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Upregulated *ex vivo* expression of stress-responsive inflammatory pathway genes by LPS-challenged CD14+ monocytes in frail older adults

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Abstract

Frailty has been increasingly recognized as an important clinical syndrome in old age. The frailty syndrome is characterized by chronic inflammation, decreased functional and physiologic reserve, and increased vulnerability to stressors, leading to disability and mortality. However, molecular mechanisms that contribute to inflammation activation and regulation in frail older adults have not been investigated. To begin to address this, we conducted a pathway-specific gene array analysis of 367 inflammatory pathway genes by lipopolysaccharide (LPS)-challenged $CD14^+$ monocytes from 32 community-dwelling frail and age-, race-, and sex-paired nonfrail older adults (mean age 83 years, range 72–94). The results showed that *ex vivo* LPS-challenge induced average 2.0-fold or higher upregulated expression of 116 genes in frail participants and 85 genes in paired nonfrail controls. In addition, frail participants had 2-fold or higher upregulation in LPS-induced expression of 7 stress-responsive genes than nonfrail controls with validation by quantitative real time RT-PCR. These findings suggest upregulated expression of specific stress-responsive genes in monocyte-mediated inflammatory pathway in the syndrome of frailty with potential mechanistic and interventional implications.

Keywords

Frailty; Monocyte-mediated inflammatory; pathways; Gene expression; Stress-responsive genes; Geriatrics; Aging

1. Introduction

The worldwide rapid expansion of the aging population and growing care needs for frail older patients demand better understanding of the pathogenesis of frailty in old age. As such, frailty has been increasingly recognized as an important clinical syndrome in older adults (Fried and

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Walston, 2007; Fried et al., 2005). It is characterized by decreased functional and physiologic reserve and increased vulnerability to stressors, leading to serious adverse health outcomes including disability, dependency, and mortality (Bandeen-Roche et al., 2006; Fried and Walston, 2007; Fried et al., 2001, 2004, 2005; Lipsitz, 2002). The estimated prevalence of this syndrome is 7–10% among community-dwelling adults aged 65 and older, and up to 25–40% of those aged 80 years and above (Fried et al., 2001, 2004). Substantial evidence suggests that chronic inflammation is a cardinal pathophysiologic feature and likely involved in the pathogenesis of this syndrome (Fried et al., 2005; Leng et al., 2002, 2007; Walston et al., 2002). For example, frailty is associated with increased interleukin-6 (IL-6) and C-reactive protein (CRP) levels and white blood cell (WBC) counts (Leng et al., 2002, 2007; Walston et al., 2002). In addition, frail older adults had higher lipopolysaccharide (LPS)-stimulated IL-6 production by peripheral mononuclear cells than matched nonfrail controls (Leng et al., 2004a). Furthermore, it has been shown that IL-6 levels have inverse associations with hemoglobin and insulin-like growth factor (IGF)-1 levels in frail older adults; low hemoglobin and IGF-1 levels are each independently associated with frailty, as well (Cappola et al., 2003; Chaves et al., 2005; Leng et al., 2002, 2004b). These findings suggest that inflammation may contribute directly or, through other patho-physiological processes, to frailty (Cappola et al., 2003; Chaves et al., 2005; Leng et al., 2002, 2004a,b, 2007; Walston et al., 2002). However, molecular mechanisms that underlie inflammation activation and regulation in frail older adults have not been investigated.

Monocytes play a pivotal role in inflammation activation and regulation. They are the major cell type in the circulation responding to lipopolysaccharide, an endotoxin from Gram(−) bacterial infections most commonly experienced by older adults (Fried and Walston, 2007; Fried et al., 2005). LPS-activated monocytes produce a plethora of biomediators, leading to systemic inflammation including sepsis (Hotchkiss and Karl, 2003). The purpose of this study was to evaluate expression of inflammatory pathway genes by $CD14^+$ monocytes isolated from frail older adults and challenged *ex vivo* with LPS. We hypothesized that frail older adults would have upregulated monocytic expression of stress-responsive inflammatory pathway genes compared to their nonfrail counterparts. To test this hypothesis, we conducted a gene expression analysis utilizing inflammatory pathway-specific gene array with confirmation by quantitative real time RT-PCR testing of identified genes in community-dwelling frail and age-, race-, and sex-paired nonfrail older adults.

