

## Research Paper

# Gene and Protein Expression Profiles in a Mouse Model of Collagen-Induced Arthritis

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## Abstract

The risk of rheumatoid arthritis (RA), an autoimmune disease, in the elderly population increases along with that of atherosclerosis, cardiovascular disease, type 2 diabetes, and Alzheimer's disease. Identifying specific biomarkers for RA can clarify the underlying molecular mechanisms and can aid diagnosis and patient care. To this end, the present study investigated the genes and proteins that are differentially expressed in RA using a mouse collagen-induced arthritis (CIA) model. We performed gene microarray and proteome array analyses using blood samples from the mice and found that 50 genes and 24 proteins were upregulated and 48 genes were downregulated by more than 2-fold in the CIA model relative to the control. The gene microarray and proteome array results were validated by evaluating the expression levels of select genes and proteins by real-time PCR and western blotting, respectively. We found that the level of integrin  $\alpha 2$ , which has not been previously reported as a biomarker of RA, was significantly increased in CIA mice as compared to controls. These findings provide a set of novel biomarkers that can be useful for diagnosing and evaluating the progression of RA.

Key words: collagen-induced arthritis; microarray; proteome analysis; biomarker; integrin  $\alpha 2$ .

## Introduction

The incidence of rheumatoid arthritis (RA) is rising in the elderly population; according to a report by the National Institutes of Health, approximately 1.3 million adults are afflicted with RA [1]. It is estimated that up to 1% of the global population has been diagnosed with RA. The symptoms include swelling, pain, and joint stiffness from the knuckles to the knees. RA can also affect other organs such as lungs and heart, and is a progressively debilitating disease that can dramatically reduce the quality of life. The exact cause of RA is unknown, although it is assumed that both genetic and environmental factors are involved [2, 3]. It has been reported that RA is related to the binding of autoantibodies to the host synovium [4], qualifying RA as an autoimmune disease. The incidence of RA is higher in women, suggesting that sex hormones influence disease etiology [5]. Cigarette smoking and dust are also proposed risk factors for RA [6, 7]. Similar to

atherosclerosis, cardiovascular disease, and non-insulin-dependent diabetes, RA is an age-associated disease [8, 9]. Rheumatoid factor (RF) and circulating anti-cyclic citrullinated peptide levels are biomarkers for RA diagnosis; however, only a subset of patients expresses both factors [4, 10]. Patients are also diagnosed based on symptoms and family history [11].

A DBA1/J mouse model of collagen-induced arthritis (CIA) is widely used for the study of RA [12, 13]. These mice exhibit the pathological features of RA, including synovial hyperplasia, inflammatory cell infiltration, and cartilage erosion [14]. Transferring CIA mouse serum to healthy mice induces arthritis via passive immunity [15, 16].

Tumor necrosis factor (TNF)- $\alpha$  is a key cytokine involved in RA. Transgenic mice overexpressing human TNF- $\alpha$  develop RA, and treatment of arthritic mice with anti-TNF- $\alpha$  antibody prevents disease

development [17, 18]. Interleukin (IL)-1, a component of TNF- $\alpha$  signaling, plays an important role in cartilage erosion [18, 19]. Several genes have been linked to RA susceptibility [20, 21], including signal transducer and activator of transcription (STAT)4, which is a risk factor for systemic lupus erythematosus [22] and is associated with IL-12/23 and interferon (IFN)- $\alpha/\beta$  in T cell signaling [23]. Despite these findings, there are few specific biomarkers that are useful for diagnosing and monitoring the progression of RA.

To address this issue, we analyzed the gene and protein expression profiles of RA using the CIA model. A previous gene expression profiling study using CIA mice reported that major histocompatibility complex class I, II, basigin, fibroblast activation protein, cathepsin K, cluster of differentiation (CD)53, RAF-1, glucagon, and retinal taurine transporter contribute to CIA susceptibility or severity [24]. In the present study, we identified the integrin  $\alpha 2$  gene (*Itga2*) as an additional and novel biomarker for RA.

## Materials and Methods

### Materials

Antibodies for western blotting were purchased from Bio-Rad (Hercules, CA, USA). The ProteomeProfiler Mouse Cytokine Array Panel A (ARY006) was from R&D Systems (Minneapolis, MN, USA). Collagen (Chondrex, 20022) and complete (Chondrex, 7001) and incomplete (Chondrex, 7002) Freund's adjuvant were purchased from Central Lab. Animal Inc. (Seoul, Korea).

### Animals

Male DBA1/J mice (6–8 weeks old) were purchased from Central Lab. Animal Inc. and Orient Bio (Seongnam, Korea). Animal maintenance and experiments were in accordance with the guidelines of the Eulji University Institutional Animal Care and Use Committee (approval No. EUIACUC16-17, approval date 10 August 2016).

