	1.340201	CYP2A13	0.010832	−1.52688
NLRP3	0.011059	2.662663	EXOG	0.0476	−1.9656
PIGK	0.014052	1.876857	GPC3	0.010239	−1.53284
RNASE4	0.007783	2.112123	IGFBP2	0.044615	−1.30936
TPP1	0.033834	1.607275	IL21	0.03692	−2.36894
XCL1	0.003066	1.997939	KLKB1	0.023261	−1.42336
			LIPF	0.020219	−1.48391
			MSMB	0.003153	−2.3955
			NAGLU	0.018426	−1.20719
			PHLPP1	0.000438	−2.88351
			PLA2G2A	0.001974	−2.3566
			PSG1	0.003256	−2.95988
			RAB26	0.017058	−1.88871
			SOSTDC1	0.014098	−1.25801
			SPINK1	0.002876	−2.26369
			TTR	0.026675	−1.29499
			WNT2	0.008818	−1.73443

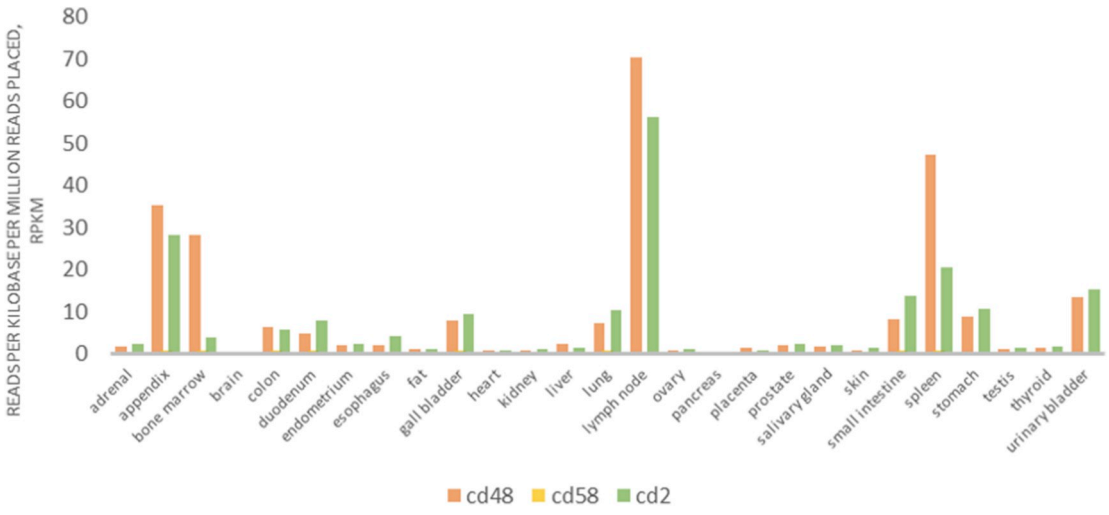

Fig. 4a. New RNA-seq (RNA-sequencing) data from Human Protein Atlas (https://www.proteinatlas.org) indicated that CD48 and CD58 are expressed in every one of 27 tissue examined; and that their ligand (CD2) is also expressed in every one of 27 tissues examined, which are correlated with the CD2 protein expression data collected in the GeneCards database shown above.

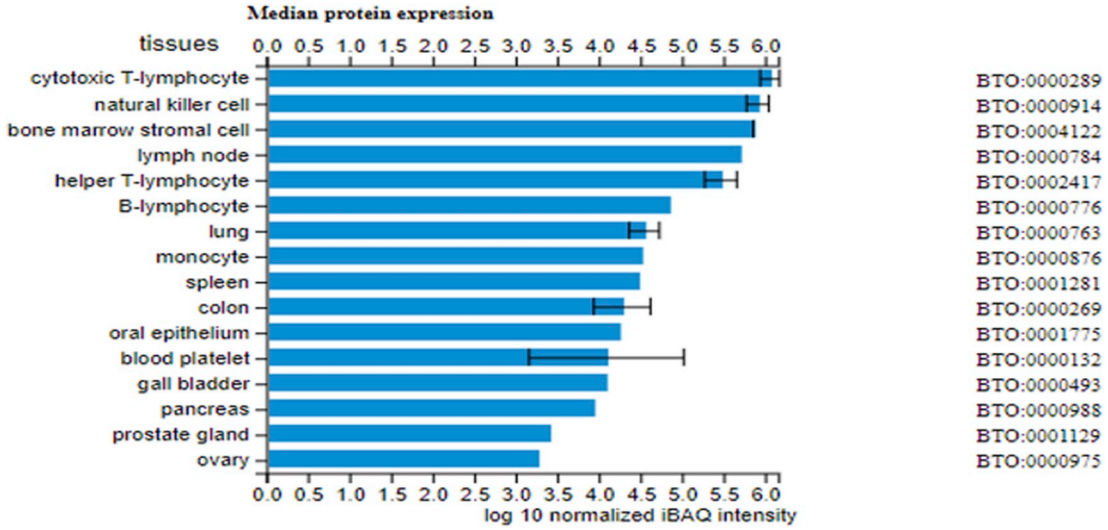

Fig. 4b. CD2 protein can be highly enriched in cytotoxic T-lymphocyte, natural killer cell, bone marrow stromal cell, helper T-lymphocyte, B lymphocyte and monocyte according to Proteomics Database (https://www.proteomicsdb.org/).