2. Materials and methods

2.1. Human subjects

Community-dwelling older adults were recruited from outpatient medical clinics, senior centers, and residential retirement communities in Baltimore, MD. The validated and widely utilized 5-item frailty criteria were employed for screening, which include exhaustion, slowed performance (by walking speed), weakness (by grip strength), weight loss, and low physical activity (Fried et al., 2001). Individuals with a critical mass of three or more of the five components were defined as frail, those with one or two components as prefrail, and those with none of the five components as nonfrail. Exclusion criteria included Parkinson's disease, stroke with residual hemiparesis, symptomatic congestive heart failure, malignancy, uncompensated endocrine disorders, rheumatoid arthritis or any other inflammatory conditions, or use of immune modulating drugs including oral steroids. Persons with significant cognitive deficit (Folstein mini-mental status exam score below 18/30) were also excluded because this study was focused on physical frailty (based on the frailty criteria described above) rather than cognitive dysfunction. In addition, most of these individuals had difficulties in providing informed consent. The Johns Hopkins Institutional Review Board approved the study protocol. Written informed consent was obtained from all participants.

2.2. Monocyte preparation, LPS-challenge, and RNA isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh peripheral blood samples by centrifugation over Ficoll–Hypaque density gradient (specific density, 1.077 g/ml) for 10 min at 600 × *g* at room temperature. PBMCs were washed three times with PBS containing 2 mM EDTA and 0.5% bovine albumin (pH 7.4) and $CD14^+$ monocytes were purified using a MACS monocyte isolation kit (Miltenyi Biotec, Auburn, CA), according to the manufacturer's instructions. This was followed by an additional 1 h-incubation in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Gaithersberg, MD) at 37 °C in a humidified 5% CO_2 incubator, after which non-adherent cells were removed by repeated rinsing with serum-free RPMI 1640. The purity of monocyte preparations was assessed by flow cytometry using phycoerythrin (PE)-conjugated anti-CD14 monoclonal antibody (Becton Dickinson, San Jose, CA).

Freshly isolated $CD14^+$ monocytes at the density of 1×10^6 cells/ml were incubated in the absence or presence of LPS (Sigma Chemical Co, St. Louis, MO) at the concentration of 1.0 μg/ml in RPMI 1640 medium containing 10% FBS, 100 μg/ml streptomycin, and 100 U/ml penicillin at 37 °C and 5% CO_2 for 3 h. The dose and duration of *ex vivo* LPS-challenge was selected based on previous gene profiling studies of human monocytes (Hashimoto et al., 1999; Suzuki et al., 2000; Williams et al., 2002). Total RNA was extracted from unchallenged and LPS-challenged monocytes using Trizol (Invitrogen/Life Technologies, Carlsbad, CA) following the manufacturer's instructions. The concentration and quality of purified RNA samples were routinely determined by spectrophotometer and electrophoresis.

To minimize potential laboratory variability, blood samples from pairs of frail and nonfrail participants were collected in the same morning and subsequent experiments were performed in parallel by the same investigator using the same reagents and equipment. All reagents and plastic wares were endotoxin-free except for LPS preparation itself.