### In vivo experiments

Bovine type II collagen was used to induce arthritis in mice as previously described [16]. Briefly, bovine type II collagen (2 mg/ml) was mixed at a 1:1 volume ratio with complete Freund's adjuvant. Each mouse was injected with 100 mg of bovine type II collagen in 0.1 ml of emulsion. A booster injection of 100 mg of bovine type II collagen was administered subcutaneously as a solution in 0.1 ml of incomplete Freund's adjuvant 14 days later. Mice were continuously observed for swelling of the distal joints after the primary immunization. Arthritis developed

between 34 and 40 days after the primary immunization based on the arthritis score [16] (data not shown). At the end of the experiment, blood and paws were collected from each mouse. Whole blood was stored in a PAXgene tube (Qiagen, Valencia, CA, USA) at  $-80^{\circ}\text{C}$  until RNA and protein extraction. Paws were fixed in 10% buffered formalin, decalcified in 10% formic acid, and then embedded in paraffin. Sagittal serial sections of the whole paws were cut and stained with hematoxylin and eosin for light microscopy examination.

### RNA extraction, cDNA synthesis, and quantitative real-time (qRT-)PCR

Total RNA was extracted using the QIAamp RNA Blood Mini kit (Qiagen) according to the manufacturer's protocols, and 1  $\mu\text{g}$  was used for cDNA synthesis with the SensiFAST cDNA Synthesis kit (Bioline, Taunton, MA, USA), with a primer annealing step at  $25^{\circ}\text{C}$  for 10 min, followed by reverse transcription at  $42^{\circ}\text{C}$  for 15 min, inactivation at  $85^{\circ}\text{C}$  for 10 min, and storage at  $4^{\circ}\text{C}$ . qRT-PCR was performed on an ABI StepOnePlus system (Applied Biosystems, Foster City, CA, USA). Forward and reverse primer sequences were as follows: IL-1 $\beta$ , 5'-GCTCATCTGGGATCCTCTCC-3' and 5'-CCTGCC TGAAGCTCTTGTTG-3' [54]; IL-6, 5'-ACGGCCTTCC TACTTCACA-3' and 5'-CATTTCACGATTTCCCA GA-3' [55]; TNF- $\alpha$ , 5'-GCCTCTTCTCATTCTGCTT G-3' and 5'-CTGATGAGAGGGAGGCCATT-3' [55]; integrin  $\alpha 2$ , 5'-CGCTCCTTCIGTCATCAAGAGTGT C-3' and 5'-GGAATGTGGATAGTCACCAATGCC-3' [56]; and  $\beta$ -actin, 5'-CGTGCGTGACATCAAAGAGA A-3' and 5'-TGGATGCCACAGGATTCCAT-3' [55].  $\beta$ -Actin was used as an internal control to normalize target gene expression levels, which were determined with the  $2^{-\Delta\Delta\text{CT}}$  method [57].

### Protein extraction and western blotting

Blood from control and CIA mice was mixed with radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Waltham, MA, USA) containing protease inhibitor (GE Healthcare, Little Chalfont, UK). After incubation on ice for 20 min, samples were centrifuged at  $15,000 \times g$  and  $4^{\circ}\text{C}$  for 15 min. The supernatant was used to determine the protein concentration with the Quick start Bradford reagent (Bio-Rad). A total of 100  $\mu\text{g}$  of extracted protein was used for immunoblotting. Samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. After incubation with 5% skim milk in Tris-buffered saline with Tween 20 (TBST) composed of 10 mM Tris (pH 8.0), 150 mM NaCl, and 0.05% Tween 20 for 1 h, the membrane was incubated

overnight at 4°C with antibodies against the following proteins: IL-1 $\beta$  (#12242) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; #5174) (both from Cell Signaling Technology, Danvers, MA, USA); TNF- $\alpha$  (ab66579) and integrin  $\alpha$ 2 (ab133557) (both from Abcam, Cambridge, MA, USA); IL-6 (sc-1265-R) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membrane was washed four times for 5 min and incubated for 2 h with a 1:10,000 dilution of horseradish peroxidase-conjugated anti-mouse or -rabbit antibody. The membrane was washed six times with TBST for 10 min and developed with the enhanced chemiluminescence system (GE Healthcare) and blue X-ray film (Agfa HealthCare NV, Mortsel, Belgium) according to the manufacturer's protocols. After the transfer, the gel was stained with Coomassie Blue reagent (Bio-Rad). GAPDH was used as the loading control. The membrane was stained with Ponceau S (Sigma-Aldrich, St. Louis, MO, USA) after immunoblotting.

### Microarray

Blood was collected from mice in a PAXgene blood RNA tube (PreAnalytiX, Hombrechtikon, Switzerland) and RNA was isolated using the PAXgene Blood RNA kit (PreAnalytiX) according to the manufacturer's protocol. RNA purity and integrity were determined based on the optical density 260/280 ratio on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Microarray analysis with a GeneChip Mouse Gene 2.0 ST Array was performed Macrogen Co. (Seoul, Korea). Raw data were extracted using Affymetrix Expression Console software and were filtered when  $P < 0.05$ . The statistical significance of expression data

was determined with the independent Student's *t* test based on fold change, where the null hypothesis was that no difference existed between the two groups. Gene enrichment analysis and functional annotation were performed based on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

### Proteome array

The ProteomeProfiler Mouse Cytokine Array Panel A (R&D Systems) was used according to the manufacturer's protocols to obtain protein expression profiles using 50- $\mu$ l blood samples. Spot density was determined using HLIImage software (Western Vision Software, Salt Lake City, UT, USA).

