Genomics and proteomics of immune modulatory effects of a butanol fraction of *echinacea purpurea* in human dendritic cells

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Abstract

**Background:** *Echinacea* spp. extracts and the derived phytocompounds have been shown to induce specific immune cell activities and are popularly used as food supplements or nutraceuticals for immuno-modulatory functions. Dendritic cells (DCs), the most potent antigen presenting cells, play an important role in both innate and adaptive immunities. In this study, we investigated the specific and differential gene expression in human immature DCs (iDCs) in response to treatment with a butanol fraction containing defined bioactive phytocompounds extracted from stems and leaves of *Echinacea purpurea*, that we denoted [BF/S+L/Ep].

**Results:** Affymetrix DNA microarray results showed significant up regulation of specific genes for cytokines (IL-8, IL-1β, and IL-18) and chemokines (CXCL 2, CCL 5, and CCL 2) within 4 h after [BF/S+L/Ep] treatment of iDCs. Bioinformatics analysis of genes expressed in [BF/S+L/Ep]-treated DCs revealed a key-signaling network involving a number of immune-modulatory molecules leading to the activation of a downstream molecule, adenylate cyclase 8. Proteomic analysis showed increased expression of antioxidant and cytoskeletal proteins after treatment with [BF/S+L/Ep] and cichoric acid.

**Conclusion:** This study provides information on candidate target molecules and molecular signaling mechanisms for future systematic research into the immune-modulatory activities of an important traditional medicinal herb and its derived phytocompounds.

**Background**

*Echinacea* spp., commonly known as purple coneflower, is indigenous to North America. The use of *Echinacea* spp. as an herbal remedy originated in the medicinal culture of North American Indians during the 17th century and was later introduced to Europe. Its use became popular again in the early 1990s and continues today. Currently, *Echinacea* extracts from whole plant or specific tissue (e.g., root

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or aerial parts) are among the top-selling medicinal or food supplement products in the United States and Europe [1-3]. Recent studies have shown that treatment with specific *Echinacea* extracts activates macrophages, natural killer cells, or other immune cells [4-6]. *Echinacea* extracts have also been reported to stimulate the secretion of cytokines such as tumor necrosis factor-alpha, interferon, interleukin-1 and interleukin-6 [7-10]. *In vivo* studies have shown that treatment with *Echinacea* extracts can increase the number of white blood cells in the circulatory system [11], enhance phagocytosis [12], and trigger the increase the number of white blood cells in the circulatory system. *Echinacea* extracts have also been reported to stimulate the secretion of cytokines such as tumor necrosis factor-alpha, interleukin-1 and interleukin-6 [7-10].

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**Results**

**Effect of [BF/S+L/Ep] extract on expression of cell surface markers in human iDCs**

Flow cytometry revealed that [BF/S+L/Ep] enhanced the expression of CD83, a key marker for DC maturation in iDCs, as compared with vehicle (0.1% DMSO) treatment (Figure 1) and the percentage of CD83+ expressing cells increased, from 20% to 45% with 10, 50 and 100 μg/ml [BF/S+L/Ep]. However, treatment with the ethyl acetate (EA) fraction of the S+L extract at 1, 10, or 50 μg/ml significantly decreased the percentage of CD83+ expressing cells from 20% to 0% (Figure 1). The observed effects were not caused by cellular mechanisms related to cytotoxicity, since MTT assay results indicated that treatment with EA partitioned fraction (BF) of the S+L tissue extracts of *E. purpurea* [BF/S+L/Ep] and cichoric acid (a major component of this fraction) through Affymetrix gene chip microarray analyses. High-resolution 2-D gel electrophoresis, MALDI-TOF mass spectrometry (MS), tandem MS-MS analysis, and bio-informatics database systems were subsequently employed for proteomics studies in parallel with the genomics studies. Results from these analyses and cell-based bioactivity-guide assays suggest that groups of differentially expressed genes, specific functional genes, and the associated molecular signaling networks can be employed as potential targets for future systematic studies of the response of human DC systems, as a response to traditional herbal medicine formulations or their derived phytocompounds.

**Figure 1**

Specific bioactivities of three subfractions of stem and leaf (S+L) extracts of *E. purpurea* in human immature dendritic cells (iDC). iDCs were treated for 24 h with the S+L tissue extracts and the derived ethyl acetate (EA), butanol (BuOH), or water fractions. Test cells were subsequently analyzed for cell-surface marker CD83 expression by flow cytometry.
(1 to 50 μg/ml) or [BF/S+L/Ep] (1 to 100 μg/ml) showed 98% to 125% cell viability as compared to vehicle treatment (data not shown). Thus, the [BF/S+L/Ep] fraction but not the EA fraction could enhance the maturation of iDCs. To reach this conclusion, it is essential for us to rule out the possibility that lipopolysaccharide (LPS), as a bacterial endotoxin contamination of the [BF+S+L/Ep] extract preparations, might have contributed to the observed results in the present CD83 assay and the subsequent functional genomics and proteomics studies. By using a LAL assay (see Methods), we have obtained a firm negative results (<0.125 EU/ml) on the presence of a significant level of LPS in our [BF+S+L/Ep] extract fraction. EchinaforceTM, a standardized commercial product from Swiss registered E. purpurea (L.) Moench fresh plant tincture, was reported to contain an endotoxin level of <0.5 EU/ml, and this tincture was used in a randomized double-blind clinical study [27]. In comparison, our plant extract fraction, [BF+S+L/Ep], was detected to contain a considerably lower level of possible endotoxin contamination (<0.125 EU/ml). We therefore are confident that a LPS effect on various bioactivities can virtually be ruled out from our present studies.

