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Research Article

Microarray Analysis of Liver Gene Expression in Apoa-I and Apoa-I Milano Knock-in Mouse Models

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Abstract

Atherosclerosis, the dominant cause of CVD, is a vascular site-specific chronic inflammation initiated in response to retained and modified lipids within arterial wall that can lead to clinically significant endpoints. Several epidemiological studies have shown that there is an independent and inverse relationship between circulating HDL-C levels and CVD. However, it has been proven difficult to successful reduce CVD risk with drugs increasing HDL-cholesterol. Patients with end-stage renal disease undergoing hemodialysis are at increased risk for CVD that is, at least in part, due to lipid abnormalities, typically called uremic dyslipidemia. The apoA-I Milano is mutant form of apoA-I, with an arginine to cysteine substitution at position 173. Carriers exhibit hypertriglyceridemia with markedly reduced HDL and apoA-I plasma levels. Evaluation of the cardiovascular status in apoA-IM carriers, compared with control subjects from the same kindred, did not reveal any evidence of increased CVD risk. Aim of this study was the identification of pivotal molecules that to a large extent differentiate two mouse models expressing the human apoA-I and the apoA-IM variant, respectively. Total RNA was isolated from mouse liver and analysed by using the Affymetrix GeneChip Mouse Gene ST system.

The expression of 1,407 known genes was significant altered, 694 upregulated and 713 downregulated, in A-I wt compared to A-IM k-in mice, most of them related to biological process involved in kidney health. In conclusion, our data, requiring validation, indicate that A-IM could be involved in the prevention of kidney disease.

Keywords: Mouse model; Apolipoprotein A-IMilano; David

Introduction

Atherosclerosis, the dominant cause of Cardiovascular Disease (CVD), is a vascular site-specific chronic inflammation initiated in response to retained and modified lipids within arterial wall that can lead to clinically significant endpoints, i.e., myocardial infarction, peripheral arterial disease, and stroke [1,2]. According to epidemiological, pathophysiological, genetic, and clinical evidence, dyslipidemia play an important pathogenic role in the development of atherosclerosis. In human and in certain genetically modified mice, circulating levels of cholesterol in Low Density Lipoproteins (LDL) are directly related to the development of atherosclerosis and subsequent CVD, while High Density Lipoprotein (HDL)-cholesterol levels are inversely related [3]. Notably, LDL-cholesterol lowering by the use of statins has become one of the most successful achievements in preventive medicine because they help to reduce CVD event rates by up to 50% in the highest dosage [4]. Furthermore, the development of atherosclerotic lesions could be decreased or even reverted in several animal models by transgenic overexpression or exogenous application of apolipoprotein A-I (apoA-I), which is the most abundant protein of HDL [5,6]. However, to date, it has been proven difficult to successful reduce CVD risk with drugs increasing HDL-cholesterol, such as fibrates, niacin or inhibitors of cholesteryl ester transfer protein [7]. Moreover, mutations in several human genes as well as targeting of several murine genes modulate HDL-cholesterol levels without changing cardiovascular risk and atherosclerotic plaque load, respectively, in the opposite direction as expected from epidemiological studies [8,9]. In this controversy it is important to note that in contrast to the pro-atherogenic role played by LDL-cholesterol, HDL-cholesterol is only a non-functional surrogate marker for estimating HDL particle number and size, without deciphering the heterogeneous composition and hence functionality of HDL [10,11]. The benefits of HDL are thought to be related to reverse cholesterol transport during which excess cholesterol is transported from the atherosclerotic plaque to the liver for degradation. This process, known as cholesterol efflux, is mediated by apolipoprotein A-I (ApoA-I), the dominant protein of HDL [12,13]. Cholesterol Efflux Capacity (CEC) has been strongly and inversely associated with the risk of ischemic events [12,14].