2.3. Inflammatory pathway-specific gene array analysis

Commercially available inflammatory pathway-specific gene array GEArray (SuperArray Bioscience Corp, Frederick, MD) was employed as previously described (Chapoval et al., 2001; Sharif et al., 2001). The array membrane contains specific oligonucleotide sequence of 367 inflammatory pathway genes and control sequences [PUC18 as negative control; β-actin and glyceraldehydes 3 phosphate dehydrogenase (GAPDH) for loading] spotted in triplets. GEArray analyses were performed according to manufacturer's protocols. Briefly, 3 μg of purified total RNA were applied for the synthesis of cDNA probes using GEArray TrueLabeling-Reverse Transcription kit (TL-RT, SuperArray Bioscience Corp.) and labeled with [α-^{32}P]-dCTP (Amersham Pharmacia BioTech, Piscataway, NJ). The ^{32}P-labeled cDNA probes were hybridized under precisely specified conditions to the array membrane. After extensive washing to minimize background noise, specific array signals were quantified by exposure to PhosphorImager screens for 3 h, recorded onto a Molecular Dynamics PhosphoImager (Amersham Biosciences Corp., Piscataway, NJ) using the manufacturer's ImageQuant program, and analyzed using GEArray Analyzer software with appropriate background subtraction and data normalization following manufacturer's instructions. The corrected and normalized signals were used to determine and compare the relative abundance of specific gene transcripts of monocyte samples between frail and nonfrail participants in pairs.

2.4. Quantitative real time RT-PCR (QPCR)

cDNAs were synthesized from purified total RNA (2 μg) samples in 20 μl reactions using random primers (Promega, Madison, WI) and Omniscript® RT kit (Qiagen, Valencia, CA) following the manufacturer's instructions. QPCR experiments were performed using the

Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA). Forward and reverse primers for candidate genes identified in GEArray analysis were purchased from SuperArray Bioscience Corp. (Frederick, MD). Each QPCR experimental set was performed using a Brilliant SYBR Green QPCR Master Mix buffer (Stratagene) containing SYBR Green I dye, SureStart Taq DNA polymerase, and a reference dye with the thermal cycling program of enzyme activation for 10 min at 95 °C and 40 cycles of 30 s denaturation at 95 °C, 45 s annealing at 60 °C, and 30 s extension at 72 °C.

2.5. Data analysis

Demographic and clinical characteristics of the study participants are presented as mean + S.D. or percentages, where appropriate. Two-sided *t* tests were employed to determine the statistical significance of a difference between the frail and nonfrail study group means (*P* values). For GEArray and QPCR data, LPS-induced upregulation in individual participants is presented as stimulated-over-unstimulated expression ratios. Differential expression between frail and nonfrail participants in pairs is presented as frail-over-nonfrail expression ratios. An expression ratio of 2.0-fold or higher is considered as a significant upregulation.

3. Results

3.1. Characteristics of the study participants

Sixty community-dwelling older adults were screened with frail to nonfrail ratio roughly 1:3, which is consistent with the prevalence of frailty previously reported (Fried et al., 2004). A total of 32, or 16 pairs, of frail and age-, race-, and sex-paired nonfrail older adults were included in and completed the study. Table 1 summarizes basic demographic and clinical characteristics of the study participants. The mean age was 83 years (range 72–94) and the majority participants were white and females. The frail and nonfrail participants had similar body mass index. They also had comparable disease profile and medication use. None of the participants was current or recent (within past 10 years) smoker, or reported illicit drug use or heavy alcohol consumption.

3.2. Inflammatory pathway-specific gene array results

As assessed by flow cytometric analysis with phycoerythrin -conjugated anti-CD14 monoclonal antibody, the purity of monocyte preparations was 98.8 ± 0.5%. LPS-challenge induced average 2.0-fold or higher upregulated expression (stimulated-over-unstimulated expression ratios) of 116 genes in frail participants and 85 genes in nonfrail controls by GEArray analysis. These genes are within several broad categories including transcription factors, signal transduction proteins, cytokines, chemokines, and cell surface receptors (data not shown). The average frail-over-nonfrail expression ratios of 12 genes by GEArray analysis are summarized in Table 2, column A. These genes consistently had average frail-over-nonfrail LPS-challenged expression ratios of 2.0 or higher in at least 12 out of the 16 study pairs (75%) examined. They include (i) *transcription factors*: hydrogen peroxide (H_2O_2)-induced clone 5 (*Hic*-5), glucocorticoid receptor DNA-binding factor 1 (GRLF1), and fas-associated via death domain (FADD); ii) *signal transduction proteins*: mitogen-activated protein kinase 10 (MAPK10) and mitogen-activated protein kinase 7 (MAP2K7); iii) *chemokines and receptors*: CXC chemokine ligand 10 (CXCL10), chemokine (C motif) ligand 1 (XCL1), vascular cell adhesion molecule 1 (VCAM-1), and CC chemokine receptor 10 (CCR10); (iv) *cytokines*: tumor growth factor β (TGF-β), lymphotoxin α (LTA), and IL-11.