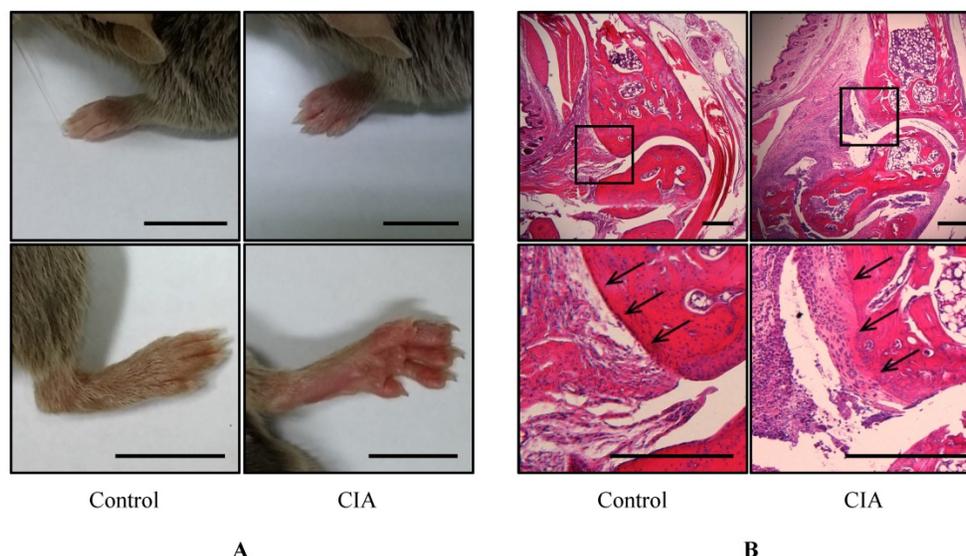
### Statistical analysis

Differences between groups were evaluated with the Student's *t* test using Excel software (Microsoft, Redmond, WA, USA).  $P < 0.05$  was considered statistically significant.

## Results

### Murine model of arthritis

To identify potential biomarkers of RA, we established a mouse CIA model by injecting male DBA1/J mice with bovine type II collagen. Pathological changes were observed after 49 days; the mice had swollen paws and ankles typical of arthritis (Figure 1A). Histological examination of the mouse foot revealed increased inflammation and immune cell infiltration (Figure 1B), and the cartilage boundaries appeared crushed. These results confirm that RA was induced in the CIA mice after 49 days.



**Figure 1.** Gross morphological and histological examination of CIA. (A) Gross observation of mouse paws. Shown are the fore paws (top) and hind paws (bottom) of control ( $n = 12$ ) and CIA ( $n = 14$ ) mice at 14 weeks of age. Scale bars = 10 mm. (B) H&E staining of sagittal sections of control and CIA mouse joints. Lower panels show enlarged views of the areas delineated by a box in the upper panels. Arrows indicate cartilage boundaries. Scale bars = 1 mm.