Patients with End-Stage Renal Disease (ESRD) undergoing Hemodialysis (HD) are at increased risk for CVD that is [15], at least in part, due to lipid abnormalities, typically called uremic

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dyslipidemia [16]. Uremic dyslipidemia typically is characterized by increased levels of triglycerides and lipoprotein (a) [Lp(a)], and low concentrations of HDL-cholesterol, and relatively normal or even lower levels of LDL-cholesterol. Therefore, while in the general population therapeutic strategies have focused on lowering LDL-C, primarily by the use of statins, this strategy has not been fruitful in patients with ESRD as indicated by the results of 4D, AURORA and SHARP trials. Hence, understanding the mechanisms responsible for HDL deficiency and dysfunction are critical steps in devising effective therapies aimed at improving HDL-C level and function in ESRD. In addition to HDL and ApoA-I deficiency, detailed analysis of HDL particles in patients with ESRD has revealed that the proportion of lipid-poor premature HDL is increased and maturation of cholesterol ester-poor HDL to cholesterol ester-rich HDL-2 is impaired in the ESRD population. These findings are clinically relevant because small-sized HDL particles alone and combined with elevated high sensitivity C-Reactive Protein (hsCRP) concentrations have been found to be independent predictors of reduced survival in patients with ESRD [15]. Furthermore, hepatic production, plasma concentration and activity of LCAT are consistently reduced in patients with ESRD [17,18]. Given the critical role that LCAT plays in HDL maturation, ESRD-induced LCAT deficiency further contributes to the reduction of plasma HDL concentration and impaired HDL maturation. These mechanisms lead to altered HDL particle composition in patients with ESRD that may lead to atherosclerosis and cardiovascular disease. This was shown by Holzer et al. using mass spectrometry and biochemical analyses to assess the composition of HDL particles in patients with ESRD. They found a significant increase in quantity of acute phase protein serum amyloid A1, albumin, lipoprotein-associated phospholipase A2, and apoC-III in HDL from patients with ESRD when compared with normal individuals. Furthermore, these HDL particles contained reduced phospholipid and increased triglyceride content and had an impaired ability to promote cholesterol efflux from macrophages [17,19].

ApoA-I-Milano (A-IM) is the first variant of APOA-1 discovered in 1974 in individuals from Limone sul Garda [20,21]. This mutation is caused by a cysteine to arginine substitution at position 173 (R173C) [22]. Interestingly, although carriers of this variant exhibited a lipid profile characterized by low HDL-cholesterol (HDL-C) levels and moderate hypertriglyceridemia, A-IM seems to confer protection against the development of atherosclerosis and cardiovascular disease [23]. This finding was also confirmed in several experimental studies and first clinical trials, where atherosclerosis regression was demonstrated [5,23].

However, in more recent investigations, infusion of sHDL did not show a convincing clinical benefit [24]. Taken together, these data justify the rational of testing infusion therapies with cholesterol-poor HDL (i.e., synthetic HDL, sHDL), as a treatment for patients with atherosclerotic disease. This therapeutic approach showed very promising results in pre-clinical studies and first clinical trials, where atherosclerosis regression was demonstrated [22,23].

Aim of this study was the identification of pivotal molecules that to a large extent differentiate two mouse models expressing the human apoA-I and the apoA-IM variant, respectively.

Materials and Methods

Animals

Previously generated homozygous knock-in (k-in) mouse lines expressing human apoA-I (hA-I wt k-in) or human apoA-IM (hA-IM k-in) were used for the study [19,25]. Mice from each line were kept under standard laboratory conditions (12 hours light cycle, temperature 22 ± 1°C, humidity 55 ± 5%), with free access to standard chow and tap water. Blood was collected after an overnight fast from the retro-orbital plexus into tubes containing 0.1% (w/v) EDTA and centrifuged in a microfuge for 10 min at 8,000 rpm at 4°C. Serum total and unesterified cholesterol were measured by enzymatic methods (Horiba ABX, Montpellier, France) (39). Triglyceride (TG) concentrations were corrected for the free glycerol present in serum as described (Horiba ABX, Montpellier, France) [26,27]. HDL-cholesterol levels were measured after precipitation of apoB-containing lipoproteins with PEG 8,000 (20% w/v) in 0.2 M glycine (pH 10) [28,29]. Human apolipoprotein concentrations were determined by immunoturbidimetric assays, using a sheep antiserum specific for human apoA-I (Hoffmann La Roche, Basel; Switzerland) which also recognizes apoA-IM [30,31].

