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ORIGINAL RESEARCH

Persistent Inflammatory Pathways Associated with Early Onset Myocardial Infarction in a Medicated Multiethnic Hawaiian Cohort

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Abstract: In spite of current standard therapies to target the major pathomechanisms in myocardial infarction (MI), inflammatory gene expression patterns have been consistently revealed in MI patients. In a multiethnic cohort, we aimed to identify MI-associated pathomechanisms that may be unresponsive to medical treatment to improve diagnosis and therapy. Gene expression profiles in whole blood were analyzed in medicated Asian, African American and Caucasian patients living in Hawaii with a history of early MI and age, ethnicity, risk factor and medication-matched controls. PANTHER ontological and Ingenuity Pathway analysis and functional evaluation of the consistently differentially expressed genes identified coordinated up-regulation of genes for inflammation (LGALS3, PTX3, ZBTB32, BCL2L1), T-cell activation (IL12RB1, VAV3, JAG1, CAMP), immune imbalance (IL-8, IL2RA, CCR7, AHNAK), and active atherosclerosis (NR1H4, BIN1, GSTT1, MARCO) that persist in MI patients in spite of concerted treatment efforts to control vascular pathology. Furthermore, significant ethnic differences appear to exist within the active disease mechanisms that need to be further investigated to identify key targets for effective medical intervention.

Keywords: Atherogenesis, inflammation, immune imbalance, microarray, myocardial infarction

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Background

Cardiovascular disease, most commonly manifested as myocardial infarction (MI), is a significant health problem worldwide. In the United States, MI accounts for one out of every three deaths, and in spite of advances in treatment, remains a pre-eminent health problem.[1]

Atherogenesis and formation of atherosclerotic plaques precede MI and numerous efforts have been directed at the development of biomarkers for the diagnosis of atherosclerosis and the atherogenic process.[2] Recent studies confirmed that circulating blood cells upon encounter of a pathological vascular environment develop a gene expression signature of the vascular disease phenotype.[3] Atherosclerosis, coronary artery disease, and MI-associated gene expression patterns have been consistently identified in peripheral blood cell types, including monocytes,[4] platelets,[5] and pro-inflammatory leukocytes that directly contribute to the development of atherosclerosis.[6] Whole blood gene expression signatures have similarly been confirmed to correlate with the extent of coronary stenosis,[7] coronary artery disease, and the type and degree of vascular injury, and correctly mirror pathophysiology in the vascular wall.[8] However, most of the gene expression patterns established in predominantly Caucasian patient groups have not been evaluated in ethnically diverse populations.

Medications commonly used in the prevention and treatment of atherosclerosis and MI include aspirin (ASA), β-adrenergic blockers (β-blocker),[9] angiotensin converting enzyme inhibitors (ACEI),[10] angiotensin receptor type 1 blockers (ARB)[11] and 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins),[12] that target inflammation, cholesterol metabolism and hypertension, the major pathomechanisms, in atherosclerosis and MI. In spite of the wide-spread and long-term use of these therapies, however, circulating blood derived inflammatory gene signatures have been consistently noted in various MI patient cohorts and suggest that certain inflammatory pathways causally relevant to the development of atherosclerosis and MI may be unresponsive to anti-inflammatory medications.[8] Furthermore, the observed persistent inflammatory pathways while demonstrate common key elements, also reveal considerable diversity in the most significant differentially regulated genes among various MI patient cohorts.

In order to define regulatory pathways associated with MI in an ethnically diverse patient population, and identify inflammatory and atherogenic pathways that may be unresponsive to the currently applied standard therapies for the prevention and treatment of MI, we analyzed gene expression profiles in whole blood samples in a multiethnic patient cohort with a history of early MI (EMI) and compared these to an age, ethnicity, risk factor and medication-matched control cohort (MCON) identified and enrolled through the VA Pacific Islands Healthcare System.

Methods

Study subjects

Following a protocol approved by the VA Pacific Islands Healthcare System (VAPIHCS), University of Hawaii IRBs, and the United States Army Medical Research and Materiel Command Human Research Protection Office, age, gender and ethnicity matched groups of early MI (EMI) and medication-matched control (MCON) subjects were recruited that had no evidence of chronic or active infectious or autoimmune diseases. Using the VAPIHCS data warehouse, eligible subjects were identified based upon the following criteria:

Inclusion criteria

The EMI patient group included individuals that had an ICD-9 code for MI before age 50 for men and 55 for women, that occurred more than 6 months prior to enrollment, and were using any combination of cardiac-related medications including ASA, ACEIs, ARBs, β-blockers and statins. The MCON group included individuals that did not have an ICD-9 code for coronary heart disease, but were taking any combination of the same medications.

Exclusion criteria

1) treatment of active infection within the last month, 2) a clinical history of chronic infection or rheumatologic disease, 3) active use of non-steroidal anti-inflammatory drugs (other than aspirin) for analgesia or inflammatory conditions, 4) a history of an MI within the last 6 months, 5) an absolute monocyte count <125 cells/μl within the last year, 6) anemia defined as hemoglobin <11 g/dL or

HCT < 33% within the last year, 7) CRP ≥ 2.0 mg/L or ESR ≥ 30 mm/hr. Potential subjects with active pregnancy and those with dementia or severe mental illness were excluded based upon review of the medical records.

Eligible subjects were invited for an interview and for informed, consenting subjects a detailed questionnaire was used to record baseline characteristics, including past medical history, compliance with medication use and habits. Routine clinical laboratory testing included a complete blood count with differential, comprehensive metabolic profile, erythrocyte sedimentation rate, C-reactive protein level and in females β-human chorionic gonadotropin level. From selected individuals blood samples were collected for RNA extraction. As no women met the study entry criteria, our recruitment process yielded two age and ethnicity matched study groups with 10 men in each group.

RNA purification and microarray hybridization

A description of the RNA isolation, labeling, double-stranded cDNA synthesis, and hybridization onto Applied Biosystems Human Survey Genome Microarray 2.0 is provided in the Supplementary Material.