**Compound identification and fingerprint analysis of the bioactive [BF/S+L/Ep] fraction**

The overall metabolite profile of [BF/S+L/Ep] of E. purpurea was analyzed using RP-HPLC, detected at A330 or A254 (Figure 2A). Seven phytocompounds (1-7) namely hypoxanthine (1), chlorogenic acid (2), caffeic acid (3), cichoric acid (4), quercetin 3-O-rhamnopyranosyl-(1→6)-galactoside (5), kaempferol 3-O-rhamnopyranosyl-(1→6)-galactoside (6), and rutin (7) were identified by a combination of chromatography on RP-18 silica gel, Sephadex gel, and C18 RP-HPLC. Structures were elucidated by 1H-nuclear magnetic resonance (NMR), 13C-NMR and MS, and confirmed with previous data [28-34]. LC/electrospray ionization-MS was further employed to establish the chemical fingerprints with two index compounds of [BF/S+L/Ep]. The RP-HPLC chromatogram detected at 330 nm of [BF/S+L/Ep] was shown in Figure 2B (a) and the mass spectra of the specific compounds were further characterized by using their pseudo molecular weight at m/z 497 [M + Na]+, Tg = 36.8 min, compound 4, Figure 2B (b); and m/z 611 [M + H]+, Tg = 40.3 min, compound 7, Figure 2B (c). These result demonstrate that compounds 4 and 7 in [BF/S+L/Ep] can be readily identified by comparing their corresponding peaks (retention time) and MS data.

The levels of cichoric acid (4) and rutin (7) were quantitatively determined for use as candidate index compounds for the [BF/S+L/Ep] fraction. Standard calibration curves (peak area versus concentrations) prepared for cichoric acid and rutin ranged from 0.125 to 1 mg/ml. Absorbance of the compounds at 330 nm varied linearly within this range. The amount of cichoric acid and rutin were determined as 8.4% (w/w) and 22.3% (w/w), respectively, of the dry weight of the [BF/S+L/Ep] fraction. Together, the results show that this [BF/S+L/Ep] phytocompound mixture was effectively standardized and defined with a specific metabolite profile containing several candidate index compounds (Figure 2). The standardized [BF/S+L/Ep] fraction was then systematically used in the following bioactivity assays to evaluate its immune regulatory effects in human iDCs.

**Affymetrix DNA microarray analysis of differential gene expression patterns in human iDCs in response to treatment with [BF/S+L/Ep] or cichoric acid**

For DNA microarray analysis, iDCs differentiated from primary monocytes in vitro were incubated with [BF/S+L/Ep] or cichoric acid for 4 or 24 h to characterize early- or late-responsive genes. A total of nine oligo-DNA chips were hybridized to determine the transcriptome profiles in human iDCs. The array data was normalized for identifying differentially expressed genes, clustering and annotation. We selected 382 genes for clustering by gap statistic analysis. Figure 3 shows a heat map of differential gene expression patterns and a plot of the resultant 6 possible gene clusters. The K-means clustering revealed that some genes exhibited a consistent response, for example with increased expression at both 4 h and 24 h after treatment with [BF/S+L/Ep]. Alternatively, some genes highly expressed at 4 h but not at 24 h after treatment, or at 24 h but not at 4 h post-treatment, and these may reflect other sets of specific and differential transient gene expression patterns.

Approximately 119 genes were up-regulated by ≥ 2-fold change in expression at 4 h post-treatment with [BF/S+L/Ep], with 20 genes showing 11- to 31-fold change in expression (Table 1A). About 20 genes were down-regulated by ≥ 2-fold change in expression at 4 h post-treatment (Table 1A). Most notably, four up-regulated genes (CCL2 [MCP1], CCL5 [RANTES], NF-κB activator and IL-8), and two down-regulated genes, IFN-α/β and leukocyte immunoglobulin-like receptor [LILRB3], are well known to be involved in various key immune-modulatory activities. Changes in expression noted after 24 h treatment are summarized in Table 1B. Treatment with cichoric acid, a key principle component of the [BF/S+L/Ep] fraction, gave a different expression profile in iDCs. A total of 81 genes and 46 genes were up-regulated and down-regulated, respectively, with 2-fold change in expression at 4 h post-treatment (data not shown). The top 20 up- or down-regulated genes, with 2.6- to 18.0-fold increase or 0.23- to 0.45-fold decrease in expression at 4 h were chosen for further analysis (Table 2A). At 24 h after treatment, 79 genes were up-regulated and 81 down-regulated with ≥ 2-fold change in expression (data not shown). The top 20
Chemical fingerprints and candidate index compounds identified in the [BF/S+L/Ep] fraction of *E. purpurea*. (A) Metabolite profile at 330 and 254 nm and the structures of compounds 1-7 identified from [BF/S+L/Ep]. (B) RP-HPLC chromatogram of [BF/S+L/Ep] at 330 nm (a) analysis on ESI-MS to obtain m/z ratios of (b) m/z 497, and (c) m/z 611 for compounds 4 and 7, respectively.
Figure 3
Comparative analysis of differential gene expression patterns in immature dendritic cells treated with [BF/S+L/Ep] and specific phytocompounds by K-means clustering analyses. Analysis of test iDC samples (including vehicle control [+DMSO only]) involved comparing the treatment values with untreated values (i.e., zero hour-treated). The left panel shows the heat map of the resulting clustered gene expression. The number of genes involved and the mean profile for each cluster is in the right panel. The mean profile was superimposed with error bars showing ± 1 standard error of the mean.
### Table 1: Effect of [BF/S+L/Ep] on gene expression in human iDCs