**Table 1.** Genes differentially expressed in the blood of control and CIA mice

Increased genes			Decreased genes								
No.	Gene symbol	Fold change	No.	Gene symbol	Fold change	No.	Gene symbol	Fold change	No.	Gene symbol	Fold change
1	<i>Irf7</i>	24.7	26	<i>Olfr1386</i>	2.2	1	<i>Sec24a</i>	-3.9	25	<i>Rps2</i>	-2.2
2	<i>Isg15</i>	6.6	27	<i>Olfr1502</i>	2.2	2	<i>Cd27</i>	-3.3	26	<i>Il7r</i>	-2.2
3	<i>Ifit1</i>	6.1	28	<i>Cyp2d26</i>	2.2	3	<i>Cd8b1</i>	-3.3	27	<i>Ccnd2</i>	-2.2
4	<i>Oas3</i>	4.9	29	<i>Mir423</i>	2.2	4	<i>Ccl5</i>	-3.2	28	<i>Rgs14</i>	-2.2
5	<i>Mir107</i>	4.2	30	<i>Ppp1r15a</i>	2.2	5	<i>Cd3g</i>	-3.2	29	<i>Pck2</i>	-2.2
6	<i>H2-Q8</i>	3.4	31	<i>Spta1</i>	2.2	6	<i>Cd3e</i>	-2.9	30	<i>Cd59a</i>	-2.2
7	<i fn1<="" i=""></i>	3.2	32	<i>Rps15</i>	2.2	7	<i>Lat</i>	-2.7	31	<i>Tcf7</i>	-2.2
8	<i>C1ra</i>	3.0	33	<i>Vwf</i>	2.2	8	<i>Hist1h2ba</i>	-2.7	32	<i>Rps11</i>	-2.2
9	<i>Ifih1</i>	2.9	34	<i>Pla2g2a</i>	2.2	9	<i>Ncr1</i>	-2.7	33	<i>Ugcg</i>	-2.1
10	<i>Fpr2</i>	2.9	35	<i>Cmpk2</i>	2.1	10	<i>Eif4a1</i>	-2.6	34	<i>Rpl6</i>	-2.1
11	<i>Ctca3a1</i>	2.7	36	<i>F5</i>	2.1	11	<i>Rps8</i>	-2.6	35	<i>Olfr373</i>	-2.1
12	<i>Olfr774</i>	2.7	37	<i>Fos</i>	2.1	12	<i>Cd4</i>	-2.6	36	<i>Olfr705</i>	-2.1
13	<i>Hist1h2bj</i>	2.7	38	<i>Olfr917</i>	2.1	13	<i>Il2rb</i>	-2.5	37	<i>Cd209a</i>	-2.1
14	<i>Ifi204</i>	2.6	39	<i>Ptpn11</i>	2.0	14	<i>Ddx5</i>	-2.5	38	<i>Lck</i>	-2.1
15	<i>Fads2</i>	2.6	40	<i>C3</i>	2.0	15	<i>Rpl30</i>	-2.5	39	<i>Klrd1</i>	-2.1
16	<i>Plxna4</i>	2.5	41	<i>Hist2h4</i>	2.0	16	<i>Mirlet7f-2</i>	-2.5	40	<i>Olfr1501</i>	-2.1
17	<i>Sp100</i>	2.5	42	<i>Flna</i>	2.0	17	<i>Ppp2r2d</i>	-2.5	41	<i>Rpl37a</i>	-2.1
18	<i>Vcl</i>	2.3	43	<i>Cks1b</i>	2.0	18	<i>Atp1b3</i>	-2.4	42	<i>Mir103-2</i>	-2.0
19	<i>Tuba3b</i>	2.3	44	<i>Olfr1057</i>	2.0	19	<i>Ube3c</i>	-2.4	43	<i>Ppil2</i>	-2.0
20	<i>Dusp3</i>	2.3	45	<i>Olfr726</i>	2.0	20	<i>Olfr875</i>	-2.4	44	<i>Ddx46</i>	-2.0
21	<i>Hist1h2aa</i>	2.3	46	<i>Olfr1298</i>	2.0	21	<i>Rmrp</i>	-2.3	45	<i>Stat4</i>	-2.0
22	<i>H2-T24</i>	2.3	47	<i>Igtp</i>	2.0	22	<i>Ctsw</i>	-2.3	46	<i>Itgb7</i>	-2.0
23	<i>Rps6ka2</i>	2.3	48	<i>Mir7-1</i>	2.0	23	<i>Slc40a1</i>	-2.3	47	<i>Bcl2a1c</i>	-2.0
24	<i>Iiga2</i>	2.3	49	<i>Thbs1</i>	2.0	24	<i>Sec61b</i>	-2.2	48	<i>Olfr1299</i>	-2.0
25	<i>Sort1</i>	2.2	50	<i>Olfr38</i>	2.0						

### Gene expression profiling

Gene expression analysis was performed using RNA from blood samples from control and CIA mice. In total, 98 genes showed  $\geq 2$ -fold change in blood mRNA expression in CIA as compared to control mice (Table 1 and Table S1). Of these genes, 50 were upregulated and 48 were downregulated. The expression levels of eight genes [*interferon regulatory factor (Irf)7*; *interferon-stimulated gene 15*; *interferon induced protein with tetratricopeptide repeats 1*; *2'-5'-oligoadenylate synthetase 3*; *microRNA 107*; *histocompatibility 2, Q region locus 8*; *fibronectin 1*; and *complement component 1, r subcomponent A*] were  $> 3$ -fold higher in CIA than in control mice, with the level of *Irf7* showing a  $> 24$ -fold difference. The expression of five genes [*Sec24a*, *Cd27*, *Cd8b1*, *chemokine (C-C motif) ligand (Ccl)5*, and *Cd3g*] was decreased by  $> 3$  fold in CIA as compared to control mice. A KEGG pathway analysis of 98 genes whose expression differed by  $\geq 2$ -fold between the two groups revealed that *Cd4* was associated with seven different KEGG pathways (Tables 1 and 2). Genes in five of nine analyzed pathways were downregulated in CIA. For example, Th1 and Th2 cell differentiation in the immune system category showed downregulation of seven genes. Of the 26 analyzed pathways, five contained only genes that were upregulated in CIA, such as those related to mitogen-activated protein kinase signaling, extracellular matrix (ECM), and focal adhesion. All of

the analyzed pathways had  $P < 0.05$ .