Data analysis

Chemiluminescence detection, image acquisition and primary data analysis were performed using the Applied Biosystems 1700 Chemiluminescent Microarray Analyzer. Chemiluminescent images were auto-gridded, spot and spatially normalized, signals were quantified, corrected for background and the final images and feature data processed using the Applied Biosystems 1700 Chemiluminescent Microarray Analyzer software v1.1. A novel algorithm that adaptively thresholds fold change was used to detect a global signal for differential expression between the EMI and MCON sample groups. Data obtained from each chip was first vectorized to form a column of an expression data matrix where the MCON and EMI sample data segregated into two disjoint groups. Each row of the data matrix represented the expression profile of a gene across the EMI and MCON sample groups. The data matrix was quantile normalized to suppress low-frequency systematic error and log2 transformed to equalize variation in fold change over intensity. Box and whiskers plots, pair-wise correlation plots and histograms of log2 expression were generated from raw microarray data to assess data quality. A Ratio-Intensity (RI) scatter plot of mean log2 ratio (MLR) versus mean log2 expression (MLE) was generated where each gene was represented by a point in (MLE, MLR) space. The horizontal MLE-axis was then divided into 51 quantile bins to decouple variation in MLR from MLE where each bin contained approximately 650 genes. Assuming that only a small percentage of genes in a bin were truly differentially expressed, ie, that the signal for differential expression was sparse, a threshold was computed for each bin based on the Donoho-Johnstone universal threshold (DJUT) of wavelet denoising theory[13,14] and Classical Multi-Dimensional Scaling (CMDS or PCA) analysis. The genes that exceeded the DJUT threshold for each bin, together, formed a global estimate of the signal for differential expression.

The differentially expressed genes were analyzed using clustered heatmaps[15] and PCA analysis.[16] Knowledge-based PANTHER (www.pantherdb.org) and Ingenuity's Pathway Analysis (IPA, Ingenuity Systems, Inc, Redwood City, CA) databases were used to identify sets of functionally related genes and signaling pathways significantly overrepresented among genes differentially expressed in EMI samples.

Quantitative real-time PCR

1 μg of total RNA from selected samples was reverse transcribed into cDNA using Superscript III cDNA Synthesis Kit (from Invitrogen, Carlsbad, CA). 100 ng of cDNA template for each sample was amplified with Applied Biosystems TaqMan Gene Expression Assays (Foster City, CA) for the corresponding gene. Gene expression values were calculated relative to GAPDH using the $2^{-\Delta\Delta CT}$ method.[17] All assays were done in triplicate and data are reported as mean ± standard error of the mean.

Results

Among medicated early myocardial infarction (EMI) subjects and controls without coronary artery disease or MI on the same medications (MCON), prospective matching ensured close baseline characteristics

including body weight, body mass index and rates of cardiac risk factors including hypertension, diabetes, hyperlipidemia, cigarette use, and family history of heart disease (Supplementary Table 1) and laboratory results for clinical parameters (not shown). Blood derived EMI and MCON RNA samples were processed for microarray analysis. Evaluation of the quality of microarray results using box-and-whisker plots (Fig. 1A), pair-wise correlation plots (not shown) and histograms (Fig. 1B) identified sample MCON4 as markedly different and excluded it from further data analysis.

Differentially expressed genes in EMI

In the EMI samples, 1,203 genes were found differentially expressed (Fig. 1C). The expression value for each differentially expressed gene was plotted in a heatmap to identify groups of genes with