| Gene Name                          | Common Name | Accession No | Fold Change (From 31 to 11-fold) |
|------------------------------------|-------------|--------------|----------------------------------|
| *(A) Up-regulated at 4 h post-treatment* |
| EH-domain containing 1             | EHD1        | NM_006795    | 31.81                            |
| chemokine (C-C motif) ligand 2      | CCL2        | NM_002982    | 22.15                            |
| cryptochrome 1 (photolyase-like)    | CRY1        | NM_004075    | 21.65                            |
| BCL-6 interacting corepressor      | BCOR        | NM_017745    | 21.62                            |
| tumor necrosis factor receptor superfamily, member 4 | TNFRSF4 | NM_003327    | 19.10                            |
| Xenopus prevents mitotic catastrophe 2 homolog | XPMC2H | NM_020385    | 17.54                            |
| basic transcription element binding protein 1 | BTEB1 | NM_001206 | 17.45 |
| Janus kinase 2 (a protein tyrosine kinase) | JAK2 | NM_004972 | 16.40 |
| wingless-type MMTV integration site family, member 5A | WNT5A | NM_003392 | 15.42 |
| discs, large (Drosophila) homolog 1 | DLG1 | NM_004087 | 15.22 |
| RAB35, member RAS oncogene family | RAB35 | NM_006861 | 14.79 |
| hypothetical protein DKFZp564O0523  | DKFZP564O0523 | AL136619 | 14.17 |
| triple functional domain (PTPRF interacting) | TRIO | NM_007118 | 14.12 |
| zinc finger and BTB domain containing 1 | ZBTB1 | NM_014950 | 13.75 |
| chemokine (C-C motif) ligand 5      | CCL5        | NM_002985    | 13.62                            |
| TRAF family member-associated NF-κB activator | TANK | BC003388 | 13.12 |
| Interleukin 8                       | IL8         | NM_005884    | 12.16                            |
| HSPC034 protein                    | LOC51668    | NM_016126    | 11.37                            |
| suppression of tumorigenicity       | ST7         | NM_013437    | 11.37                            |
| chemokine (C-X-C motif) ligand 2    | CXCL2       | NM_002089    | 10.77                            |
| *(B) Up-regulated at 24 h post-treatment* |
| transducin (beta)-like 2 isoform 1  | TBL2        | NM_012453    | 0.50                             |
| decorin isoform a preproprotein    | DCN         | NM_001920    | 0.50                             |
| nucleolar protein family A, member 2 | NOLA2 | NM_017838 | 0.50 |
| leukocyte immunoglobulin-like receptor | LLRB3 | NM_006864 | 0.50 |
| calpain 3 isoform a                 | CAPN3       | NM_000070    | 0.49                             |
| interferon, alpha 16                | IFNA16      | NM_002173    | 0.49                             |
| chromosome 5 open reading frame 4   | CSORF4       | NM_016348    | 0.49                             |
| tripartite motif-containing 45      | TRIM45      | NM_025188    | 0.49                             |
| A kinase (PRKA) anchor protein 7 isoform alpha | AKAP7 | NM_004842 | 0.49 |
| carbonic anhydrase IV precursor     | CA4         | NM_000717    | 0.49                             |
| uncoupling protein 3 isoform UCP3L  | UCP3        | NM_003356    | 0.49                             |
| NICE-4 protein                     | NICE-4      | NM_014847    | 0.48                             |
| alpha 2 type V collagen preproprotein | COL5A2 | NM_000393 | 0.48 |
| SWI/SNF-related matrix-associated actin-dependent regulator of chromatin a3 | SMARCA3 | NM_003071 | 0.48 |
| CD27-binding (Siva) protein isoform 1 | SIVA | NM_006427 | 0.48 |
| prostate derived STE20-like kinase PSK | PSK    | NM_016151 | 0.48 |
| stanniocalcin 2                     | STC2        | NM_003714    | 0.48                             |
| arginine-glutamic acid dipeptide (RE) repeats | RERE | NM_012102 | 0.48 |
| stimulated by retinoic acid gene 6  | FLJ12541    | NM_022369    | 0.48                             |
| zinc finger protein 228             | ZNF228       | NM_013380    | 0.48                             |

### Fold Change (From 0.49 to 0.47-fold)

| Gene Name                          | Common Name | Accession No | Fold Change |
|------------------------------------|-------------|--------------|-------------|
| protein tyrosine phosphatase, non-receptor type 2 | PTPN2 | NM_002828 | 0.48 |
| solute carrier family 26, member 4 | SLC26A4 | NM_000441 | 0.48 |
| adaptor-related protein complex 4, sigma 1 subunit | AP4S1 | NM_007077 | 0.48 |
| zinc finger protein RINZF          | RINZF       | NM_023929    | 0.48 |
| calbindin 1, 28kDa                | CALB1       | NM_004929    | 0.48 |
| chromosome 9 open reading frame 10 | C9orf10 | AF214738 | 0.48 |
| secreted frizzled-related protein 1 | SFRP1 | NM_003012 | 0.48 |
| cholinergic receptor, nicotinic, alpha polypeptide 5 | CHRNA5 | NM_000745 | 0.48 |
| chorea acanthocytosis             | CHAC        | AB023703     | 0.48 |