of CKD and ESRD; and *third*, whether CKD and ESRD differentially modulate the secretomic changes via disease stage-specific pathways. To fill in these important knowledge gaps, in this study, we used cutting-edge molecular database mining approaches that we pioneered in 2004 [25,113,138,139] and analyzed PBMC secretomic (all the signal peptide sequence-containing secreted protein genes) changes in induced by CKD and ESRD. Our data analyses have made for the first time the following significant findings: *1)* 86.7% middle class (molecular weight >500 Daltons) uremic toxins (UTs) were encoded by secretomic genes (SGs); *2)* Upregulations of SGs in PBMCs in patients with ESRD (121 SGs) were significantly higher than that of CKD (44 SGs); and among ESRD specifically modulated 975 SGs, ESRD upregulated 116 SGs (11.9%) but downregulates 859 SGs (88.1%), respectively; *3)* ESRD-upregulated SGs had 2 folds higher percentages of the cytoplasm and nucleus subcellular groups than the controls; and had the higher percentages of five out of 13 SG functional groups including enzyme, kinase, peptide, transcription regulator, and transmembrane in comparison to the controls. Transcriptomic analyses of PBMC secretome have advantages to identify more comprehensive secretome than conventional secretomic analyses; *4)* Although CKD-, and ESRD-upregulated SGs were highly diversified in signaling, ESRD-induced SGs had strong proinflammatory pathways; *5)* Proinflammatory cytokines-based middle class UTs such as interleukin-1β (IL-1β) and IL-18 promote ESRD modulation of SGs; *6)* ESRD-upregulated co-stimulation receptors CD48 and CD58 increase secretomic upregulation in the PBMCs; may not be even limited in the PBMCs, CD48/CD58-CD2 signaling can be actually magnified enormously in tissues; *7)* Classically activated macrophages (M1)-, and alternatively activated macrophages (M2)- macrophage polarization signals contribute to ESRD- and CKD-upregulated SGs; *8)* ESRD- and CKD-upregulated SGs in PBMCs contain senescence-promoting regulators by upregulating proinflammatory growth factor IGFBP7 and downregulating anti-inflammatory cytokine TGF-β1 and leukocyte telomere length stabilizer SERPINE1/PAI-1; and *9)* Reactive oxygen species (ROS) pathways play much bigger roles in ESRD-upregulated SGs (11.6%) than that in CKD-upregulated SGs (6.8%); and half of ESRD-upregulated SGs are ROS-independent.

Clinical and epidemiological studies have identified more than 10 risk factors in accelerating CKD progression and potential transition to ESRD as shown in Table 9A. However, the molecular pathways driving the pathogenesis of ESRD remained poorly characterized. Based on our