3.3. Validation by quantitative real time RT-PCR

Validating results from QPCR experiments are shown in Table 2, column B. Among the 12 candidate genes identified by GEArray, 7 genes encoding transcription factors (*Hic*-5), signal

transduction proteins (MAPK10 and MAP2K7), chemokines (CXCL10 and XCL1), and cytokines (TGF-β and LTA) had 2.0 or higher frail-over-nonfrail expression ratios. Three genes including GRLF-1, FADD, VCAM-1, did not show consistent 2.0 frail-over-nonfrail ratios. Expression of CCR10 and IL-11 was undetectable in at least one or more paired QPCR experiments.

Fig. 1 illustrates the typical QPCR product curves of monocytic expression of *Hic*-5 in a pair of frail and nonfrail participants (panel A), demonstrating upregulation in its LPS-challenged expression in frail participant [frail, LPS (+)] compared to that in the paired nonfrail control [nonfrail, LPS (+)]. In the absence of LPS-challenge, no significant difference was observed between frail and nonfrail subjects [frail, LPS (−) *vs* nonfrail, LPS (−)]. Monocytic expression of GAPDH, a house-keeping gene, did not show any significant difference among its product curves under all four experimental conditions (panel B).

4. Discussion

This exploratory study has observed, for the first time, upregulated *ex vivo* expression of 7 stress-responsive inflammatory pathway genes by LPS-challenged $CD14^+$ monocytes from community-dwelling frail older adults compared with that from paired nonfrail controls. With careful control of major demographic variables and comparable clinical profiles in the paired subjects, the observed differential gene expression is less likely influenced by these variables or common clinical conditions rather than frailty.

Consistent with previous studies that have reported increased monocytic activation in older adults (De et al., 2004; Ono et al., 2001; Sadeghi et al., 1999), our GEArray analysis showed upregulation in *ex vivo* LPS-challenged monocytic expression of significant number of inflammatory pathway genes, more in frail participants than in nonfrail controls. In addition, 7 genes with upregulated expression in frail older adults are stress-responsive genes that are known to play an important role in stress responses in various study settings (Barcellos-Hoff, 2005; Jia et al., 2001; Li et al., 2000; Sasaki et al., 2001; Viswanathan and Dhabhar, 2005). This is consistent with the cardinal feature of frailty that frail elderly manifest increased vulnerability to stressors. Clinically, frail older adults may experience LPS exposure surge(s) during Gram (−) bacterial infections, such as urinary tract infection and urosepsis.

Hic-5 is an important transcription factor and co-activator for androgen and glucocorticoid receptors (Heitzer and DeFranco, 2006; Jia et al., 2001). Studies have demonstrated its role in oxidative stress, cellular senescence, and skeletal muscle atrophy (Hu et al., 1999; Shibanuma et al., 1994; Zdanov et al., 2006), potentially important biological processes in aging and frailty. Data from the present study suggest *Hic*-5 upregulation as a potential link between inflammation and these biological processes in the syndrome of frailty. Two genes encode signal transduction proteins MAPK10 and MAP2K7, suggesting that upregulated gene expression is related to early events in monocyte activation and its mediated inflammatory pathways in frailty. Alternatively, this could be explained by the brief LPS-challenge that might not be able to detect frailty-associated differential expression of late responsive genes, such as IL-6 (Leng et al., 2004a,b). Chemokines play a critical role in regulating leukocyte recruitment, trafficking, and activation in inflammation (Charo and Ransohoff, 2006). CXCL10 has been implicated in multiple sclerosis and several other inflammatory conditions (Charo and Ransohoff, 2006; Gerard and Rollins, 2001; Lazzeri and Romagnani, 2005; Sorensen et al., 2001). LTA, a TNF superfamily cytokine with specific binding to TNF receptors, activates NF-kB and promotes chemokine production (Gramaglia et al., 1999; Gray et al., 1984). Therefore, upregulated expression of CXCL10, XCL-1, and LTA can potentially promote inflammation activation and further perpetuate chronic inflammation in frail older adults. The observed upregulation in the expression of TGF-β, a potent anti-inflammatory cytokine, is