*Fold Change: Fold Change in gene expression levels after treatment with [BF/S+L/Ep] compared to control conditions.*
up- or down-regulated genes, with 5.36- to 18.9-fold increase or 0.13- to 0.37-fold decrease in expression at 24 h were chosen for further evaluation (Table 2B). As shown in Table 3, among the groups of genes up- or down-regulated by cichoric acid versus \([BF/S+L/Ep]\) treatment for 24 h, an overlapping expression pattern was observed for 31 genes.

**Putative signaling networks/pathways involved in the modulatory effect of \([BF/S+L/Ep]\) on iDCs**

To identify the possible putative signal transduction pathways, we analyzed, for 4 hr and 24 hr treatments, the top 20 up-regulated genes in human iDCs in response to treatment with \([BF/S+L/Ep]\) using TRANSPATH software. Signal transduction pathways involving the MCP-1, IL-8, CCL5, JAK2 and TRIO genes with ≥ 5-fold change in expression on treatment (Table 1A) was identified. Signalizing pathway networking and gene function analyses led to the hypothesis that treatment of iDC with \([BF/S+L/Ep]\) may activate the cyclic AMP and PKC pathways leading to the regulation of a key downstream molecule, adenylate cyclase 8 (AC8), in a \(Ca^{2+}\) dependent manner (Figure 4).

**Construction of proteome maps and identification of differentially expressed proteins in human iDCs**

Treatment of iDCs with 75 μg/ml \([BF/S+L/Ep]\) or 50 μg/ml cichoric acid resulted in differential expression of 43 proteins (Figures 5 and Table 4) identified by MALDI-TOF-MS. These 43 proteins were further categorized by function with use of the Swiss-Prot database. Most of the proteins responding to \([BF/S+L/Ep]\) (30.2%) were cell growth- or maintenance-related proteins (Figure 6); these included structural and cytoskeleton proteins such as macrophage-capping protein, cofilin, profilin, F-actin capping protein β subunit, and laminin A/C (Table 4). Other major classes were energy pathway-related proteins or metabolic enzymes (26.4%), proteins involved in protein metabolism or degradation (18.9%), and ion channel/transport proteins (13.2%). A small number were related to signal transduction and cell communication (5.7%), and others were associated with the regulation of nucleotides and nucleic acid metabolism (5.7%). Some proteins of interest include oxidative stress-related proteins (superoxide dismutases [Mn, Cu and Zn], catalase and peroxiredoxin 6) and cytoskeleton related proteins (gelsolin, macrophage capping protein, actin related pro-
### Table 2: Effect of cichoric acid on gene expression in human iDCs

| Gene Name | Common Name | Accession No | Treatment/Control (From 18 to 2.6-fold) |
|-----------|-------------|--------------|----------------------------------------|
| discs, large (Drosophila) homolog 1 | DLG1 | NM_004087 | 18.24 |
| pre-B-cell leukemia transcription factor 2 | PBX2 | NM_002586 | 6.45 |
| inhibitor of DNA binding 4, dominant negative helix-loop-helix protein | ID4 | NM_001546 | 4.22 |
| contactin associated protein-like 2 | CNTNP2 | NM_014141 | 4.00 |
| DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 6 (RNA helicase, 54 kDa) | DDX6 | NM_004397 | 3.74 |
| guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1 | GNAI1 | NM_002069 | 3.66 |
| tropomodulin 3 (ubiquitous) | TMOD3 | NM_014547 | 3.60 |
| tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor) | TFPI | NM_002572 | 3.13 |
| tolloid-like 1 | TLL1 | NM_012263 | 3.09 |
| RAB3 GTase-activating protein | RAB3GAP | XM_040048 | 3.08 |
| histone 1, H2a | HIST1H2AL | NM_003511 | 3.07 |
| solute carrier family 4, sodium bicarbonate cotransporter, member 7 | SLC4A7 | NM_003615 | 3.00 |
| fibulin 1 | FBLN1 | NM_005286 | 2.97 |
| cytochrome P450, family 4, subfamily A, polypeptide 8 | CYP2C8 | NM_030878 | 2.86 |
| peptidyl-prolyl isomerase G (cyclophilin G) | PPIG | NM_004792 | 2.80 |
| Nijmegen breakage syndrome 1 (nibrin) | NBS1 | AK001017 | 2.76 |
| NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2, 8 kDa | NDUF2 | NM_004546 | 2.71 |
| cytosolic ovarian carcinoma antigen 1 | COVA1 | NM_006375 | 2.69 |
| zinc finger protein 305 | ZNF305 | NM_014724 | 2.67 |
| platelet-activating factor acetylhydrolase, isoform Lb, beta subunit 30 kDa | PAFH1B2 | NM_002572 | 2.60 |