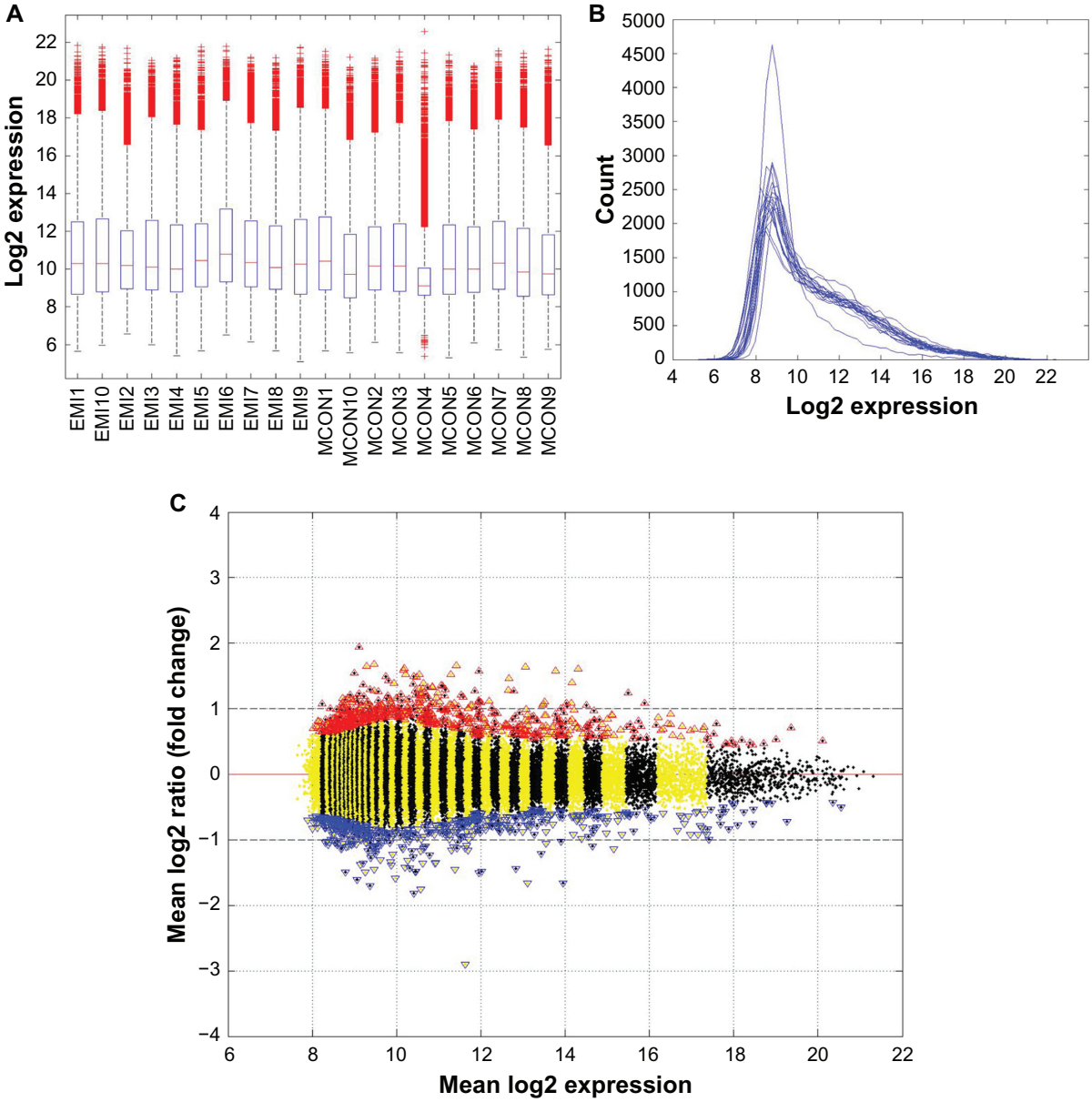

Figure 1. A) Box and whiskers plots of raw microarray data. Samples are along the horizontal axis while log2 intensity values for each gene are plotted on the vertical axis; the upper and lower ends of the box contain all genes between the first and third quartiles of log2 gene intensity; the red line in each box represents the median log2 intensity value; the upper and lower ends of the dotted vertical lines represent outliers of log2 gene intensity. **B**) Histogram of raw microarray data, showing the number of genes with a given log2 gene intensity. **C**) Ratio-intensity (RI) plot of the quantile normalized microarray data. The results of the differential expression analysis for all quantile bins are superimposed on the RI plot. The vertical black and yellow stripes represent 51 sets of approximately 650 genes having similar levels of fluorescent intensity. The dots in red triangles show the up- and those in blue the down-regulated genes.

consistent similarity in expression across the samples (Supplementary Fig. 1A). Principal component analysis (PCA) of the data clearly separated EMI from MCON individuals (Supplementary Fig. 1B) and identified samples EMI2, EMI5 and EMI7 as distinctly different. Based on PCA results and inspection of clustered heatmaps, 195 genes (Supplementary Fig. 1A, genes 540–735) were excluded from further analysis as their expression variation was contributed by only by two samples (EMI2 and EMI5) and neither subsequent PANTHER nor IPA analysis revealed any significant functional or pathway structure within these genes.

Altered immunity in early MI patients

In order to identify functionally significant EMI-associated patterns among the differentially expressed genes, PANTHER ontological analysis was performed. The biological process of "Immunity and defense" was identified as significantly overrepresented in the set of all differentially expressed genes (P = 1.4E–05) and in the subset of down-regulated genes (P = 1.39E–05) (Supplementary Table 2). Within the subset of down-regulated genes the biological process "Interferon-mediated immunity" (P = 5.72E–03) and the molecular function "Defense/immunity protein" (P = 2.12E–03) were overrepresented. PCA analysis (not shown) and gene distribution within the clustered heatmap (Supplementary Fig. 2) clearly separated EMI from the MCON group suggesting that immunity is a significant distinguishing feature for EMI subjects.

Ingenuity Pathway Analysis (IPA) of the differentially expressed genes identified "Immunological disease and response" and "Immune and lymphatic system development and function" in two of the four most significant IPA networks represented in our expression data (Supplementary Table 3), and additionally, immunity themes within the other two networks. The differentially expressed network genes and those within functional categories (described below) are referenced for their MI-associated roles in Supplementary Table 4.

Multiple active inflammatory networks in medicated MI subjects

In the EMI samples, both IPA *Network 1* and *2* (Figs. 2A and 2C) had differential gene regulation pattern with inflammatory functions. The clustered heatmaps (Figs. 2B and 2D) and PCA analysis (not shown) of these networks clearly separated EMI from MCON samples.

Within *Network 1*, the up-regulated FOS is a highly connected hub gene that promotes proliferation of lymphocytes. The cell fate determinant DACH1 (up-regulated) with a role in immune response is connected to FOS, Ap1, and TGF-β. JAG1 (up-regulated) is known to be increased in MI patients and is a ligand for NOTCH-1 that has a role in T-cell maturation and activation. PTX3 (up-regulated) is an acute phase protein that rapidly increases during inflammation with a protective function in cardiovascular disease. CCR7 (down-regulated) is a g-protein coupled receptor chemotactic to leukocytes linked to multiple organ autoimmunity.

In *Network 2*, CD40LG (down-regulated) is a hub gene expressed on T cells with a central role in atherosclerosis. Elevated CD40LG is a prognostic marker for cardiovascular disease and its down-regulated state in our EMI subjects indicates a response to medications. However, another hub gene, BCL2L1, with an anti-apoptotic and immune activating role, is up-regulated. IL12RB1, also up-regulated, is known to be expressed during chronic inflammation and in MI it contributes to regulation of the cytokine profile of Th1 lymphocytes. LGALS3 and CAMP (both up-regulated) induce chemotaxis of inflammatory cells.

Network 3 displays features of immune imbalance, cell survival, chemotactic, and angiogenic functions (Fig. 2E). Its clustered heatmap and PCA analysis (not shown) also differentiates EMI from the MCON group (Fig. 2F). IL-8 (up-regulated) is a hub gene in this network that, through several parallel mechanisms, is a mediator of neutrophil chemotaxis. The down-regulated IL-2RA increases susceptibility for autoreactive T-cells and may indicate an imbalanced immune reaction.[18] The down-regulated WNT1 shows a potential for deficient cell protection and cardiomyocyte survival via PI3K/AKT signaling. WNT5A (up-regulated) has an important role in inflammatory macrophage activation. VEGFB with significant role in neovascularization during inflammation and repair was found down-regulated.

Network 4 is dominated by stress response, cardiac hypertrophy and pro-inflammatory themes (Fig. 2G) with its clustered heatmap (Fig. 2H) and