intriguing and may suggest a built-in regulatory mechanism in the effort of controlling inflammation cascade in the early stage of monocyte-mediated inflammatory pathways in frailty (Lawrence et al., 2002).

The reason for significant results from gene array analysis of five genes (GRLF-1, FADD, VCAM-1, CCR10, and IL-11) not being confirmed by QPCR is unknown. It may represent different sensitivity and specificity of the two methods, and thus, further emphasize the importance of array results to be confirmed by another independent method.

The strengths of this study include that this was a hypothesis-driven study with a clear focus on inflammatory pathways. We chose pathway-specific gene array over microarray because of the complex nature of the frailty syndrome, numerous potential confounding factors in older adults, and genes and expressed sequence tags (ESTs) with unknown physiologic function identified in previous microarray and serial analysis of gene expression (SAGE) analysis in young volunteers (Suzuki et al., 2000; Williams et al., 2002), which would make the interpretation of microarray data difficult in this setting.

This study has three limitations. First, no frailty-associated downregulation in LPS-challenged monocytic expression of inflammatory pathway genes was observed in this study. This could be due to LPS stress dose being “super physiologic”, as it may mask or negate any downregulation under physiologic conditions. In addition, relatively small sample size and stringent consistency requirement could limit our ability to detect less robust differential gene expression, upregulation and downregulation alike. However, since TGF-β plays a major role in down-regulating inflammation, its elevated expression might suggest a down-regulatory mechanism in frailty. Secondly, monocyte-mediated inflammatory pathways represent only part of the cellular and molecular mechanisms that can potentially contribute to chronic inflammation in frail older adults. For example, other cell types including adipocytes, endothelial cells, and fibroblasts can produce IL-6 and contribute to elevated IL-6 levels in frail older adults (Maggio et al., 2006). In addition, we have recently reported increased frequency of T cells expressing CC chemokine receptor 5 (CCR5), a type-1 pro-inflammatory T cell phenotype, in frail older adults (De et al., 2008)). Therefore, findings from this study should be interpreted in the context of the complexity of the biological mechanisms that regulate inflammation activation and resolution. Thirdly, results from this study cannot establish a causal role of these upregulated stress-responsive inflammatory pathway genes in the pathogenesis of frailty. Alternative explanations including that upregulation of innate immunity in frail older individuals as a compensatory response or an epiphenomenon should also be considered. In this regard, further longitudinal and/or interventional studies will be required. Despite these limitations, findings from this study do support our original hypothesis and advance our understanding of the molecular mechanisms that contribute to the activation and regulation of monocyte-mediated inflammatory pathways in frail older adults. They also provide a basis for further investigation into the expression and regulation of the specific genes identified in frailty. For example, further investigations into the expression and regulation of CXCL10 and *Hic*-5 are currently underway in our laboratory. Recent studies have suggested CXCL10 and MAP kinase inhibitor-based strategies as promising treatment modalities for multiple sclerosis, rheumatoid arthritis, and other inflammatory conditions (Maggio et al., 2006; Sweeney and Firestein, 2006). Therefore, findings from this study, if further confirmed, may have significant clinical implications in future development of novel interventional strategies for frailty in older adults.