All animals were sacrificed under general anaesthesia with 2% isoflurane (Forane, from Abbot Laboratories Ltd, Illinois, USA) and blood was removed by perfusion with Phosphate-Buffered Saline (PBS). At necropsy, the livers were immediately removed, snap-frozen in liquid nitrogen and stored at -80°C prior to analyses [13].

Procedures involving animals and their care were conducted in accordance with institutional guidelines that are in compliance with national (D.L. No. 26, March 4, 2014; G.U. No. 61, March 14, 2014) and international laws and policies (EEC Council Directive 2010/63, September 22, 2010: Guide for the Care and Use of Laboratory Animals, United States National Research Council, 2011). The study was approved by the Italian Ministry of Health (Progetto di Ricerca Protocollo 2012/4).

RNA isolation

Total RNA was isolated from mouse liver using the NucleoSpin RNA extraction kit (Macherey-Nagel) according to the manufacturer's instructions. RNA concentration and purity were estimated from the optical density at 260 nm and 280 nm, respectively (Nanodrop ND-1000, NanoDrop Technologies, Wilmington, DE) [26,32,33].

Global gene expression analysis by complementary DNA (cDNA) microarray

Total RNA were labeled with cyanine 3 dye using the QuickAmp Labeling Kit (Agilent Technologies). RNA concentration, dye incorporation, and quality were analyzed using a UV–VIS spectrophotometer. Fluorescently labeled cRNA was hybridized to whole mouse genome expression microarrays following the manufacturer's protocol (Affymetrix GeneChip Mouse Gene ST system). Normalization of gene expression data and filtering of probe sets by expression levels, flags, and errors were performed using GeneSpring software. Significant differences in gene expression among the two mouse lines were analyzed by analysis of variance. Genes exhibiting expression changes between the two mouse lines were detected by Tukey's significant difference test.

Gene ontology and pathway enrichment analyses

To identify the Differentially Expressed Genes (DEGs) determined with BRB-ArrayTools, GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed via the Database for Annotation, Visualization and Integrated Discovery (DAVID 6.8; http://david.abcc.ncifcrf.gov/) [18,19]. GO terms [categorized into Molecular Function (MF), Biological Process (BP) and Cellular Component (CC)] and KEGG pathways with $P<0.05$

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were considered significantly enriched by the DEGs.

Statistical analysis

The results were presented as mean ± SD. Statistical analysis has been performed using ANOVA. A FDR threshold of 0.05 has been chosen to select significant changes. P<0.05 was considered statistically significant.

Results

Lipid and apolipoprotein plasma concentrations

As expected, total cholesterol in A-IM k-in mice was significantly lower than that measured in A-I wt k-in animals (52.9 ± 5.9 mg/dl vs. 132.0 ± 34.6 mg/dl, p<0.001). Plasma HDL cholesterol concentrations in A-IM k-in mice were substantially lower than those observed in A-I k-in (-63%, 28.1 ± 5.0 mg/dl vs. 75.5 ± 14.9 mg/d, p<0.001). In addition, a significant increase in plasma unesterified to esterified cholesterol ratio was observed in A-IM k-in compared to A-I k-in mice (0.69 ± 0.13 vs. 0.41 ± 0.02, p<0.001), suggesting impaired cholesterol esterification in the former. In contrast to changes in cholesterol concentrations, plasma triglyceride levels were similar in all the mouse lines analysed (data not shown). As expected, the apoA-IM concentrations in A-IM k-in mice was approximately 50% of apoA-I in A-I k-in mice (100.7 ± 19.4 mg/dl vs. 213.7 ± 41.8 mg/dl, p<0.001.