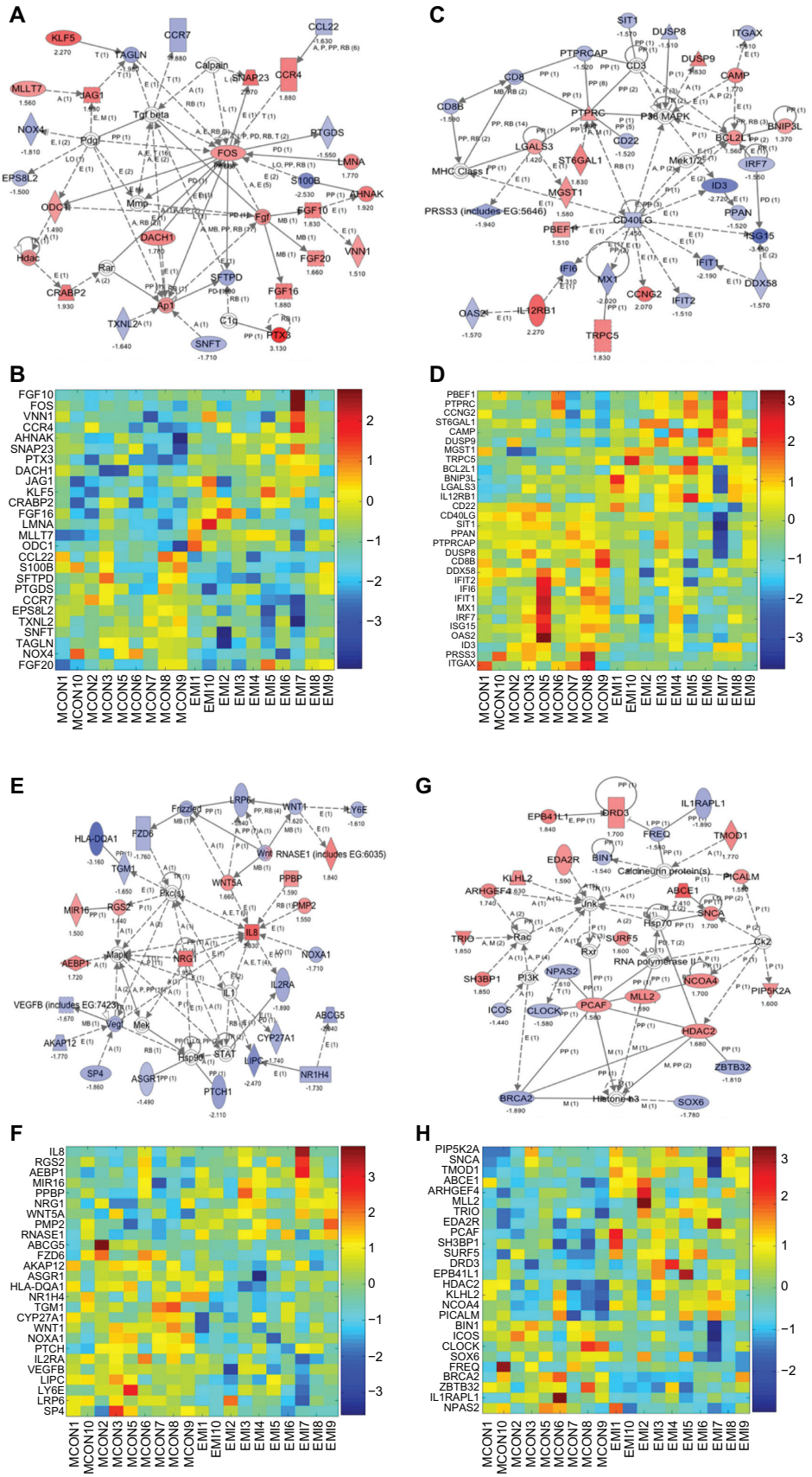

Figure 2. A) Ingenuity Networks 1–4 (**A**, **C**, **E**, **G**) and their clustered heatmaps (**B**, **D**, **F**, **H**). Red color denotes up- and blue down-regulated genes. Solid lines show direct, dotted lines indirect interactions. Arrowheads indicate direction of interactions. Genes with the most connections are hub-genes.

PCA (not shown) separating EMI from MCON. One of the hub genes, HDAC2 (up-regulated), is an integrator of Hsp70 mediated stress response pathways during heart remodeling in cardiac hypertrophy. BIN1 (down-regulated) has functions in apoptosis and T cell mediated inflammation. TRIO (increased) orchestrates cell-matrix and cytoskeletal rearrangements. SOX6 (decreased) is known to regulate cardiomyocyte development and is a candidate gene for myopathy. IL1RAPL1 (interleukin-1 receptor accessory protein-like-1, down-regulated), belongs to a novel class of the IL-1/Toll receptor family and interacts with neuronal calcium sensor-1 (NCS-1). FREQ (NCS-1) down-regulated in all EMI cases, is a calmodulin-related protein that controls inflammatory reactions.

Immune imbalance, coordinated up-regulation of inflammation and active atherogenesis

In order to gain insight into the regulation of the immunity-related and inflammatory processes in EMI, genes within PANTHER biological process "Immunity and defense" and within IPA *Networks 1–4* were plotted on clustered heatmap diagrams. Genes with the most consistent differential expression were identified using clustered heatmaps by two independent evaluators that scored the number of up- or down-regulated genes in the EMI and MCON groups and compared the original expression values.

In EMI, forty-four genes were found most consistently differentially expressed. Among these four major functional categories were identified: 1) inflammation-related, 2) immune imbalance, 3) atherosclerosis, LDL, and cholesterol related genes, and 4) those with a yet unknown function in cardiovascular disease.

Based on expression changes of genes in the inflammation-related category, a pattern of coordinated up-regulation and an overall increase of pro-inflammatory processes emerged. Pro-inflammatory genes LGALS3, ROCK1, and PTX3, were consistently up-regulated, while the anti-inflammatory SNFT, and ZBTB32 (PLZP), were consistently down-regulated. Additionally, pro-apoptotic genes NOXA1 and NGFR that would reduce the number of inflammatory cells were consistently down-regulated, while the anti-apoptotic gene BCL2L1 with immune activating effects was consistently up-regulated. Genes that increase T-cell activation, IL12RB1, VAV3, JAG1, and CAMP, were also consistently up-regulated, while SNFT and SIT that decrease T-cell activation were down-regulated. The only pro-inflammatory molecule that was consistently down-regulated was CD40LG.

In the second functional category, down-regulation of IL2RA, SIT1 and CCR7, and up-regulation of IL12RB1, JAG1, VAV3 and AHNAK with roles T-cell activation indicated immune imbalance.

Within the atherosclerosis category, the anti-atherosclerotic genes, NR1H4, BIN1 and GSTT1, were consistently down-regulated, while the pro-atherosclerotic gene, MARCO, was consistently up-regulated and indicated ongoing atherogenesis.

In addition to genes known to contribute to coronary artery disease, we also identified consistently differentially regulated genes that have not been previously reported in association with MI including HDAC2, WNT-1, TGLN1, DACH1, HLA-DQA1 and SNCA.

Biologically relevant subset of differentially expressed EMI signature genes

Based on biological relevance and the greatest degree of differential expression in EMI patients we have identified 10 out of the 44 most consistently differentially expressed genes for subsequent analysis. A clustered heatmap and PCA analysis (Supplementary Figs. 3A and B) confirmed the ability of these 10 genes to classify the samples as either EMI or MCON. Of these genes we have validated the microarray-derived mean quantile normalized gene expression data (Fig. 3A) for PTX3, LGALS3, CAMP, IL12RB1, JAG1, IL2RA, CCR7, GSTT1, and NGFR using quantitative real time-PCR normalized to GAPDH (Fig. 3B).