### Protein expression profiling

Changes in protein levels in CIA mice were evaluated with a proteome array using whole blood. Because most genes identified by the KEGG pathway analysis were related to the immune system and immune-related diseases, we used the ProteomeProfilier Mouse Cytokine Array Panel A for protein expression profiling. We found that the levels of all 40 cytokines were slightly increased in the blood of CIA as compared to control mice (Table S2), with 24 showing a  $> 2$ -fold increase (Table 3B). These genes were grouped into 18 categories based on the KEGG classification scheme (Table 3A). Most of the upregulated cytokines were associated with cytokine-cytokine receptor interaction, Janus kinase-STAT signaling, and helper T cell (Th)17 differentiation pathways, whereas seven were associated with RA and the hematopoietic cell lineage. The cytokines associated with the RA pathway included IL-17; IL-23; IL-1 $\beta$ ; monocyte chemoattractant protein (MCP)-5; TNF- $\alpha$ ; regulated upon activation, normally T-expressed, and presumably secreted (RANTES); IL-6; and IL-16. IL-2 expression showed the greatest difference between CIA and control mice. The levels of IL-1 $\beta$ , TNF- $\alpha$ , and IL-6, which are the major pro-inflammatory cytokines [25], were 6.41, 3.35, and 2.41-fold higher, respectively; IL-17 and -23, which are involved in Th17 cell differentiation [26], were upregulated by

13.09- and 7.21-fold, respectively; and RANTES, also known as Ccl5 [27, 28], was upregulated 2.5-fold in the RA model.

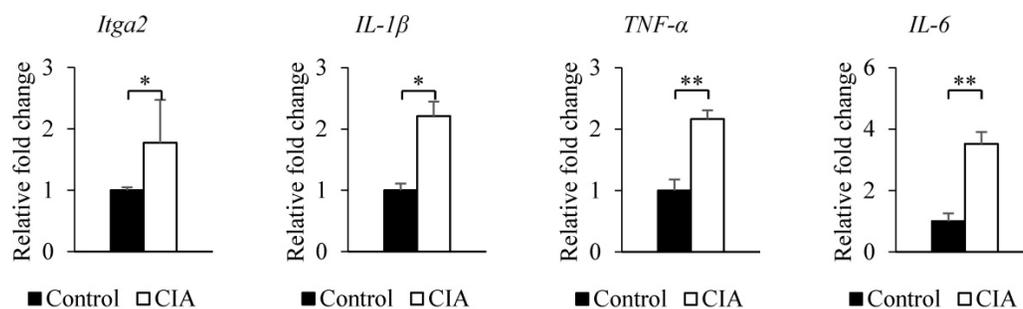
### Validation of differentially expressed genes and proteins

The results from the gene expression microarray and proteome array were validated by qRT-PCR and western blot analysis of selected genes and proteins, including integrin  $\alpha 2$ , IL-1 $\beta$ , TNF- $\alpha$ , and IL-6, that showed  $\geq 2$ -fold difference in expression relative to

the control. Consistent with the gene microarray results, *Itga2* expression was significantly higher in CIA than in control mice by qRT-PCR (Figure 2). A similar result was obtained for the genes encoding IL-1 $\beta$ , TNF- $\alpha$ , and IL-6. Western blot analysis revealed that blood protein levels of integrin  $\alpha 2$ , IL-1 $\beta$ , TNF- $\alpha$ , and IL-6 were increased in CIA as compared to control mice (Figure 3). Thus, the qRT-PCR and western blotting results support the validity of the microarray and protein array data.