#### (A) Up-regulated at 4 h post-treatment

- **Down-regulated at 4 h post-treatment (From 0.23 to 0.45-fold)**

| Gene Name | Common Name | Accession No | Fold Change |
|-----------|-------------|--------------|-------------|
| glycerol kinase | GK | NM_000167 | 0.23 |
| SMC4 structural maintenance of chromosomes 4-like 1 (yeast) | SMC4L1 | AL136877 | 0.31 |
| ATPase, Ca²⁺-transporting, type 2C, member 1 | ATP2C1 | AB037768 | 0.36 |
| megakaryocyte-associated tyrosine kinase | MKT | NM_002378 | 0.37 |
| ubiquitin specific protease 24 | USP24 | XM_165973 | 0.37 |
| SEC24 related gene family, member D (S. cerevisiae) | SEC24D | NM_014822 | 0.38 |
| talin 1 | TNN | NM_006289 | 0.39 |
| methionyl aminopeptidase 2 | METAP2 | NM_006838 | 0.39 |
| protein kinase, cAMP-dependent, regulatory, type II, beta | PRKAR2B | NM_002736 | 0.42 |
| endothelial differentiation, G-protein-coupled receptor, 2 | EDG2 | NM_001401 | 0.42 |
| integrin, beta 8 | ITGB8 | NM_002214 | 0.42 |
| uridine monophosphate synthetase | UMP | NM_000171 | 0.42 |
| ribosomal protein L13 | RPL13 | AA789278 | 0.43 |
| DNA cross-link repair 1B | DCLRE1B | NM_022836 | 0.43 |
| chloride channel, calcium activated, family member 2 | CLCA2 | NM_006375 | 0.43 |
| caspase 2, apoptosis-related cysteine protease | CASP2 | NM_032982 | 0.44 |
| apoptotic chromatin condensation inducer in the nucleus | AAIN | NM_014724 | 0.45 |
| peptidoglycan recognition protein-l-beta precursor | PGLYRP1BETA | NM_020393 | 0.45 |

#### (B) Up-regulated at 24 h post-treatment

| Gene Name | Common Name | Accession No | Fold Change |
|-----------|-------------|--------------|-------------|
| Zinc finger protein 229 | ZNF229 | AC084239 | 18.93 |
| adaptor-related protein complex 4, sigma 1 subunit | AP4S1 | NM_007077 | 14.10 |
| chromosome 8 open reading frame 17 | C8orf17 | NM_020237 | 12.13 |
| DKKF2P566K023 protein | DKKF2P566K023 | NM_015485 | 11.07 |
| glycine receptor, alpha 1 | GLRA1 | NM_000171 | 10.81 |
| frizzled homolog 3 (Drosophila) | FZD3 | NM_017412 | 10.31 |
| secreted frizzled-related protein 1 | SFRP1 | NM_003012 | 10.11 |
| chorea acanthocytosis | CHAC | AB032320 | 9.56 |
| actin, alpha, cardiac muscle | ACTA1 | NM_055159 | 9.51 |
| tripartite motif-containing 45 | TRIM45 | NM_055189 | 8.26 |
| phosphodiesterase 4D, cAMP-specific | PDE4D | AP012073 | 8.23 |
protein 3, coronin-1C, beta adducin, vimentin, WD-repeat protein 1, T-complex protein 1 epsilon subunit, T-complex protein 1-beta subunit, cofilin, F-actin capping protein beta subunit 2, actin-related protein 2/3 complex subunit 2, profilin-1). These specific proteins may warrant further evaluation because of their apparent roles in modulation of DC maturation and function.

Confirmation of up- or down-regulation of specific proteins

Western blot analysis was conducted to confirm some of the up-regulated proteins observed from proteomic analysis. The expression of Mn-SOD (SOD2) was increased 2.65- to 1.99-fold with \( [BF/S+L/Ep] \) and 2.35- and 1.67-fold with cichoric acid treatment at 12 and 24 h, respectively, as compared with vehicle controls, which is consistent with results from 2-D gel electrophoresis (Figure 7, Table 4). The levels of cofilin determined by 2-D gel electrophoresis (Figure 8A) were slightly decreased, by 0.75- and 0.84-fold, with \( [BF/S+L/Ep] \) and greatly decreased, by 0.09- and 0.42-fold, with cichoric acid treatment at 12 or 24 h, respectively. Interestingly, confirmation by Western blot analysis revealed only a 0.5- and 0.32-fold decrease in cofilin level at 12 and 24 h with cichoric acid treatment (Figure 8B). Both assay systems revealed reduced effect of \( [BF/S+L/Ep] \) on cofilin expression in iDCs (0.75- and 0.84-fold, respectively) (Figure 8A Vs 8B). Nevertheless, these results confirmed our finding that the expression of cofilin in iDCs can be substantially reduced by treatment with cichoric acid and may be slightly inhibited by \( [BF/S+L/Ep] \). Rutin, another major index component of the \( [BF/S+L/Ep] \), effectively inhibited the expression of cofilin, by 0.4- and 0.6-fold at 12 and 24 h, respectively, in iDCs (Figure 8B). Treatment with 100 ng/ml lipopolysaccharide (LPS, used as a positive control) conferred an approximately 0.5- to 0.4-fold decrease in cofilin expression as compared to controls (Figure 8B).