Discussion

In this study of peripheral blood cell gene expression profiling in a prospectively matched cohort of Asian, African-American and Caucasian patients living in Hawaii, diagnosed with early myocardial infarction, and undergoing standard post-MI therapy, integrated functional analysis of the differentially expressed genes identified pro-inflammatory gene signatures. CD40LG that is actively involved in the pathophysiology of

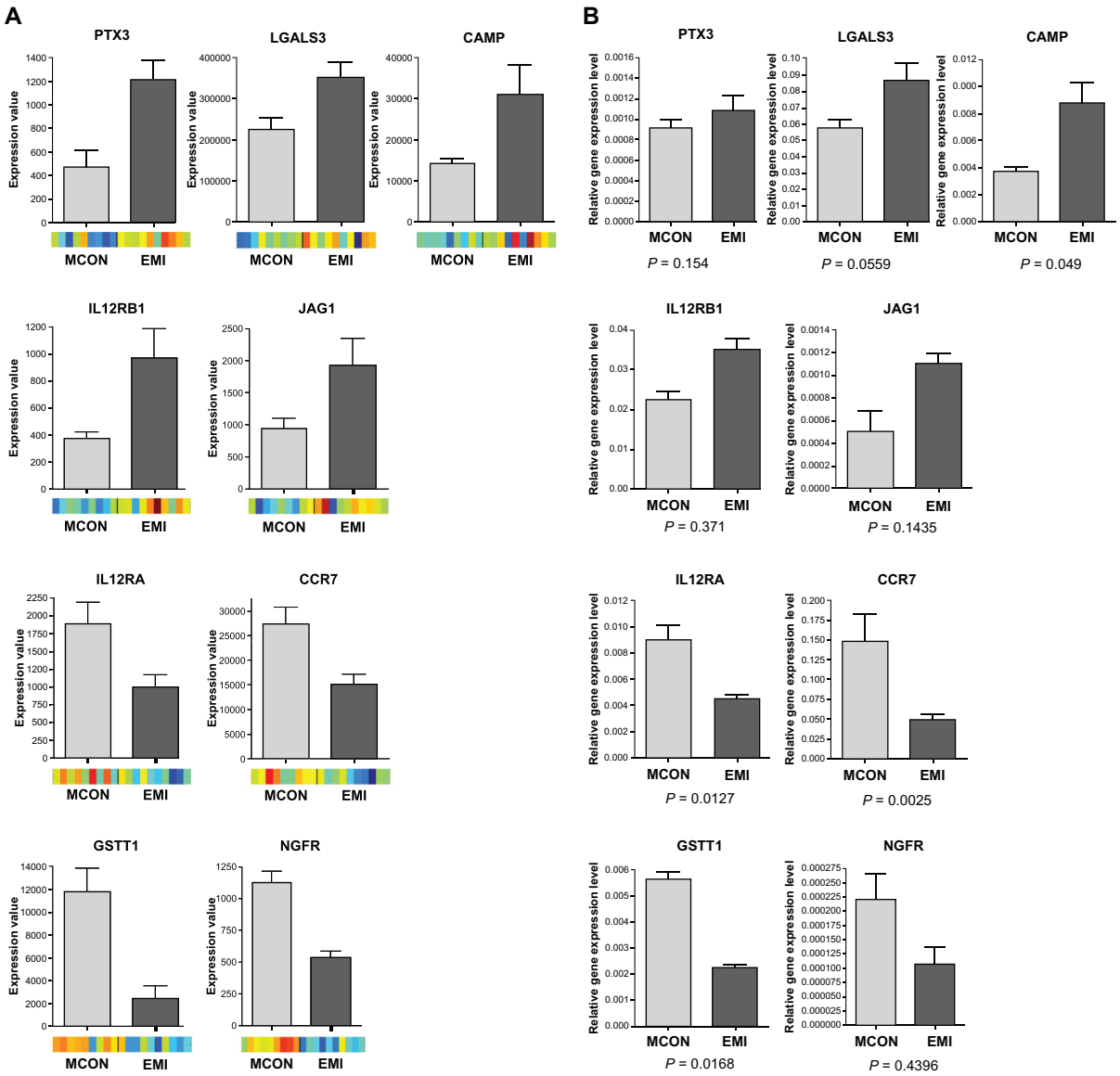

Figure 3. A) Average microarray expression levels (±standard error) and clustered heatmaps of signature genes. Blue hues represent down- and red up-regulation. **B**) Quantitative real-time PCR validation of the mRNA levels for the same genes. Expression values were calculated relative to GAPDH levels, assays were performed in triplicate and data are shown as mean ± standard error of the mean.

coronary artery syndromes was down-regulated in response to therapy.[19] However, prominent signatures for active inflammation involving FOS, DACH1, PTX3 and JAG1 were still present. Inflammatory functions were also represented by disregulated IL12RB1, LGALS3, CAMP, and BCL2L1, an immune imbalance pattern included IL-8 and the IL-2RA/IL-2 system, and additionally WNT1 disregulation indicated likely compromised cardiomyocyte survival. The hub gene HDAC2 revealed an overall stress response, and we also noted gene signatures consistent with cardiac hypertrophy and myopathy.

Functional analysis of the 44 most consistently differentially expressed genes further confirmed immune imbalance, coordinated differential regulation of inflammatory pathways and active atherosclerosis as the persisting main disease mechanisms in medicated EMI patients. Of these genes the pro-inflammatory LGALS3 and PTX3 and the T-cell activating IL12RB1, JAG1 and CAMP were consistently up-regulated, while the pro-apoptotic, anti-inflammatory NGFR, auto-immunity protective CCR7, IL2RA and the anti-atherosclerotic GSST1 consistently down-regulated. Altered peripheral blood expression of a similarly

small number[20] of genes have been shown to be a signature significantly associated with the severity of coronary artery disease[7] and predictive for the severity of atherosclerosis.[8] The novel, differentially expressed and early MI-associated genes that we have identified include HDAC2 an integrator of stress response pathways during heart remodeling, WNT1 with a known role in cardiomyocyte survival, TGLN1 (TAGLN) an actin-binding SM22/transgelin-related protein, DACH1 a cell fate determinant, SNCA linked to multiple system atrophy, and HLA-DQA1 a regulator of immune-specific response to anti-myocardium antibodies.