**Table 2.** KEGG pathway analysis of differentially expressed genes identified by microarray analysis

No.	KEGG classification	Pathway	Number of significant genes	Increased genes	Decreased genes	P value
1	Immune system	Th1 and Th2 cell differentiation	7		<i>Stat4, Lck, Il2rb, Cd4, Lat, Cd3e, Cd3g</i>	< 0.001
2		Th17 cell differentiation	6		<i>Lck, Il2rb, Cd4, Lat, Cd3e, Cd3g</i>	< 0.001
3		T cell receptor signaling pathway	6		<i>Lck, Cd4, Lat, Cd3e, Cd3g, Cd8b1</i>	< 0.001
4		Antigen processing and presentation	5	<i>H2-Q8, H2-T24</i>	<i>Klrd1, Cd4, Cd8b1</i>	< 0.001
5		Hematopoietic cell lineage	5		<i>Il7r, Cd4, Cd3e, Cd3g, Cd8b1</i>	< 0.001
6		Complement and coagulation cascades	5	<i>C1ra, Vwf, F5, C3</i>	<i>Cd59a</i>	< 0.001
7	Immune diseases	Natural killer cell mediated cytotoxicity	4		<i>Klrd1, Lck, Ncr1, Lat</i>	< 0.001
8		NOD-like receptor signaling pathway	4	<i>Irf7, Oas3, Ifi204</i>	<i>Ccl5</i>	< 0.01
9		RIG-I-like receptor signaling pathway	3	<i>Irf7, Isg15, Ifih1</i>		< 0.01
10	Immune diseases	Primary immunodeficiency	5		<i>Lck, Il7r, Cd4, Cd3e, Cd8b1</i>	< 0.001
11		Systemic lupus erythematosus	5	<i>Hist1h2bj, Hist1h2aa, C3, Hist2h4</i>	<i>Hist1h2ba</i>	< 0.001
12	Signal transduction	Graft-versus-host disease	3	<i>H2-Q8, H2-T24</i>	<i>Klrd1</i>	< 0.01
13		PI3K-Akt signaling pathway	10	<i>Fn1, Itga2, Vwf, Thbs1</i>	<i>Pck2, Ppp2r2d, Itgb7, Ccnd2, Il7r, Il2rb</i>	< 0.001
14	Signaling molecules and interaction	Jak-STAT signaling pathway	4		<i>Stat4, Ccnd2, Il7r, Il2rb</i>	< 0.01
15		MAPK signaling pathway	4	<i>Dusp3, Rps6ka2, Fos, Flna</i>		< 0.01
16		NF-kappa B signaling pathway	3		<i>Bcl2a1c, Lck, Lat</i>	< 0.05
17	Cellular community	Cell adhesion molecules (CAMs)	5	<i>H2-Q8, H2-T24</i>	<i>Itgb7, Cd4, Cd8b1</i>	< 0.001
18		ECM-receptor interaction	4	<i>Fn1, Itga2, Vwf, Thbs1</i>		< 0.001
19		Cytokine-cytokine receptor interaction	4		<i>Il7r, Il2rb, Ccl5, Cd27</i>	< 0.01
20	Cell growth and death	Focal adhesion	6	<i>Fn1, Vcl, Itga2, Vwf, Flna, Thbs1</i>		< 0.001
21		Apoptosis	4	<i>Tuba3b, Spta1</i>	<i>Bcl2a1c, Ctsw</i>	< 0.01
22	Transport and catabolism	Phagosome	9	<i>H2-Q8, C1ra, Tuba3b, H2-T24, Itga2, C3, Thbs1</i>	<i>Sec61b, Cd209a</i>	< 0.01
23		alpha-Linolenic acid metabolism	2	<i>Fads2, Pla2g2a</i>		< 0.05
24	Translation	Ribosome	7	<i>Rps15</i>	<i>Rps2, Rpl30, Rpl37a, Rpl6, Rps11, Rps8</i>	< 0.01
25		Neurotrophin signaling pathway	3	<i>Rps6ka2, Sort1, Ptpn11</i>		< 0.05
26	Sensory system	Olfactory transduction	12	<i>C1ca3a1, Olfr774, Olfr1386, Olfr1502, Olfr726, Olfr1298, Olfr38</i>	<i>Olfr1299, Olfr1501, Olfr705, Olfr373, Olfr875</i>	< 0.001



**Figure 2.** Validation of microarray and proteome array results by qRT-PCR. The expression of each gene shown in the figure was confirmed by qRT-PCR using specific primers.  $\beta$ -Actin served as an internal control. Data represent the mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$

**Table 3A.** Proteins differentially expressed in the blood of control and CIA mice. (A) Classification of upregulated proteins

No.	Classification	Identified proteins	Number of proteins
1	Cytokine-cytokine receptor interaction	IL-2, IL-27, IL-17, MIP-1beta, IL-23, IL-1beta, IL-1ra, MIP-2, MCP-5, TARC, IL-3, TNF-alpha, IP-10, MIG, BLC, I-TAC, RANTES, IL-4, IL-6, IL-5, IL-10	21
2	Jak-STAT signaling pathway	IL-2, IL-27, IL-23, IL-3, IL-4, IL-6, IL-5, IL-10	8
3	Th17 cell differentiation	IL-2, IL-27, IL-17, IL-23, IL-1beta, IL-1ra, IL-4, IL-6	8
4	Rheumatoid arthritis	IL-17, IL-23, IL-1beta, MCP-5, TNF-alpha, RANTES, IL-6, IL-16	7
5	Hematopoietic cell lineage	IL-1beta, IL-1ra, IL-3, TNF-alpha, IL-4, IL-6, IL-5	7
6	NOD-like receptor signaling pathway	IL-1beta, MIP-2, MCP-5, TNF-alpha, RANTES, IL-6	6
7	Graft-versus-host disease	IL-2, IL-1beta, TNF-alpha, IL-6, IL-10	5
8	NF-kappa B signaling pathway	MIP-1beta, IL-1beta, IL-1ra, TNF-alpha, BLC	5
9	T cell receptor signaling pathway	IL-2, TNF-alpha, IL-4, IL-5, IL-10	5
10	PI3K-Akt signaling pathway	IL-2, IL-3, IL-4, IL-6	4
11	Th1 and Th2 cell differentiation	IL-2, IL-4, IL-5	3
12	MAPK signaling pathway	IL-1beta, IL-1ra, TNF-alpha	3
13	Systemic lupus erythematosus	TNF-alpha, IL-10	2
14	HIF-1 signaling pathway	TIMP-1, IL-6	2
15	RIG-I-like receptor signaling pathway	TNF-alpha, IP-10	2
16	Apoptosis	IL-3, TNF-alpha	2
17	Natural killer cell mediated cytotoxicity	TNF-alpha	1
18	Antigen processing and presentation	TNF-alpha	1