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Abbreviations

***Hic*-5**
hydrogen peroxide-induced clone 5

GRLF1
glucocorticoid receptor DNA-binding factor 1

FADD
fas-associated via death domain

MAPK10
mitogen-activated protein kinase 10

MAP2K7
mitogen-activated protein kinase 2K7

CXCL10
CXC chemokine ligand 10

VCAM-1
vascular cell adhesion molecule 1

XCL1
chemokine (C motif) ligand 1

CCR10
CC chemokine receptor 10

TGF-β
transforming growth factor β

LTA
lymphotoxin α

IL-11
interleukin-11

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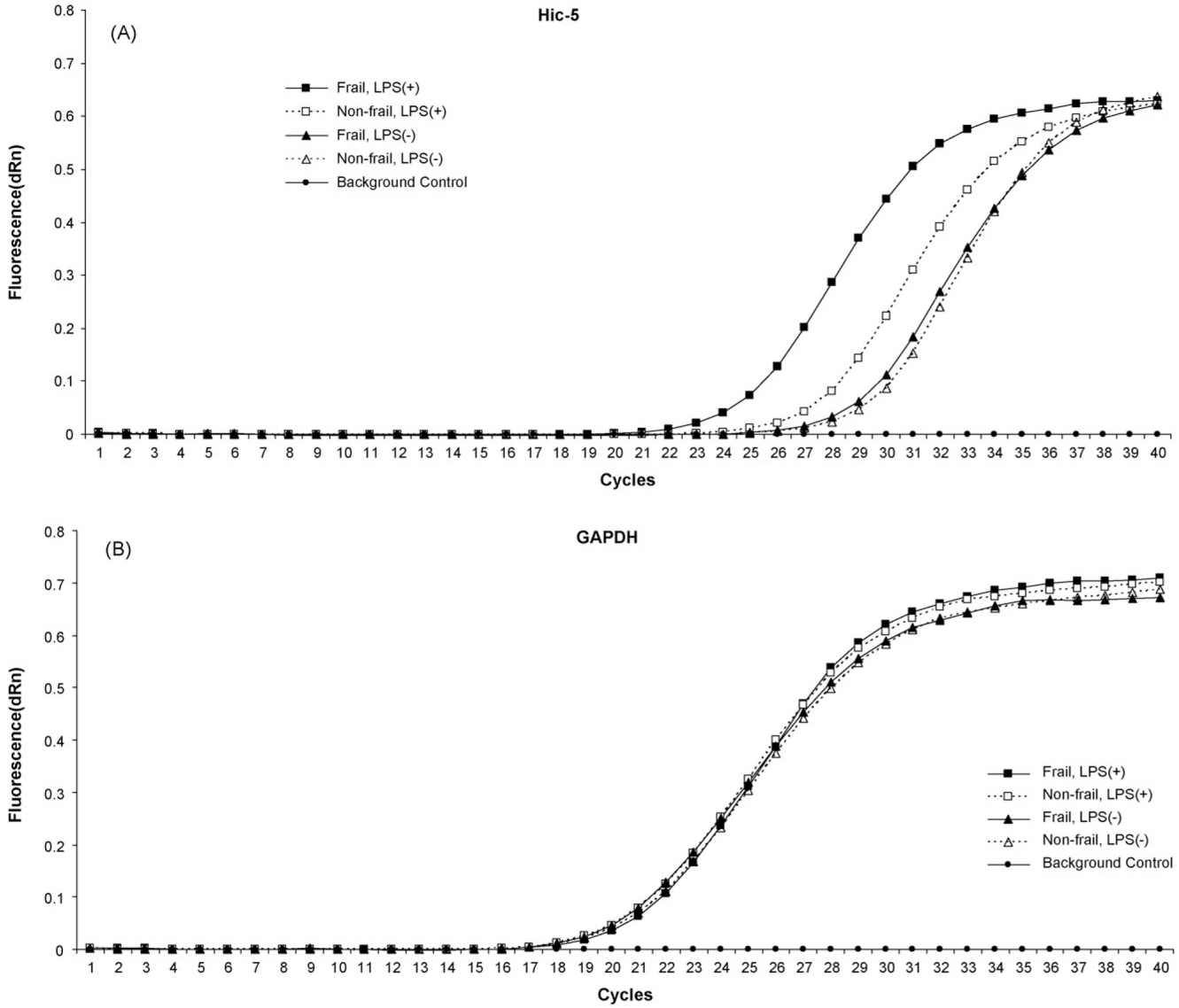

Fig. 1.