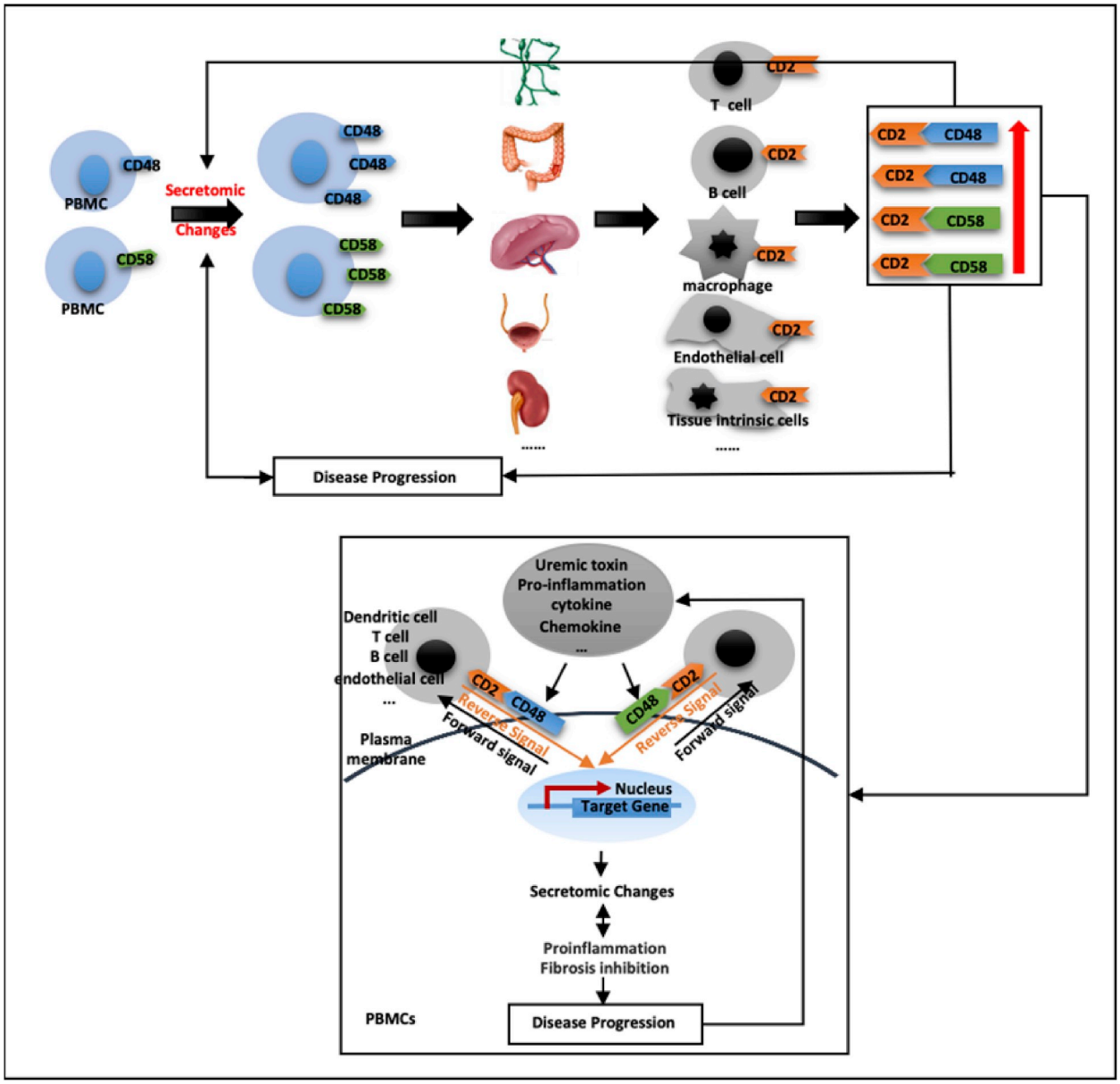

Fig. 4c. Novel mechanism. Co-stimulation receptors CD48 and CD58 can initiate signaling cascades via their interactions with their ligand CD2 to amplify the expression changes of SGs upregulated in the PBMCs in patients with ESRD.

findings, we propose a new working model (Fig. 8), under the stimulation of uremic toxins, more than 121 novel secreted proteins are significantly upregulated in innate immune cells, PBMCs, in patients with ESRD, which makes PBMCs the major cell types in upregulating secretomes. This is the first time for us to understand that in addition to uremic toxins identified, significant secretomic changes may play highly significant roles in driving ESRD pathogenesis. In addition, since some PBMC secretome proteins identified with transcriptomic approach are localized in the subcellular locations other than the supernatants of cultured cells and plasma that conventional secretomic analyses sampled and examined, therefore, our data have also demonstrated that transcriptomic analyses of PBMC secretome have advantages to identify more comprehensive secretome than conventional secretomic analyses [69]. To determine the mechanisms underlying the SGs, we identified several novel molecular mechanisms: *first*, UTs play significant roles in upregulating PBMC SGs in patients with CKD and ESRD; and ESRD-induced SGs have strong proinflammatory pathways. In addition, our IPA results indicate that SGs in PBMCs upregulated in ESRD have a novel proinflammatory signaling pathway overlapped with that of UTs, role of cytokines in mediating communication between immune cells (Fig. 3A). We also used the Cytoscape and found that UTs pathways and ESRD-upregulated pathways share 20 regulatory or regulators. These results have demonstrated for the first time that ESRD-upregulated PBMC SGs have synergistic effects with that of UTs, which contribute to the disease progression significantly; *second*, we found strong functional evidence that proinflammatory cytokines-based middle class UTs such as interleukin-1β (IL-1β) and IL-18 promote ESRD modulation of SGs, which also serve as a new working model for UTs and PBMC secretome interactions in patients with ESRD and CKD; *third*, as a novel membrane protein mechanism, ESRD-upregulated co-stimulation receptors CD48 and CD58 increase secretomic upregulation in the PBMCs; may not be even limited in the PBMCs, CD48/CD58-CD2 signaling can be actually magnified enormously in tissues; *fourth*, as another novel membrane Toll-like receptor-mediated signaling and interferon-g receptor signaling mechanisms, we found that classically activated macrophages (M1)-, and alternatively activated macrophages (M2)- macrophage

Table 6
A Eight out of 44 SGs (18.18%) upregulated in CKD were found in M1 macrophage polarization dataset; and six out of 44 SGs (13.64%) upregulated in CKD were found in M2 macrophage polarization. 15 out of 121 SGs (12.4%) upregulated in ESRD were found in M1 macrophage polarization dataset; 16 out of 121 SGs (13.2%) were found in M2 macrophage polarization. These results suggest that macrophage polarization pathways participate CKD-, and ESRD-upregulated secretomic changes in the PBMCs in patients with CKD and ESRD (PMID: 30827512).