**Table 3B.** Densitometry analysis of upregulated proteins (n = 24)

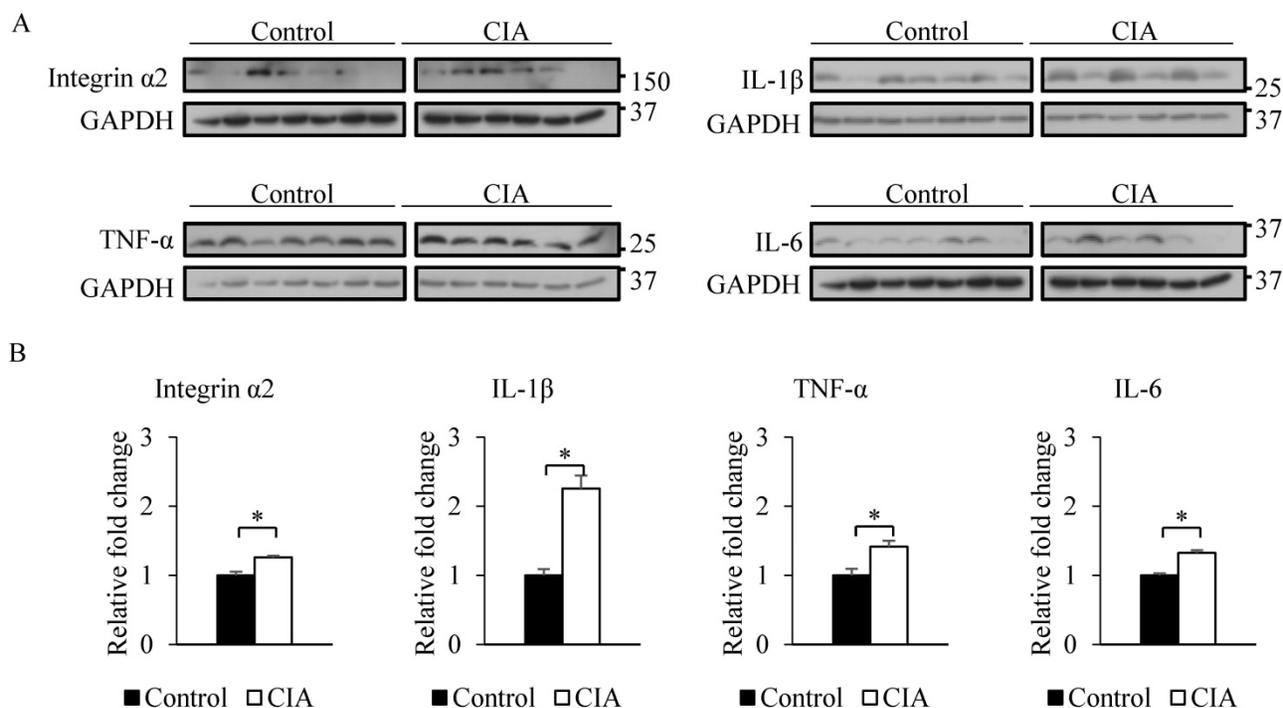
No.	Protein name	Relative fold change
1	IL-2	27.4
2	IL-27	19.3
3	IL-17	13.1
4	MIP-1beta	10.2
5	IL-23	7.2
6	IL-1beta	6.4
7	IL-1ra	4.6
8	MIP-2	4.2
9	MCP-5	4.1
10	TIMP-1	4.0
11	TARC	3.9
12	IL-3	3.9
13	TREM-1	3.8
14	TNF-alpha	3.4
15	IP-10	2.9
16	MIG	2.9
17	BLC	2.7
18	I-TAC	2.5
19	RANTES	2.5
20	IL-4	2.4
21	IL-6	2.4
22	IL-16	2.2
23	IL-5	2.2
24	IL-10	2.0

## Discussion

RA initially occurs as non-specific inflammation in the joints; however, other organs are also affected in 15%–25% of individuals [29]. Following T cell activation, chronic inflammation occurs accompanied by tissue injury due to activation of the pro-inflammatory cytokines IL-1 and -6 and TNF- $\alpha$ . Our microarray results showed that *Itga2* and *Irf7* were upregulated, whereas *cd4* was downregulated in CIA mice. A KEGG pathway analysis indicated that pathways related to the immune system were highly

represented among the differentially expressed genes. Accordingly, IL-2, -27, -17, IL-1 $\beta$ , -6, and TNF- $\alpha$  levels were > 2-fold higher in the RA model relative to control mice, which was confirmed by qRT-PCR and western blotting.