Discussion

Results of some clinical studies are controversial about the effects of the traditional herbal medicine *Echinacea* extracts [14-19], even though recent studies seemed to provide some support for its possible beneficial effects for treatment of the common cold [20,21]. In addition, studies from animal experiments have shown that alkamides form *Echinacea* extracts may confer immune-modulatory activities [35,36]. Among these different observations, we believe it is important to evaluate systematically the specific and multiple effects of *Echinacea* phytocompounds on human immune cells, at the cellular and molecular
levels. Previously, we reported that un-fractionated, crude extracts of *E. purpurea* plant tissues could affect specific cell-surface markers of human DCs [25], which suggests the potential of using human DCs as an experimental system for in-depth study of the effect of *Echinacea* phyto-compounds on important human immune cell systems.

In the current study, we evaluated and characterized the possible immune-modulatory effect(s) of phytocompounds from *E. purpurea*, as an organic solvent-fractionated phytocompound mixture or a single major component (cichoric acid), on human DCs. Using transcriptome and proteome experimental approaches, we analyzed the global and differential gene expression patterns at the mRNA and protein levels in human monocyte-derived iDCs treated with test phytocompounds.

Previous studies have demonstrated that DCs play important roles in initiating and regulating innate as well as adaptive immunities [22]. Under normal conditions, DCs are present in most tissues in a relatively immature state, but with inflammation, irritation or danger signals from foreign antigens or environment, iDCs undergo rapid changes and initiate a cascade of activities to defend the body system. The phenotypic and functional characteristics of DCs are intimately and dynamically linked to their stage of differentiation and maturation [37]. Human DCs

| Genes up-regulated                                      | Genebank  | Fold Change in Treatment with [BF/S+L/Ep] (From 23.9 to 2.7-fold) | Fold Change in Treatment with Cichoric Acid (From 14 to 3.1-fold) |
|--------------------------------------------------------|-----------|-----------------------------------------------------------------|-----------------------------------------------------------------|
| adaptor-related protein complex 4                      | NM_007077 | 23.91                                                           | 14.10                                                           |
| secreted frizzled-related protein 1                    | NM_003012 | 10.17                                                           | 10.11                                                           |
| cholinergic receptor                                    | NM_00745  | 9.95                                                            | 6.02                                                            |
| chorea acanthocytosis                                   | AB023703  | 9.91                                                            | 9.56                                                            |
| tripartite motif-containing 45                         | NM_025188 | 8.34                                                            | 8.26                                                            |
| zinc finger protein (ZFD25)                            | NM_016220 | 5.27                                                            | 5.35                                                            |
| SUMO-1-specific protease                               | NM_015571 | 5.02                                                            | 4.56                                                            |
| nuclear respiratory factor 1                           | NM_005011 | 4.97                                                            | 3.39                                                            |
| pleckstrin homology-like domain                         | NM_007350 | 4.79                                                            | 3.36                                                            |
| protein phosphatase 2                                  | NM_004576 | 4.56                                                            | 6.75                                                            |
| chromosome 19 open reading frame 2                     | NM_003796 | 3.91                                                            | 3.27                                                            |
| SMCS structural maintenance of chromosomes 5-like 1 (yeast) | NM_015110 | 3.87                                                            | 5.78                                                            |
| myeloid/lymphoid or mixed-lineage leukemia              | NM_004641 | 3.70                                                            | 5.34                                                            |
| aryl hydrocarbon receptor interacting protein-like 1   | NM_014336 | 3.60                                                            | 3.70                                                            |
| left-right determination, factor B                      | NM_020997 | 3.53                                                            | 4.14                                                            |
| ATP-binding cassette, sub-family A                     | NM_005502 | 3.35                                                            | 5.36                                                            |
| RBP1-like protein                                      | NM_016374 | 3.07                                                            | 3.26                                                            |
| solute carrier family 26, member 10                    | NM_133489 | 2.89                                                            | 3.05                                                            |
| insulin induced gene 1                                 | NM_00542  | 2.87                                                            | 3.12                                                            |
| ceroid-lipofuscinosis, neuronal 8                       | NM_018941 | 2.72                                                            | 3.10                                                            |

| Genes down-regulated                                   | Gene bank (From 0.08 to 0.38-fold) | (From 0.13 to 0.4-fold) |
|--------------------------------------------------------|-------------------------------------|------------------------|
| Interferon-inducible protein p78 (mouse)               | NM_002462 0.09                     | 0.13                   |
| Interferon-inducible protein 44                        | NM_006417 0.16                     | 0.16                   |
| Interferon, alpha-inducible protein (clone IFI-1SK)    | NM_005101 0.19                     | 0.20                   |
| Mitochondrial ribosomal protein S14                   | NM_022100 0.27                     | 0.31                   |
| 2'-5'-oligoadenylate synthetase 2, 69/71 kDa          | NM_016817 0.27                     | 0.24                   |
| Myxovirus (influenza virus) resistance 2 (mouse)       | NM_002463 0.29                     | 0.30                   |
| XIAP associated factor-1                               | NM_017523 0.30                     | 0.33                   |
| SPI10 nuclear body protein                             | NM_080424 0.33                     | 0.35                   |
| Signal transducer and activator of transcription 1, (91 kDa) | NM_007315 0.33 | 0.29                   |
| zinc finger antiviral protein                          | NM_020119 0.34                     | 0.36                   |
| Solute carrier family 35                               | NM_012243 0.36                     | 0.38                   |

Human immature DCs were treated for 24 h with test samples and assayed for differential gene expression.
have been recently recognized as a promising cell system for ex vivo/in vitro tissue culture manipulations for potential clinical application as tumor vaccines for cancer therapy [23].