The short lists of differentially expressed genes in our multiethnic EMI cohort proved different from the signature genes reported for coronary artery disease in Caucasian cohorts.[7,8] Cardiovascular disease susceptibility and SNP frequencies in cardiovascular disease genes are known to vary among populations.[21] In a recent study, the biological processes of apoptosis, lipoprotein metabolism and immunity showed the greatest difference among Africans, Europeans and South Asians.[22] Significant differences in allele frequencies were noted for Toll-like receptors,[23] immune response involving IL-2, IL-6, and IL-10,[24,25] and apoptosis including BCL2L1, IL-4, and IL-6 among Blacks, Whites, and Asians.[22] In Japanese, Taiwanese[26] and Korean populations, elevated BRCA1-associated protein, BRAP, was shown to contribute to increased MI risk.[27] These observations are in agreement with the differential representation in our EMI cohort of a IL-1/Toll receptor family member, IL1RAPL1, numerous interleukins and IL-receptors, BCL2L1, BRCA1 and the immune response regulator HLA-DQA1 that was recently reported in association with cardiomyopathy in Chinese patients.

Chronic local and systemic inflammation and immune disregulation[28] play prominent roles in the pathogenesis of MI. However, given that our EMI patients have been under long-term anti-inflammatory and anti-atherosclerotic therapy, the robust representation of inflammation, immune imbalance, and active atherogenesis gene expression signatures was unanticipated. In a recent study of coronary artery stenosis patients with a comparable percentage of subjects receiving ASA, ACEIs, β-blockers and statins, differential blood expression pattern of 160 genes also revealed altered regulation of immunity, persistent inflammation and apoptosis.[8] The anti-atherosclerotic and anti-inflammatory statins are one of the most important medications in cardiovascular diseases that favorably affect platelet adhesion, endothelial function, plaque stability and cholesterol synthesis, and were thought to also inhibit TNF-α, IL-1 and IL-8.[29] However, emerging evidence revealed that statins did not have significant effect on markers of inflammation and platelet activation,[30] release of IL-1β and IL-6, and that actually increased the release of TNF-α and IL-8. Our data, particularly the robust representation of IL-8-associated inflammation in medicated EMI patients, support these observations.

Conclusion

Our results demonstrate that active pathogenesis including multiple inflammatory pathways, immune imbalance, stress response and atherogenesis persists in EMI patients in spite of concerted treatment efforts to protect these subjects from continued vascular pathology. Furthermore, while persistent inflammation, undetected by standard laboratory tests, appears to be the most significant ongoing pathomechanism in this cohort, ethnic differences exist among Asian, African American and Caucasian MI patients in the active inflammatory pathways. Further ethnic cohort-based studies are needed, therefore, to determine the critical ethnicity-specific pathology profiles, identify optimal candidate targets and develop effective treatment modalities for post-MI patients with a focus on inflammatory, immune, and atherogenic pathways that are unresponsive to current therapies.

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Disclosures

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published elsewhere. The authors and peer reviewers report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

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Supplementary Material

RNA purification and microarray hybridization

Total RNA was isolated from EMI and MCON peripheral blood samples using the Qiagen PAXgene RNA Kit (Valencia, CA). Globin mRNA was reduced using the GLOBINclear-Human Globin removal kit from Ambion (Austin, TX). RNA concentration was determined with a NanoDrop ND-1000 Spectrophotometer (Wilmington, DE) and RNA quality assessed using an Agilent Bioanalyzer 2100 (Santa Clara, CA). 1 µg of total RNA with a RIN (RNA Integrity Number) of ≥7 from each sample was processed using Applied Biosystems Chemiluminescent RT-IVT Labeling Kit V 2.0 (Foster City, CA) to generate digoxigenin (DIG, Roche, Indianapolis, IN)-labeled cRNA for microarray hybridizations. Double-stranded cDNA was synthesized from RNA template and in vitro-transcribed resulting in approximately 100-fold amplified DIG-labeled cRNA. 10 µg of cRNA for each sample were hybridized onto Applied Biosystems Human Survey Genome Microarray 2.0 (Foster City, CA) that contains 32,878 60-mer oligonucleotide probes to represent a set of 28,098 human genes and more than 1000 control probes.

Table S1. Clinical characteristics of early Myocardial Infarction (EMI) and medicated control (MCON) cohorts. *P*-values were calculated from the t-test for continuous variables and the McNemar's test for categorical covariates.

	EMI (n = 10)	MCON (n = 10)	*P*-value
Age, average ± SD, y	51.5 ± 5.79	51.5 ± 5.79	1.0000
Ethnicity, %			
Black	20	20	1.0000
Asian	40	40	1.0000
Caucasian	40	40	1.0000
Weight ± SD, kg	89.5 ± 13.0	94.6 ± 15.1	0.1873
Body mass Index ± SD	28.7 ± 4.6	29.7 ± 3.5	0.7103
Hypertension, %	50	70	0.6831
Diabetes mellitus, %	30	20	1.0000
Hyperlipidemia, %	80	70	1.0000
Smoking status, %	20	10	1.0000
Family history, %	10	10	0.4795

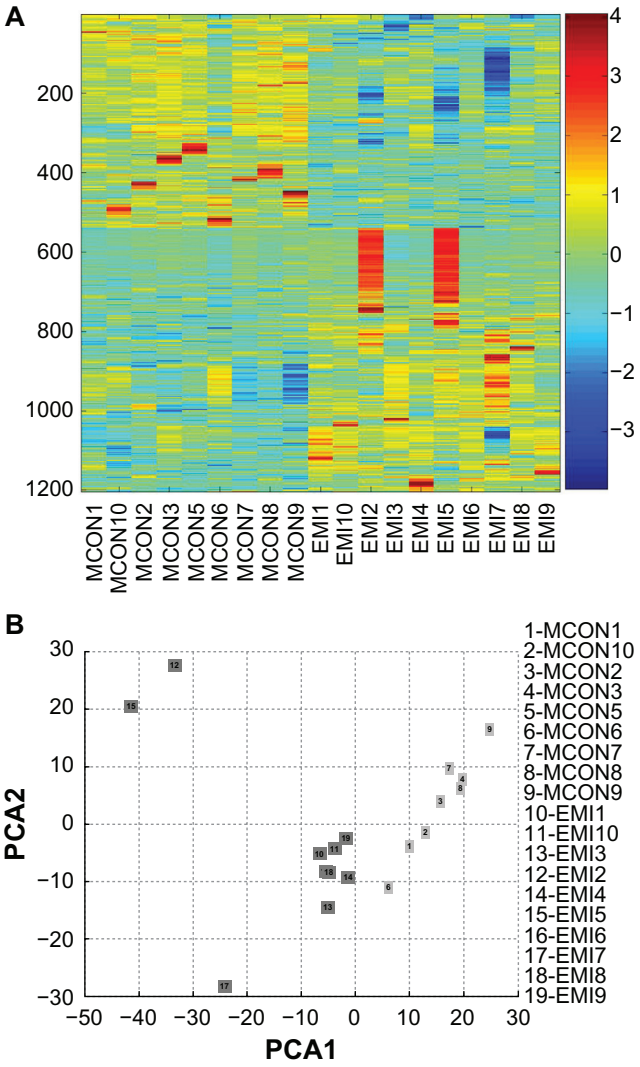

Figure S1. A) Clustered heatmap of all differentially expressed genes in EMI compared to MCON samples. Genes are on the vertical axis and each column correlates with a specific subject. **B**) Principal component analysis (PCA) of all differentially expressed genes separates the EMI and MCON groups.