Integrin  $\alpha 2$  is a component of the very late-activation antigen 2 complex (integrin  $\alpha 2\beta 1$ ) [30] and binds to collagen via the I-domain [31, 32]. Integrin  $\alpha 2\beta 1$  is expressed only by effector Th1 and Th17 cells and attaches to collagen I/II-expressing cells of the synovial matrix [32, 33], resulting in the stimulation of T cell receptor-dependent IL-17 production [34]. IL-17 secreted by Th17 cells induces the production of pro-inflammatory cytokines such as IL-1 and -6 and TNF- $\alpha$  by macrophages, chondrocytes, and fibroblast-like synoviocytes, and was found to cause bone erosion via expression of receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) in fibroblast-like synoviocytes and osteoclasts [35]. Blockade of integrin  $\alpha 2\beta 1$  reduced synovial inflammation, cartilage destruction, and bone loss in the joints of CIA mice [36]. According to the RNA microarray results and KEGG pathway analysis, integrin  $\alpha 2$  is involved in phosphoinositide 3-kinase (PI3K)-Akt signaling, ECM receptor interaction, focal adhesion, and phagosome formation. PI3K-Akt signaling maintains basic cellular functions such as proliferation and differentiation [37], and inhibition of this pathway is a therapeutic strategy for RA treatment. Although the exact role of integrin  $\alpha 2$  in the pathways identified by KEGG analysis is unclear, our results suggest that integrin  $\alpha 2$  plays an important role in RA etiology, and is thus a candidate biomarker for RA diagnosis.



**Figure 3.** Validation of microarray and proteome array results by western blotting. (A) Western blotting was performed using blood samples from control and CIA mice. GAPDH served as an internal control. (B) Relative fold change in band intensity of target proteins normalized to GAPDH level. Data represent the mean  $\pm$  SEM. \* $p < 0.05$

*Irf7* was another gene that was identified by microarray analysis as being upregulated in RA. IRF7 regulates the transcription of IFN-stimulated genes such as IFN- $\beta$ , RANTES, and IFN- $\gamma$ -inducible protein 10 that are expressed in the joints of RA patients [38-40]. *Irf7* knockdown was found to decrease IFN-stimulated response element promoter activity [41], resulting in a decrease in the expression of genes associated with Th17 cell differentiation; however, this was accompanied by an increase in IL-17 secretion by Th17 cells. Further research is needed to resolve this discrepancy.

IL-1 and TNF- $\alpha$  are involved in joint inflammation and erosion in RA [42]. TNF- $\alpha$ -induced upregulation by TNF- $\alpha$  in synovial T cells was shown to increase RANKL expression and stimulate osteoclastogenesis in RA [43]. IL-27 is produced by antigen-presenting cells and regulates T cell differentiation and function [44]; it has pro- or anti-inflammatory functions depending on the disease stage [45, 46]. IL-27 levels were found to be higher in RA patients than in healthy individuals [47]. IL-27 $R\alpha^{-/-}$  mice showed reduced severity of proteoglycan-induced arthritis [46], whereas injection of exogenous IL-27 improved RA symptoms in the CIA model [45]. Consistent with these earlier studies, we found here that IL-27 was upregulated in CIA as compared to control mice. In contrast, we observed that IL-2 expression was also increased in the RA

model, although previous reports suggest that the IL-2 level is lower in rheumatoid synovial fluid, synovial tissue, and peripheral blood of RA patients than in those of control subjects [48, 49]. This discrepancy may be due to differences between species.

Our proteome array results showed that RANTES (or CCL5) was upregulated in CIA as compared to control mice. In contrast, the *Ccl5* transcript (encoding RANTES) showed the opposite trend. RANTES is a chemotactic factor that recruits monocytes, memory T cells, and natural killer cells [50-52]. Others have reported higher RANTES levels in CIA mice relative to controls [53]. Therefore, additional research is necessary to clarify the exact role of RANTES in RA.

In summary, we found that integrin  $\alpha 2$ , IL-1 $\beta$  and -6, and TNF- $\alpha$  were upregulated in a mouse model of RA. In particular, integrin  $\alpha 2$  was identified for the first time as a potential biomarker that can expedite RA diagnosis and be used to monitor disease progression.

## Abbreviations

RA, rheumatoid arthritis; CIA, collagen-induced arthritis; RF, rheumatoid factor; TNF, tumor necrosis factor; IL, interleukin; STAT, signal transducer and activator of transcription; IFN, interferon; CD, cluster of differentiation; *Itga2*, integrin  $\alpha 2$  gene; KEGG,

Kyoto encyclopedia of genes and genomes; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ECM, extracellular matrix; CAMs, cell adhesion molecules; Th cell, helper T cell; MCP, monocyte chemoattractant protein; RANTES, regulated upon activation, normally T-expressed, and presumably secreted; integrin  $\alpha 2\beta 1$ , very late-activation antigen 2 complex; RANKL, receptor activator of nuclear factor- $\kappa$ B ligand; PI3K, phosphoinositide 3-kinase.

## Supplementary Material

Supplementary tables.

<http://www.medsci.org/v15p0077s1.pdf>

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## Authors' Contributions

Sun-Yeong Gwon and Ho Joong Sung conceived and designed the experiments; Sun-Yeong Gwon performed the experiments; Sun-Yeong Gwon and Ho Joong Sung analyzed the data; Ho Joong Sung contributed reagents/materials/analysis tools; Sun-Yeong Gwon, Ki-Jong Rhee and Ho Joong Sung wrote the paper.

## Competing Interests

The authors have declared that no competing interest exists.

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