Liver differentially expressed genes

The differentially expressed genes in A-I wt and hA-IM k-in mouse lines were detected by mRNA microarray assay. The expression of 1,880 genes was significantly altered between the two mouse lines. As shown in Figure 1, 1,407 out of 1,880 were known genes, of which 694 upregulated and 713 downregulated in A-I wt compared to A-IM k-in mice, and 473 were unknown genes.

Gene functional classification analysis

The gene functional classification analysis of upregulated genes detected 19 clusters with an Enrichment Score (ES) between 4.7 and 0.09. In Figure 2 are reported a cluster with ES of 2.94 containing different members of collagen genes, and a cluster with ES of 2.09 containing several members of A Disintegrin and Metalloproteinase with Thrombospondin Motifs (ADAMTS) genes, respectively.

The downregulated genes showed 21 clusters of genes with ES between 2.47 and 0.27. The gene groups of ES of 1.43 and 1.29 contain solute carriers family and ATP-binding cassette, sub-family members, respectively (data not shown).

GO analysis

Gene ontology function enrichment analysis was performed using the DAVID platform. Figure 3 listed the highest 10 Gene Ontology (GO) upregulated significant functions in A-I wt compared to hA-IM k-in mouse line, divided in GO biological process (GO_BP, Figure 3A), GO cellular component (GO_CC, Figure 3B) and GO molecular function (GO_MF, Figure 3C). Figure 4 listed the highest 10 GO downregulated significant functions in A-I wt compared to hA-IM k-in mouse line, divided in GO biological process (GO_BP, Figure 4A), GO cellular component (GO_CC, Figure 4B) and GO molecular function (GO_MF, Figure 4C).

KEGG pathway enrichment analysis

All the 5 KEGG significant pathways (Table 1) obtain by functional annotation analysis in upregulated DEGs contained members of collagen genes. Moreover, Tenascin C gene is involved in EMC-receptor interaction, PI3K-Akt signalling and focal adhesion pathways. Tenascin C gene, that is associated with kidney health, is 1.178 fold increased in A-I wt mice compared to A-IM animals.

Table 2 shows the KEGG significant pathways found by DAVID analysis in A-I wt vs. A-IM downregulated genes. In 8 out of 10 KEGG pathways jun oncogene (Jun) and thymoma viral proto-oncogene 2 (Akt2) are involved, the former being the most downregulated gene (-3.596, p<0.012) and the second being -1.124 fold in A-I wt vs. A-IM k-in mice. Furthermore, among the KEGG non-significant pathways we found that, as shown in Table 3, almost all are CD36 antigen (Cd36)-related pathways. It is also important to highlight that the vanin gene (Vnn1) having a fold chance of -3.192 is a key factor of the Pantothenate and CoA biosynthesis.

Discussion

As already published the two k-in mouse lines, i.e., A-I wt and A-IM k-in, have a lipid/lipoprotein profile that, in many respects, is similar to that of human carriers, i.e., characterized by low plasma total and HDL-C levels, compared to A-I k-in mice [21].

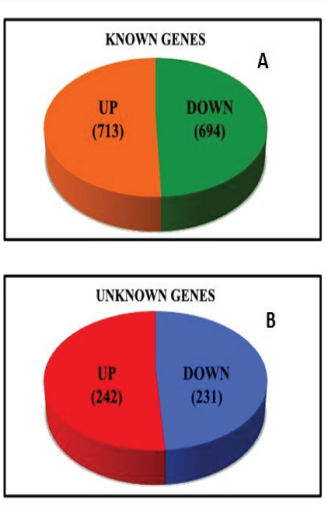

Figure 1: Numbers of known (top) and unknown (bottom) upregulated and downregulated differentially expressed genes in the A-Iwt k-in vs. A-IM k-in mouse models.