Representative QPCR experiments demonstrating differential expression of *hic*-5 by $CD14^+$ monocytes *ex vivo* under four conditions (panel A): frail, LPS (+)—monocytes from a frail participant with LPS-challenge; nonfrail, LPS (+)—monocytes from the paired nonfrail control with LPS-challenge; frail, LPS (−) and nonfrail, LPS (−)—monocytes from frail or paired nonfrail participant without LPS-challenge, respectively. Expression of glyceraldehyde 3 phosphate dehydrogenase (GAPDH), a house-keeping gene, under these conditions is shown in panel B as the control.

Table 1

Demographic and clinical characteristics of the study participants.

Characteristics	Frail (*N* = 16)	Nonfrail (*N* = 16)	P[a]
Age (years)	83 ± 5 (range 72–94)		
Race (% white)	93.7%		
Sex (% female)	87.5%		
Body mass index (kg/m^2)	26.5 ± 5.5	27.1 ± 4.6	.73
Comorbid chronic conditions			
Total # of diagnosis	4.1 ± 1.7	4.0 ± 1.7	.92
Specific diseases			
Hypertension	50.0%	81.3%	
Osteoporosis	43.8%	37.5%	
Osteoarthritis	25.0%	31.3%	
Hypercholesterolemia	43.8%	18.8%	
Congestive heart failure (controlled)	37.5%	25.0%	
Hypothyroidism (compensated)	31.3%	31.3%	
Coronary heart disease (asymptomatic)	18.8%	25.0%	
Malignancy (reported cured)	12.5%	37.5%	
Medication usage			
Total # of medications	4.3 ± 1.9	3.8 ± 1.7	.39
Specific medications:			
Aspirin	37.5%	31.3%	
β-Blockers	43.8%	31.3%	
Diuretics	37.5%	43.8%	
Calcium-channel blockers	25.0%	50.0%	
ACE inhibitors	31.3%	31.3%	
HMG-CoA reductase inhibitors	31.3%	25.0%	
Thyroid (T_4) supplement	31.3%	31.3%	

[a] *P* values were determined by two-sided *t* test between the two means.

Table 2

LPS-challenged *ex vivo* expression of specific genes with average frail-over-nonfrail ratio of 2-fold or higher by $CD14^+$ monocytes from 16 study pairs.

Genes	A. GEArray[a] (mean ± S.D.)	B. QPCR (mean ± S.D.)
Transcription factors		
Hic-5[b]	**5.4 ± 2.1**	3.4 ± 1.2
GRLF1	26.7 ± 21.9	1.6 ± 1.5
FADD	7.9 ± 4.8	1.7 ± 1.0
Signal transduction proteins		
MAPK10	**4.6 ± 1.9**	3.4 ± 1.3
MAP2K7	**2.9 ± 0.9**	4.4 ± 2.2
Chemokines & receptors		
CXCL10	**3.8 ± 0.8**	4.2 ± 1.1
XCL1	**12.6 ± 7.5**	2.8 ± 0.7
VCAM-1	10.5 ± 6.4	1.8 ± 1.7
CCR10	4.3 ± 1.8	U.D[c]
Cytokines		
TGF-β	**2.9 ± 0.6**	3.1 ± 0.9
LTA	**4.5 ± 2.4**	4.3 ± 2.1
IL-11	5.1 ± 2.9	U.D[c]

[a] Twelve genes had 2-fold or higher frail-over-nonfrail expression ratios by GEArray in at least 12 out of 16 study pairs (75%).

[b] Seven genes with verified upregulation by QPCR are highlighted as bold.

[c] U.D.: expression undetectable by QPCR in one or more study pairs.