	GEO ID	GSE85346	M2 (6)					
	M1 (8)							
	M1/M0		M2a/M0		M2b/M0		M2c/M0	
	Gene Symbol	Log FC	Gene Symbol	Log FC	Gene Symbol	FC	Gene Symbol	Log FC
CKD	ADAM23	3.347203	APCS	1.077464	C3	2.819101	S100A12	2.702538
	APCS	2.623466	CELA2B	1.065595	CELA2B	1.216281		
	CGREF1	3.072414	PLA2G5	1.280399	S100A12	4.50631		
	LAMB1	3.023362			TNFAIP6	5.233126		
	NRP2	1.740985						
	PI3	3.458997						
	S100A12	3.80053						
	TNFAIP6	8.844792						
	M1 (15)		M2 (16)					
ESRD	CD44	1.014886	CTSC	3.295263	ASGR1	1.066338	CTSC	1.434133
	CD48	1.739752			C3	2.819101	DSE	1.554709
	CXCL2	2.43399			CTSC	1.612899	HEG1	1.066944
	DSE	1.893179			CXCL2	2.70138	IL7	2.068031
	EREG	3.423501			IRAK3	1.930381	MGAT4A	1.202096
	IGFBP7	1.388798			PTX3	1.792836	S100A12	2.702538
	IL15	2.544436			RETN	4.306012	S100A8	3.187036
	IRAK3	1.341773			RNASE2	1.692809	VCAN	2.876119
	MGAT4A	1.055701			S100A12	4.50631	XCL1	1.001179
	MTHFD2	1.605983			S100A8	5.25634		
	PTX3	3.304702			VCAN	1.791611		
	S100A12	3.80053						
	S100A8	3.323343						
	TXN	1.426231						
	VEGFA	2.795328						

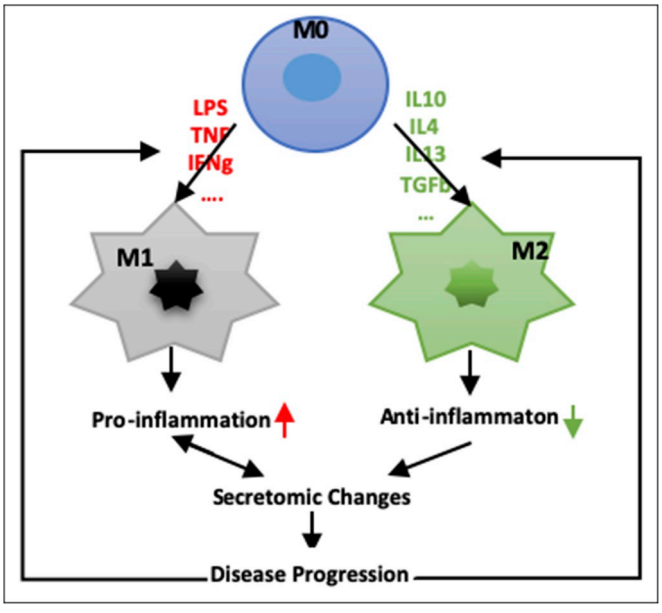

Fig. 5. Novel mechanism. Macrophage polarization pathways participate CKD-, and ESRD-upregulated secretomic changes in the PBMCs in patients with CKD and ESRD; M1-, and M2-polarization signaling pathways involving in upregulating SGs are diversified.

polarization signals contribute to ESRD- and CKD-upregulated SGs; *fifth*, ESRD- and CKD-upregulated SGs in PBMCs contain senescence-promoting regulators by upregulating proinflammatory growth factor IGFBP7 and downregulating anti-inflammatory cytokine TGF-β1 and leukocyte telomere length stabilizer SERPINE1/PAI-1. Therefore, our data have demonstrated for the first time that controlling senescence-associated inflammation cell by targeting specific inflammatory mediators may have a beneficial therapeutic effect in treatment of ESRD-related cancers, aging and inflammatory diseases [116]; and *sixth*, Reactive oxygen species (ROS) pathways play much bigger roles in ESRD-upregulated SGs (11.6%) than that in CKD-upregulated SGs (6.8%); and half of ESRD-upregulated SGs are ROS-independent.

Of note, the interactions of the pathways in our findings have been reported by other researches. These interactions may collectively lead to disease progression: *First*, cytokines exerted as key initiators, mediators and effectors for other pathway entries. Upregulated co-stimulating secretomic member CD48 can be induced under proinflammatory environment; secretion of IL2 was decreased in T cells isolated from CD48-deficient mice [140]. *Second*, co-signaling molecule CD48 and CD58 are widely reported to be closely related to cytokine synthesis: $CD48^{-}CD2$ interaction can facilitate TCR signaling to promote production of pro-inflammatory cytokines such as IL2 [141]. *Third*, macrophages as a crucial component of innate immunity, can function as scavengers to clear the interstitial environment of extraneous cellular materials and also as antigen-presenting cells to stimulate adaptive immune response. Based on functionality, resident and infiltrating macrophages can produce a set of pro-inflammatory cytokines and

Table 7
Among all SGs we investigated, no senescence regulators out of 71 were upregulated in CKD; two out of 71 senescence regulator genes such as Inhibitor Of DNA Binding 1 (ID1) and secreted protein acidic and rich in cysteine (SPARC) were downregulated in CKD; two out of 71 senescence regulator genes such as Cellular Repressor Of E1A Stimulated Genes 1 (CREG1) and Insulin Like Growth Factor Binding Protein 7 (IGFBP7) were upregulated in ESRD while five were downregulated in ESRD such as Serpin Family E Member 1 (SERPINE1), SPARC, transforming growth factor b1 (TGFB1), insulin-like growth factor (IGF1) and Insulin Like Growth Factor Binding Protein 5 (IGFBP5).