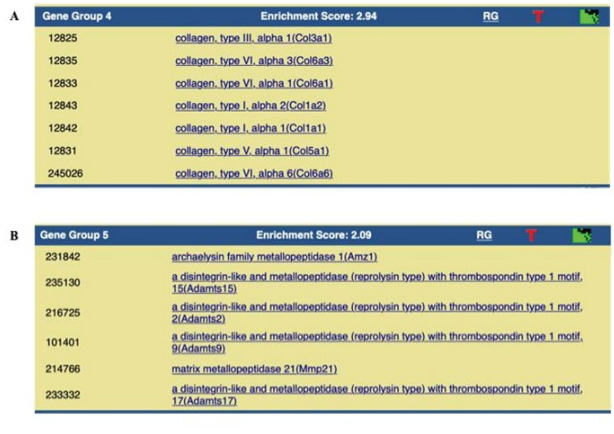

Figure 2: The gene functional classification analysis of (A) upregulated and (B) downregulated genes in A-Iwt k-in compared to A-IM k-in mouse livers.

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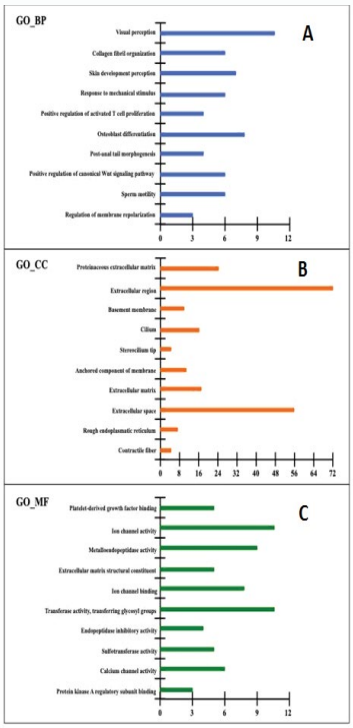

Figure 3: Top 10 most enriched GO terms among the genes upregulated in A-Iwt k-in compared to A-IM k-in mouse livers. The horizontal axis represents the number of enriched genes. The vertical axis represents the GO terms, increasing in p value from top to bottom. GO means gene ontology; BP means biological process; CC means cellular component and MF means molecular function.

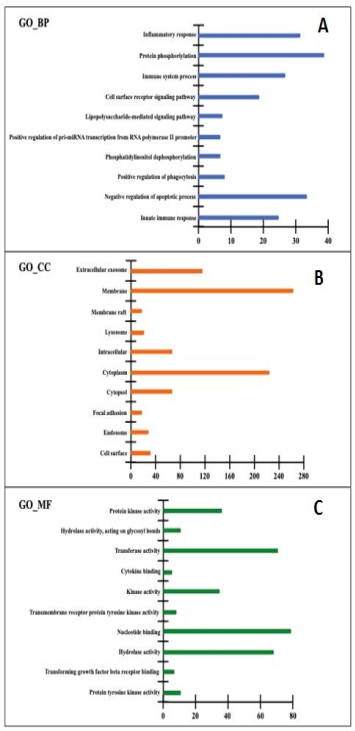

Figure 4: Top 10 most enriched GO terms among the genes down regulated in A-Iwt k-in compared to A-IM k-in mouse livers. The horizontal axis represents the number of enriched genes. The vertical axis represents the GO terms, increasing in p value from top to bottom. GO means gene ontology; BP means biological process; CC means cellular component and MF means molecular function.

In the present study using Affymetrix GeneChip Mouse Gene ST system we have compared the genetic profile of A-I wt k-in mice with that of A-IM k-in animals. The aim was the identification of pivotal biological process or cellular component or molecular function that to a large extent could differentiate the human apoA-I and it's molecular variant apoA-IM.

In the liver, the expression of 1,407 known genes was significantly altered between the two mouse lines:

694 upregulated and 713 downregulated in A-I wt compared to A-IM k-in mice. We have exploited this data and analysed the function of differentially expressed genes using bioinformatics platform, i.e., the DAVID Bioinformatics Resources.

The Gene Ontology (GO) function enrichment analysis of upregulated genes showed that among the top 10 enriched terms GO_BP there were biological processes associated to collagen fibril organization, osteoblast differentiation and positive regulation of canonical Wnt signalling pathway. The KEGG analysis revealed that Tenascin C gene could differentiate the two mouse models and a very recent study demonstrated that Tenascin C protected tubular epithelial cells against apoptosis and augmented Wnt1-mediated β-catenin activation. In fact, Tenascin C is specifically induced at sites of injury and recruits Wnt ligands, thereby creating a favourable microenvironment for tubular repair and regeneration after acute kidney injury (Chen et al., 2019, page 62).