Initially, we used the expression of a highly specific cell-surface and DC maturation maker, CD83, as a key indicator for assessing possible immune modulatory effects of Echinacea extracts on human DCs. The butanol fraction [BF/S+L/Ep] significantly stimulated but the ethylacetate (EA) fraction greatly inhibited CD83 expression in iDCs, which suggests a possible immune modulatory effect of [BF/S+L/Ep] on the maturation of iDCs and that different sub-fractions of the phytocompound mixtures/herbal extracts could confer different or even opposite biological activities. These findings thus strongly suggest the urgent need for a clear, specific and stringent definition(s) of the extraction, fractionation and plant-tissue usage of the medicinal herb preparations and the derived phytocompounds for both laboratory experimental studies and commercial applications. The lack of such definition and information has already raised public concern.

Since our results on CD83 protein expression suggested that the [BF/S+L/Ep] may stimulate the maturation of iDCs, we subjected this butanol fraction to HPLC analysis to characterize the major components and some possible active compounds. We identified seven specific phytocompounds, namely hypoxanthine, caffeic acid, chlorogenic acid, cichoric acid, rutin, quercetin 3-O-rhamnosyl-(1→6)-galactoside, and kaempferol 3-O-rhamnosyl-(1→6)-galactoside from the [BF/S+L/Ep] fraction. The content of two major compounds cichoric acid (4) and rutin (7) were quantified, and this data could be used as chemical fingerprints and index compounds for future study and standardization of the bioactive [BF/S+L/Ep] fraction of E. purpurea. Potential future application of the [BF/S+L/Ep] fraction for use as remedy, nutraceutical or food supplement would have to wait until future studies are carried out with the considerations of appropriate dosage, bio-activity and defined immuno-modulatory functions to be assayed under in vivo conditions.

Use of the Affymetrix microarray analysis revealed approximately 10% of the whole human genome effectively expressed in DCs (data not shown). A total of 400 genes (≈18% of the expressed genes) were differentially or specifically regulated during the differentiation or maturation of iDCs. The top 20 genes with significantly up-regulated expression (> 3-fold change) at 4 h post-treatment with [BF/S+L/Ep] include some important early-responsive chemokine genes such as CCL2, CCL5 and IL-8, previously reported to play important roles in inflammatory responses [38]. Although treatment with cichoric acid did not affect CD83 expression, as was observed for [BF/S+L/Ep], it nonetheless up-regulated a similar subset of early response genes. Additional analysis of genes with overlapping expression on treatment with both [BF/S+L/Ep] and cichoric acid revealed that some of the interferon-inducible proteins, including IFI44, IFI78 (Table 3), IFIT1 and the interferon alpha-inducible protein (Table 1B), were all down-regulated at 24 h post-treatment. Thus, several key chemokine genes are likely involved in the early response to treatment with specific Echinacea phytocompounds, and another set of interferon-inducible genes belong to the group of late-responsive genes. We also per-
Table 4: Up- or down-regulation of proteins in human iDCs in response to treatment with [BF/S+L/Ep] or cichoric acid