Group	Gene Symbol	p value	log FC	Function
Upregulated in CKD	N/A			
Downregulated in CKD	ID1	0.042591	−1.10654	p53/pRb signaling & cell cycle
	SPARC	0.028006	−1.00435	p53/pRb signaling & cell cycle
Upregulated in ESRD	CREG1	7.63E-05	1.463896	p53/pRb signaling & cell cycle
	IGFBP7	5.81E-05	2.059804	Interferon Signaling; insulin growth factor related
Downregulated in ESRD	SERPINE1	0.0234	−1.17965	Other Senescence Response Gene
	SPARC	0.00428	−1.93975	p53/pRb signaling & cell cycle
	TGFB1	3.62E-06	−2.48915	p53/pRb signaling & cell cycle; Cell Adhesion Molecules
	IGF1	0.000375	−2.37204	insulin growth factor related
	IGFBP5	0.000673	−1.7563	insulin growth factor related

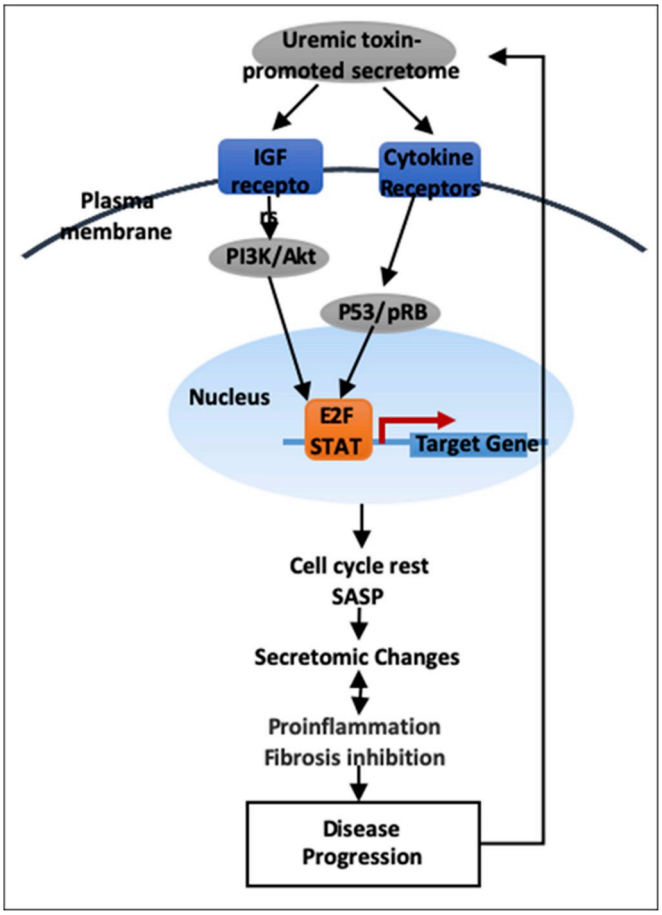

Fig. 6. Novel mechanism. Uremic toxins-promoted secretome accelerated renal disease and inflammation by inducing cellular senescence and senescence-associated secretory phenotype (SASP) according to p53 signaling and insulin growth factor (IGF) related pathways (PI3K/Akt) in ESRD.

Table 8a
Oxidative stress-related gene expressions (reactive oxygen species, ROS, regulatome) contributes to the progression of kidney dysfunction. We analyzed the gene expression of total 84 ROS regulatom84e (shown in supplement table 11) in CKD and ESRD. We found that 2 out of total 121 (1.65%) secretomic genes in ESRD while none of SGs in CKD are upregulated. Meanwhile, 8 out of 928 (0.8%) in ESRD and 1 out of 55 (1.82%) in CKD are downregulated.

Group	Gene Symbol	p value	log FC
Upregulated in CKD	N/A		
Downregulated in CKD	SOD3	0.031602	−1.04179
Upregulated in ESRD	PRDX4	3.25E-05	1.308793
	PRNP	8.80E-08	1.915437
Downregulated in ESRD	GPX3	4.56E-04	−1.13831
	GPX5	0.0101	−1.48694
	LPO	0.00185	−1.8362
	MPO	4.01E-03	−1.85159
	SOD3	0.0214	−1.23203
	IL19	0.00647	−1.56396
	IL22	0.0124	−1.3153
	APOE	4.61E-06	−1.78856

other metabolites under the stimuli such as uremic toxins in ESRD and their phenotype can be reversely reprogrammed by the different subsets of cytokines and polarized into different subsets [142–144]; *Fourth*, senescence has been induced by signaling through a bevy of critical cytokines such as TNF-a, IFN-g as an important extrinsic pathway of senescence and those cytokines initiated an inflammatory network acted both cause and consequences during senescence [145–147]. *Fifth*, redox oxygen species has been widely reported to be a marker and an inducer to deteriorate diseases which could establish the inflammatory network [148–152]. This process could be carried out directly or indirectly. Direct pathways were according to the enhancing secretion of pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-1 by activating TCR and mTOR signaling while indirect pathways was activated by imbalance of M1-and M2-macrophages, SASP, and T cell signaling [153–162]. In conclusion, these pathways cross talk directly or

Table 8b

Using the classification in Fig. 7A, four SG subsets were compared based on the regulation of these CKD and ESRD modulated SGs by reactive oxidative species (ROS) generated by NOX2 and suppressed by NRF2. The results showed that 1) ROS-suppressed SGs (29.41%) upregulated in CKD were much higher than that of total SGs control (7.14%); 2) ROS-promoted SGs (30.77%) downregulated in CKD were much higher than that in total SGs control (18.54%); 3) ROS-independent SGs (76.19%) upregulated in ESRD were much higher than that in total SGs control and that in CKD (67.18% and 52.94%), respectively. These results suggest that ROS play much bigger roles in ESRD-upregulated SGs than that in other groups of SGs; and ROS play much more significant roles in ESRD pathologies.