The GO analysis of downregulated genes showed instead that among the top 10 enriched terms GO_BP there were biological processes associated to inflammatory response, cell surface receptor signalling pathway, negative regulation of apoptotic process and innate immune response. In this case, the KEGG pathways enrichment analysis showed that the jun oncogene (Jun), the thymoma viral proto-oncogene 2 (Akt2), the CD36 antigen (Cd36) and the vanin gene (Vnn1) are involved in several of this pathways.

The AKT family of proteins are serine/threonine kinases that regulate adaptation to many cellular stress-induced processes, including survival, proliferation, migration and cytoskeletal organization [34]. Once activated, by phosphorylation Thr308 and Ser473 by 3-phosphoinositide–dependent protein kinase-1 (PDK1) and mTORC2, respectively, AKT proteins phosphorylate several substrates to regulate multiple cellular functions [35,36]. A recent study uncovered a new function for Akt proteins and showed that Akt2, but not Akt1, has a crucial role in podocyte adaptation to nephron reduction. Akt2 inactivation resulted in podocyte apoptosis and foot process effacement, which led to severe proteinuria and glomerulosclerosis [14,34]. It is well known that podocytes are highly specialized epithelial cells that together with endothelial cells and the glomerular basement membrane compose the glomerular filtration barrier [37]. Podocytes help prevent proteinuria through a complex regulation of the actin cytoskeleton of their foot processes. Moreover, another study demonstrated the involvement of Akt2 in TGFβ stimulated podocyte hypertrophy and matrix protein accumulation seen in glomerulosclerosis [38].

The highest KEGG significant pathway is the osteoclast differentiation. Inflammation and immune system alterations contribute to bone damage in many pathologies by inducing the differentiation of osteoclasts (OCs), the bone resorbing cells, that starts from monocytes/macrophages through the actions of the Macrophage-Colony Stimulating Factor (M-CSF) and the Receptor

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Activator of Nuclear factor-Kappa B Ligand (RANKL) [39]. Systemic inflammation is also a feature of advanced chronic kidney disease and in these patients it might contribute to the increased bone fracture risk. The issue is of relevance because bone fractures in CKD patients not only engender disabling orthopaedic problems, but are also associated with increased mortality [39].

Despite technical improvements in clinical care and the development of preventive strategies, the incidence of acute kidney injury has gradually increased. Renal disorders are often obvious at their later stage. Although serum creatinine and blood urea nitrogen are routinely used as the biomarkers for renal disease and injury, both markers obviously increase only at the advanced stage of renal damage, and have a lack of specificity [40]. Vanin-1 is an epithelial ectoenzyme with pantetheinase activity, which catalyzes the conversion of pantetheine into pantothenic acid (vitamin B5) and cysteamine [41,42]. In vitro and in vivo studies have shown that vanin-1 mRNA expression as well as urinary and serum concentrations of vanin 1 increased in the renal tubular cells exposed to the organic solvents. Because urinary and serum concentrations of vanin-1 elevated before the conventional markers changed, vanin-1 could be considered as an early rapid biomarker for detecting and monitoring the nephrotoxicant-induced renal tubular injury [43,44].

Finally, our data showed that 13 genes were involved into the HIPPO signalling pathway, which is best known for its function in organ size control, tissue homeostasis/regeneration, and cancer. A recent report supports the hypothesis that the Hippo pathway could play a role in autosomal dominant polycystic kidney disease pathogenesis when PKD1 is mutated [45].

On summary, even though our data are preliminary and need to be confirmed, seem to be in line with previously published results demonstrating a link between the downregulated genes found in A-I k-in mice and the pathogenesis of kidney disease.