Table S2. PANTHER ontological analysis of differentially expressed genes.

	*REFLIST (25909)	All genes (965)				Up regulated genes (456)				Down regulated genes (509)			
		#	exp.		*P*-value	#	exp.		*P*-value	#	exp.		*P*-value
Biological process													
Immunity and defense	1393	90	51.88	+	1.40E–05	34	24.52	+	1.00E+00	56	27.37	+	1.39E–05
Interferon-mediated immunity	62	9	2.31	+	9.37E–02	1	1.09	–	1.00E+00	8	1.22	+	5.72E–03
Cytokine/chemokine mediated immunity	113	11	4.21	+	5.86E–01	7	1.99	+	6.23E–01	4	2.22	+	1.00E+00
B-cell- and antibody-mediated immunity	148	13	5.51	+	6.39E–01	3	2.6	+	1.00E+00	10	2.91	+	1.21E–01
Nitrogen metabolism	30	5	1.12	+	1.79E–01	5	0.53	+	6.73E–03	0	0.59	–	1.00E+00
Nitrogen utilization	5	3	0.19	+	1.35E–01	3	0.09	+	1.53E–02	0	0.1	–	1.00E+00
Protein metabolism and modification	3063	107	114.08	–	1.00E+00	49	53.91	–	1.00E+00	58	60.17	–	1.00E+00
Protein biosynthesis	692	34	25.77	+	1.00E+00	8	12.18	–	1.00E+00	26	13.59	+	2.20E–01
Small molecule transport	136	9	5.07	+	1.00E+00	8	2.39	+	4.62E–01	1	2.67	–	1.00E+00
Receptor mediated endocytosis	108	9	4.02	+	1.00E+00	8	1.9	+	1.52E–01	1	2.12	–	1.00E+00
Signal transduction	3259	138	121.38	+	1.00E+00	84	57.36		7.04E–03	54	64.03	–	1.00E+00
Molecular function													
Defense/immunity protein	467	30	17.39	+	9.99E–02	7	8.22	–	1.00E+00	23	9.17	+	2.12E–03
Immunoglobulin	70	6	2.61	+	1.00E+00	0	1.23	–	1.00E+00	6	1.38	+	4.65E–01
Nucleic acid binding	2897	114	107.9	+	1.00E+00	42	50.99	–	1.00E+00	72	56.91	+	6.60E–01
Translation elongation factor	37	6	1.38	+	5.88E–01	6	0.65	+	1.18E–02	0	0.73	–	1.00E+00
Ribosomal protein	579	35	21.57	+	6.95E–01	9	10.19	–	1.00E+00	26	11.37	+	1.80E–02
Growth factor	115	10	4.28	+	1.00E+00	8	2.02	+	1.84E–01	2	2.26	–	1.00E+00
Transfer/carrier protein	329	14	12.25	+	1.00E+00	12	5.79	+	4.39E–01	2	6.46	–	1.00E+00

Notes: Categories in italics are sub-categories of the parent-categories. Yellow highlight denotes processes or functions that contain significant number of differentially expressed genes. *Reference list containing all genes on the ABI microarray. + denotes overrepresentation; – underrepresentation; # number of genes detected. Bonferroni correction was used for multiple comparisons.

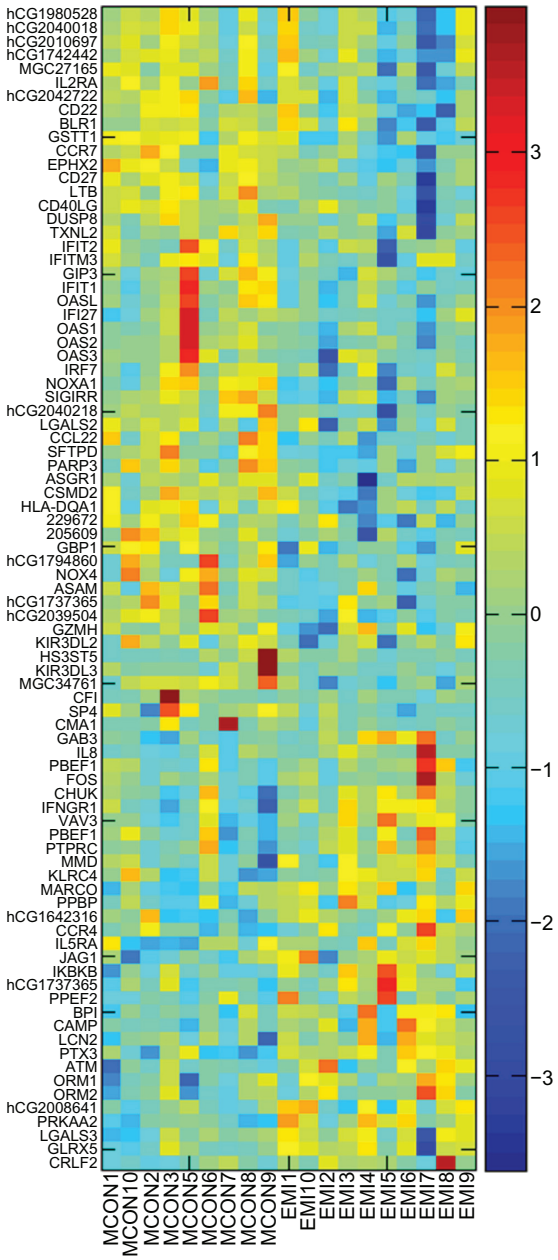

Figure S2. Clustered heatmap of genes from the "Immunity and defense" molecular function category identified by PANTHER. The color of each square represents the expression value of a gene according to the scale of the color bar on the right.

Table S3. The most significant (top) functions of the first four networks identified by Ingenuity Pathway Analysis (IPA). Score is a ranking algorithm of IPA.