Group	classification	ROS-suppressed SGs	ROS-promoted SGs	ROS-uncertain SGs	ROS-independent SGs	all ROS-related genes
ROS-related SGs	Number	144	191	3	692	1030
	Percentage	13.98%	18.54%	0.29%	67.18%	100.00%
up in CKD	Number	5	3	0	9	17
	Percentage	29.41%	17.65%	0.00%	52.94%	100.00%
down in CKD	Number	2	8	0	16	26
	Percentage	7.69%	30.77%	0.00%	61.54%	100.00%
up in ESRD	Number	6	14	0	64	84
	Percentage	7.14%	16.67%	0.00%	76.19%	100.00%
down in ERSD	Number	59	74	2	206	341
	Percentage	17.30%	21.70%	0.59%	60.41%	100.00%

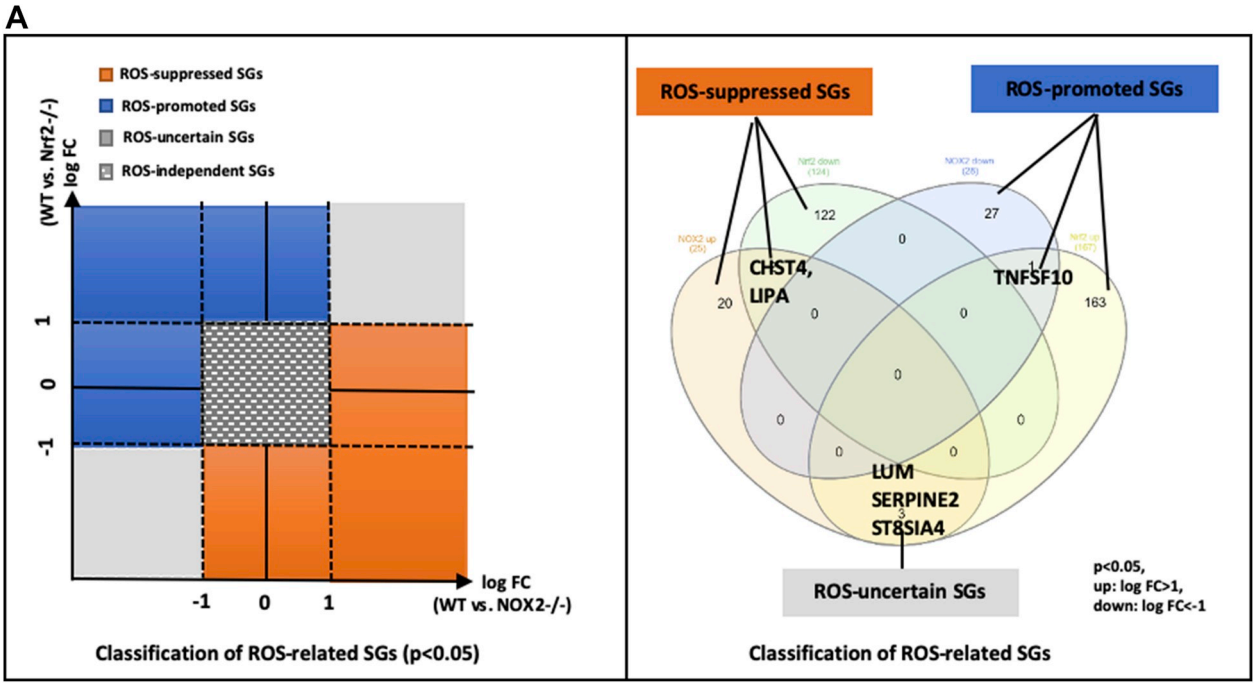

Fig. 7a. All of the 2641 SGs were testified in NOX2 and Nrf2 knockout GEO datasets (GSE7810, GSE100671) and total of 1030 SGs (39.0% of all SGs) were found in these two datasets (cutoff: P value < 0.05). We classified these 1030 SGs into 4 categories: 1) ROS-suppressed SGs, 2) ROS-promoted SGs, 3) ROS-uncertain SGs, and 4) ROS-independent SGs by analyzing expression changes in NOX2 and Nrf2 knockout GEO.

indirectly, and make ROS pathway as potential therapeutic targets to suppress disease progression.

One limitation of the current study is that due to the low throughput nature of verification techniques so that we could not verify every result we identified with the analyses of high throughput data (Table 10). We acknowledge that carefully designed *in-vitro* and *in-vivo* experimental models will be needed to verify the CKD-, and ESRD-upregulated PBMC secretomes further and underlying mechanisms we report here. Nevertheless, our findings provide novel insights on the roles of PBMC secretomes in the pathogenesis of ESRD and CKD, novel pathways underlying the multi-hit models as well as new targets for the future therapeutic interventions for CKD, ESRD and their related diseases, aging and cancers.