Author Contributions

Giulia S. Ganzetti and Elena Rigamonti performed the experiments; Elena Rigamonti, Roberta Brambilla and Cinzia Parolini analysed and interpreted the data; Giulia S. Ganzetti, Elena Rigamonti, Roberta Brambilla and Cinzia Parolini designed the study and wrote the manuscript; Cinzia Parolini supervised the research.

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Table 1: KECG significant pathways obtained by DAVID functional annotation analysis in upregulated DEGs.

Category	Term	Genes #	p-value
KEGG_PATHWAY	ECM-receptor interaction	11	2.50E-05
KEGG_PATHWAY	P13K-Akt signalling pathway	18	2.30E-03
KEGG_PATHWAY	Protein digestion and absorption	8	3.50E-03
KEGG_PATHWAY	Focal adhesion	12	6.90E-03
KEGG_PATHWAY	beta-Alanine metabolism	4	3.70E-02

Table 2: KEGG significant pathway obtained by DAVID functional annotation analysis in downregulated DEGs.

Category	Term	Genes #	p-value
KEGG_ PATHWAY	Osteoclast differentiation	15	1.20E-04
KEGG_ PATHWAY	Pathways in cancer	29	2.70E-04
KEGG_ PATHWAY	Toll-like receptor signalling pathway	11	2.70E-04
KEGG_ PATHWAY	MAPK signalling pathway	19	3.00E-03
KEGG_ PATHWAY	Inflammatory bowel disease(IBD)	8	4.30E-03
KEGG_ PATHWAY	Hippo signalling pathway	13	6.50E-03
KEGG_ PATHWAY	Chemokine signalling pathway	13	4.20E-02
KEGG_ PATHWAY	Sphingolipid signalling pathway and metabolism	9 and 5	6.9E-2 and 8.6E-2
KEGG_ PATHWAY	TGF-beta signalling pathway	7	7.70E-02
KEGG_ PATHWAY	TNF signalling pathway	8	8.70E-02

Table 3: KEGG Non-significant pathway obtained by DAVID functional annotation analysis in downregulated DEGs.

Category	TERM	GENE SYMBOL (Fold-Change)							
KEGG_ PATHWAY	Pantothenate and CoA biosynthesis	Vnn1(-3.192)							
KEGG_ PATHWAY	PPAR signaling pathway	Cd36(-1.545)	Fadp7(-1.238)	Rxrg(-1.238)					
KEGG_ PATHWAY	Phagosome	Cd36(-1.545)	Fegr2b(-1.317)	H2-T24(-1.797)	H2-B1(1.839)	H2-Dmb1	Itgb2(-1.361)	Mcr1	Tlr6(-1.274)
KEGG_ PATHWAY	AMPK signaling pathway	Cd36(-1.545)	Rab8a(1.114)	Adrala (-1.333)	Lipe(-1.276)	Ppp2r1b (-1.276)	Ppp2r2d (-1.254)	Akt2(-1.124)	
KEGG_ PATHWAY	ECM-receptor interaction	Cd36(-1.545)	Cd44(-1.423)						
KEGG_ PATHWAY	Hematopoietic cell lineage	Cd36(-1.545)	Cd38(-1.412)	Cd44(-1.423)	Cd55(-1.697)	Csflr	II lb(-1.481)	Kit(-1.799)	
KEGG_ PATHWAY	Adipocytokine signaling pathway	Cd36(-1.545)	Nfkbl(-1.189)	Nfkbib(-1.210)	Rxrg(-1.238)	Akt2(-1.124)			
KEGG_ PATHWAY	Insulin resistance	Cd36(-1.545)	Nfkbl (-1.189)	Prkcz (-1.386)	Rps6ka 3(-1.237)	Akt2(-1.124)			
KEGG_ PATHWAY	Fat digestion and absorption	Cd36(-1.545)	Mttp(-1.210)						
KEGG_ PATHWAY	Malaria	Cd36(-1.545)	Gypc(-1.418)	Itgb2(-1.361)	II lb(-1.481)	Myd88(-1.182)	Tgfb1(-1.285)		

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