#	Top functions	Score	Focus molecules
1	Cell Death, **Immunological Disease**, **Immune Response**	46	30
2	Cell Morphology, Cellular Response to Therapeutics, Connective Tissue Development and Function	37	26
3	Cellular Function and Maintenance, Hematological System Development and Function, **Immune and Lymphatic System Development and Function**	37	26
4	Gene Expression, Cellular Assembly and Organization, Cellular Movement	37	26

Table S4.

Gene symbol	References for MI-associated function
AHNAK	Matza et al. *Proc Natl Acad Sci U S A.* 2009;106:9785–90.
BIN1	Muller et al. *Nat Med.* 2005;11:312–9.
BCL2L1	Yang et al. 2007;44(6):483–94.
CAMP	Zanetti et al. *J Leukoc Biol.* 2004;75:39–48.
CCRC7	Worbs T, et al. *Trends Immunol.* 2007;28:274–80.
CD40LG	Lutgens et al. *Trends Cardiovasc Med.* 2007;17:118–23.
DACH1	Zhou et al. *Proc Natl Acad Sci U S A.* 2010;107:6864–9.
FOS	Hikasa et al. *Biochem Biophys Res Comm.* 2003;304:143–7.
FREQ (NCS-1)	Kapp-Barnea et al. *Mol Biol Cell. 2006; PMID: 16837555.*
GSTT1	Wang et al. *Mutagenesis*. 2010;25(4):365–9.
HDAC2	Trivedi et al. *Nat Med.* 2007;13:324–31.
HLA-DQA1	Liu et al. *Ann Hum Genet.* 2005;69:382–8.
IL1RAPL1	Bahi et al. *Hum Mol Genet*. 2003;PMID: 12783849.
IL-2RA	Shevach et al. *Nat Rev Immunol.* 2002;2:389–400.
IL-8	Baggiolini et al. *J Clin Invest.* 1989;84:1045–9.
IL12RB1	Trinchieri et al. *Nat Rev Immunol.* 2003;3:133–46. Guo et al. *Infect Immun.* 2009;77:3909–18.
JAG-1	Guidos et al. *J Exp Med.* 2006;203:2233–7.
LGALS3	Rabinovich et al. *Trends Immunol.* 2002;23:313–20.
MARCO	Bowdis and Gordon, *Immunol Rev*. 2009;227(1):19–31.
NGFR	Anderson et al. *Acta Physiol (Oxf)*. 2006;186(1):17–27.
NR1H4	Marzolini et al. *Mol Endocrinol*. 2007;21(8):1769–80.
NOTCH-1	Koyanagi et al. *Circ Res.* 2007;01:1139–45. Yuan et al. *Annu Rev Immunol*. 2010;28:343–65.
NOXA1	Niu et al. *Circulation*. 2010;121(4):549–59.
PTX3	Latini et al. *Circulation.* 2004;110:2349–54. Norata et al. *Trends Cardiovasc Med.* 2010;20:35–40.
ROCK1	Shi et al. *J Mol Cell Cardiol*. 2010;49(5):819–28.
SIT	Marie-Cardine et al. *J Exp Med*. 1999;189(8):1181–94.
SNCA	Al-Chalabi et al. *PLoS One.* 2009;4:e7114.
SNFT	Hildner K, *Science*. 2008 Nov 14;322(5904):1097–100.
SOX6	Cohen-Barak et al. *Nucleic Acids Res.* 2003;31:5941–8. Hagiwara et al. *N Proc Natl Acad Sci U S A.* 2000; 97:4180–5.
TGLN1	Winder et al. *Biochem J*. 2003;375:287–95.
TRIO	Debant et al. *Proc Natl Acad Sci U S A*. 1996;93:5466–71.
VAV3	Tybulewicz et al. *J Intern Med.* 2010;268:50–8.
VEGFB	Sobti et al. *Mol Cell Biochem.* 2010;341:139–48.
WNT1	Venkatesan et al. *J Med Chem.* 2010;53:2636–45.
WNT5A	Fujio et al. *FEBS Lett.* 2004;573:202–6. Pereira et al. *Curr Atheroscler Rep.* 2009;11:236–42.
ZBTB32 (PLZP)	Piazza et al. *Mol Cell Biol.* 2004;24:10456–69.

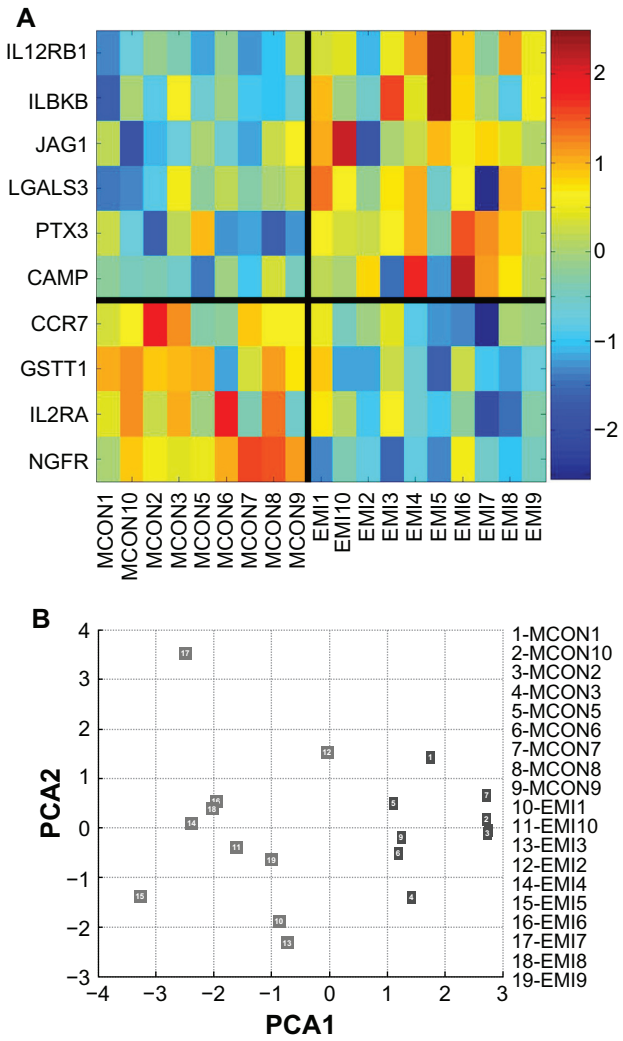

Figure S3. A) Clustered heatmap of selected genes with most significantly different expression. **B**) Principle component analysis demonstrates a clear separation of EMI and MCON samples.