Authors' contributions

RJZ carried out the data gathering, data analysis and prepared tables and figures. JS, YS, TY, LL, FS, WYY, YS, CJ, CDIV, HF, YL, KX, ML,

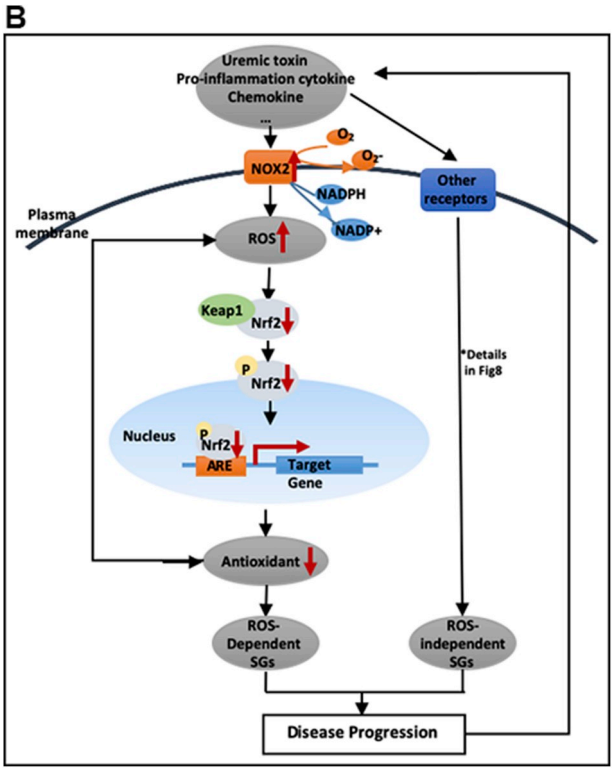

Fig. 7b. Novel Mechanism: Reactive oxygen species (ROS)-related mechanisms, regulated by ROS generating enzyme NADPH oxidase 2 (NOX2) and antioxidant transcription factor Nrf2 pathways, modulated the secretomic changes during kidney dysfunction. The modulation was closely related to the balance of ROS and antioxidants and the imbalance contributes to the alteration of ROS-dependent SGs which could promote disease progression.

JW, EC, DY, XJ, YL, RL, LW, ETC, HW aided with analysis of the data. XFY supervised the experimental design, data analysis, and manuscript writing. All authors read and approved the final manuscript.

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Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Table 9a
Multiple risk factors accelerate CKD progression to ESRD, which have been reported by clinical studies and scientific literature. Our report provides novel data analyses evidences and mechanisms to support the multi-hit model for the development of CKD.

clinical risk factor for CKD progression	scientific mechanism	PMID
ethnicity	APOL1 mutant in African	27312436
	Epigenetically heritable changes	25993323
gender	direct effects of sex steroids on kidney	29355169
	sex differences in NO metabolism and oxidative stress	29355169
	gender-differential impact of comorbidities and lifestyle risk factors	29355169
diabetes	Renal hemodynamic changes	29486908
	ischemia and inflammation	29486908
	Overactive renin-angiotensin-aldosterone system	29486908
hypertension	Global RAS activation	29144825
	renal inflammation	29144825
obesity	RAAS activation	31015582
	renal compression	31015582
	Metabolic abnormalities	31015582
gut microbiota	production of uremic toxins	30464044
	promoting translocation of bacterial component	31243394
	secreting metabolites favoring insulin resistance favoring insulin resistance and endothelial dysfunction	31243394
nephrotoxicity	inducing apoptosis, autophagy and necrosis	29341864
	Oxidant-Induced Renal Injury	15519281
	mitochondria dysfunction in the proximal tubules	29939355
Hepatitis B and C infections	glomerular immune complex deposition	28149647
	direct viral invasion of the renal parenchyma	31155101
dyslipidemia	Direct lipid-induced cellular injury	22290079
	inflammatory cytokines	29176657
proteinuria	Increased intraglomerular hydraulic pressure	22137726
	damage to glomerular filtration barrier	22137726
mineral bone disorder	VSMC dedifferentiation	28119179
	neointimal atherosclerotic calcification	28119179

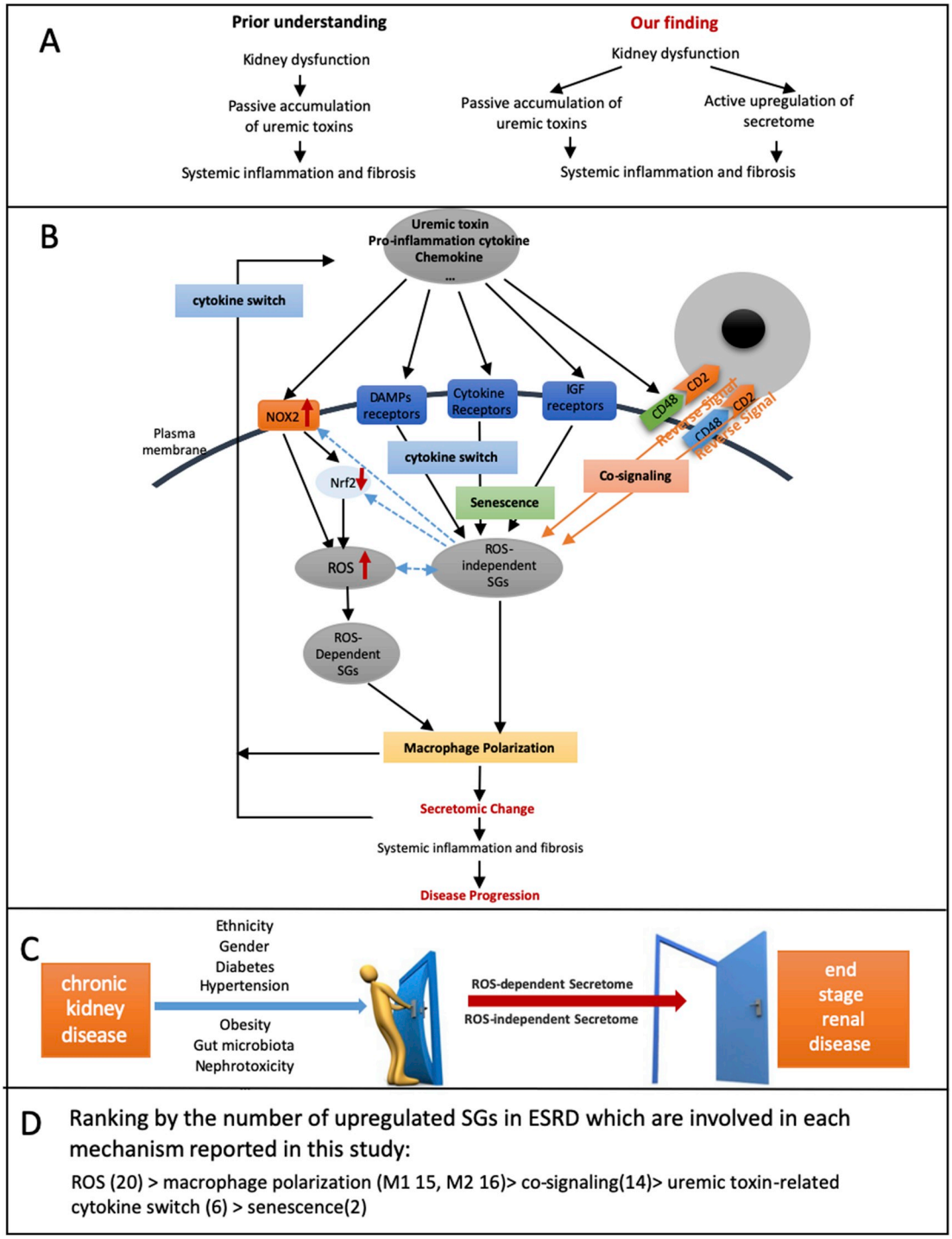

Fig. 8. A: Our new finding suggested a new model that not only passive accumulation of uremic toxins, but also other active upregulation of secretome during ESRD contributes to disease progression through proinflammatory and profibrotic pathways and molecules. **B:** This active accumulation modulated by both ROS-dependent and –independent pathways could promote systemic inflammation and fibrosis to accelerate disease progression. Uremic toxin-related cytokine switching, macrophage polarization and co-signaling by the interaction of CD48 and CD58 with CD2 were important pathways associated with ROS-independent SGs. Of note, there has been researches identified those pathways could modulate and interact with ROS-dependent pathway. **C:** The active accumulation of secretomic changes are the key mediators when combined with and modulated by other risk factors as multiple hits in the transition from CKD to ESRD. **D:** Ranking of all the mechanisms in our research by the numbers of SGs upregulated in each entry suggested that ROS serves as an important complementary role for prior knowledge of CKD progression, multiple-hit model of CKD progression.

Table 10
A novel research publication type with big-omics experimental database mining analyses leads to original new findings and generate anew hypotheses. A few aspects of comparisons were made within this study using big-omics experimental database mining approaches, the traditional literature reviews and the meta-analysis.

category	Big-omics Database mining	Traditional literature review	meta analysis
Analysis of experimental Data (NIH-Geo-DataSets with microarray experimental data, etc.)	yes	no	yes
Original new Findings	yes	no	no
Association research (gene co-expression patterns at the same pathology or stimuli)	yes	no	yes
Causative research (upstream regulator gene deficient microarrays, …)	yes	no	no
Panoramic view at multiple mechanisms and pathways	yes	yes	yes
Improvement of our understanding	yes	yes	yes
Searchable Database requirements and tools	yes	no	yes
New publication types after –omics and high throughput experimental data generation	yes	no	yes
Different focuses from original papers	yes	no	no
Use of Ingenuity Pathway Analysis (IPA) to analyze experimental data	yes	no	no
Bioinformatic prediction	no	no	no
Future experimental verification	yes	yes	yes
Summary of previous reports	no	yes	yes
Example	PMID: 22438968 [our datamining paper focusing on IL-35 (highly cited by 173 papers)]	PMID: 24060958 [a Nature Review paper of management of hypertriglyceridaemia]	PMID: 23083786 [a meta-analysis paper focusing on Effects of fibrates in kidney disease]
experimental papers verifying the findings originated from example paper	PMIDs: 26085094; 29371247	N/A	PMID: 25419705
Use of multiple NIH databases including PubMed database (https://www.ncbi.nlm.nih.gov/books/NBK143764/) yes	yes	no	no

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2020.101460.

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