| Protein Name                                      | Protein Expression Ratio | Accession Number |
|---------------------------------------------------|--------------------------|------------------|
|                                                   | [BF/S+L/Ep] (75 ug/ml)    |                  |
|                                                   | 12 h                     | 24 h             |
|                                                   | Chichoric Acid (50 ug/ml) |                  |
|                                                   | 12 h                     | 24 h             |
|                                                   | (in 0.1% DMSO, Untreated) |                  |
|                                                   |                          |                  |
| **Up-regulation in protein expression**           |                          |                  |
| Gelsolin                                          | 1.12                     | 1.1             |
|                                                   | 2.37                     | 5.09            |
| Macrophage capping protein                        | 1.39                     | 0.97            |
|                                                   | 1.13                     | 1.04            |
|                                                   | 1.31                     | 1.34            |
| Actin-related protein 3                           | 0.97                     | 0.71            |
|                                                   | 1.74                     | 1.1             |
| Coronin-IC                                        | 2.55                     | 1.1             |
| Beta aducing                                      | 1.1                      | 1.49            |
|                                                   | 1.34                     | 1.69            |
| Rho GDP-dissociation inhibitor I                  | 0.94                     | 0.16            |
|                                                   | 0.83                     | 1.49            |
| Rab GDP dissociation inhibitor beta               | 0.9                      | 1.37            |
|                                                   | 0.88                     | 1.46            |
| T-complex protein 1, epsilon subunit              | 1.29                     | 1.03            |
|                                                   | 1.17                     | 1.44            |
| Serum albumin [Precursor]                         | 1.24                     | 1.1             |
|                                                   | 1.23                     | 0.87            |
| Vimentin                                          | 2.44                     | 0.77            |
|                                                   | 1.1                      | 0.7             |
| Protein disulfide-isomerase A3 [Precursor]        | 1.09                     | 1.21            |
|                                                   | 0.93                     | 1.03            |
| Carbonic anhydrase II                             | 1.76                     | 1.16            |
|                                                   | 1.32                     | 1.01            |
| Catalase                                          | 1.14                     | 1.21            |
|                                                   | 1.28                     | 1       |
| Superoxide dismutase [Mn], mitochondrial          | 1.72                     | 1.23            |
|                                                   | 1.09                     | 0.84            |
| Peroxiredoxin                                    | 1.1                      | 1.13            |
|                                                   | 1.11                     | 1.31            |
| Biliverdin reductase A [Precursor]                | 1.21                     | 1.05            |
|                                                   | 1.32                     | 1.27            |
| Poly(rC)-binding protein 1                        | 0.87                     | 1               |
|                                                   | 0.79                     | 1.31            |
| WD-repeat protein 1                                | 1.54                     | 1.17            |
|                                                   | 1.74                     | 1.47            |
| TFIIH basal transcription factor complex helicase subunit | 0.88                     | 1.06            |
|                                                   | 0.86                     | 1.36            |
| Chloride intracellular channel protein 1          | 0.98                     | 1.04            |
|                                                   | 0.94                     | 1.32            |
| Potassium voltage-gated channel subfamily H member 5 | 0.89                     | 1.07            |
|                                                   | 1.14                     | 1.68            |
| **Down-regulation in protein expression**         |                          |                  |
| Cofilin, non-muscle isoform                       | 0.75                     | 0.84            |
|                                                   | 0.09                     | 0.42            |
| F-actin capping protein beta subunit              | 0.99                     | 1.09            |
|                                                   | 0.87                     | 1.23            |
| Actin-related protein 2/3 complex subunit 2       | 0.83                     | 0.53            |
|                                                   | 0.94                     | 0.58            |
| Profilin-I                                        | 0.84                     | 0.76            |
|                                                   | 0.6                      | 0.51            |
| Lamin A/C                                        | 0.81                     | 0.69            |
|                                                   | 0.45                     | 0.76            |
| Phosphoglycerate mutase 1                         | 1.3                      | 0.83            |
|                                                   | 0.89                     | 1.76            |
| Superoxide dismutase [Cu-Zn]                     | 0.84                     | 0.99            |
|                                                   | 0.78                     | 1.48            |
| Dihydrolipooyl dehydrogenase, mitochondrial [Precursor] | 0.97                     | 0.61            |
|                                                   | 1.12                     | 0.82            |
| Galactose mutarotase                              | 1.08                     | 1.05            |
|                                                   | 1.32                     | 1.46            |
| Delta3, 5-delta2, 4-dienoyl-CoA isomerase, mitochondrial [Precursor] | 1.24                     | 0.84            |
|                                                   | 1.23                     | 0.22            |
| Dihydropyrimidinase related protein-2             | 0.96                     | 1.22            |
|                                                   | 0.77                     | 1.74            |
| 26S proteasome non-ATPase regulatory subunit 13    | 0.94                     | 1.32            |
|                                                   | 0.95                     | 1.42            |
| 60S acidic ribosomal protein P0                    | 1.07                     | 1.02            |
|                                                   | 0.7                      | 1.13            |
| Heterogeneous nuclear ribonucleoprotein H         | 1                        | 0.7             |
|                                                   | 0.74                     | 0.52            |
|                                                   |                          |                  |
formed gene-clustering analysis to classify the gene expression patterns in human iDCs. A total of 382 genes were grouped into six clustering sets on the basis of their expression in response to [BF/S+L/Ep] (Figure 3). In contrast, cichoric acid and vehicle treatment produced no clustering differences, which suggests the lack of a multidomain or substantial effect of cichoric acid on most expressed genes in iDCs.

We have identified a hypothetical signaling network involving [BF/S+L/Ep]-induced early response genes such as CCL2 (MCP-1), IL-8, CCL5, JAK2 and TRIO (Figure 4). This signaling network revealed possible activation of cAMP and PKC pathways leading to the regulation of AC8 through a Ca\(^{2+}\) receptor, calmodulin. Previous studies have demonstrated that activation of cAMP induces DC maturation and migration [39]. PKC plays an important role in DC differentiation and antigen presenting function [40]. In addition, Ca\(^{2+}\) dependent pathways have been shown to regulate DC maturation and migration [41]. Our results on increased expression of CD83 maturation marker coupled with candidate signal transduction pathways strongly suggest that [BF/S+L/Ep] may enhance the iDC maturation and function, which warrants further systematic investigation.

2-D gel electrophoresis revealed 100 of the 1,300 detectable protein spots (~7.7%) affected in iDCs after 12- or 24-h treatment with [BF/S+L/Ep]. We suggest that this 7.7% change in iDC protein levels may reflect a marked change in protein expression, since the other cell types we have studied in parallel (e.g., primary T-cells) showed only a 3% to 5% change (data not shown). We observed significant up-regulation of antioxidant defense enzymes such
as Mn-SOD, catalase and peroxiredoxin 6 in iDCs in response to [BF/S+L/Ep] and cichoric acid. Endogenous antioxidants have been reported to play key role in dendritic cell survival, and ability to induce T cell activation and regulate the polarity of immune responses [42,43]. Several cytoskeletal and actin-binding proteins (Table 4) were significantly up-regulated in [BF/S+L/Ep] or cichoric acid treated iDCs. In DC, the changes in actin cytoskeleton components are essential for the formation of its characteristic dendrites and veils, as well as an immunological synapse necessary for antigen presentation [44]. In addition, actin cytoskeleton rearrangement is important for the motility and migration of cells and may influence the migration of DC to lymph nodes during their maturation [45]. Results from 2-D gel electrophoresis (Figures 7A and 8A) and Western blot analyses (Figures 7B and 8B) confirmed in general the trend of changes in differential expression of Mn-SOD and cofilin. We therefore hypothesize that a specific group of phyto-compounds in Echinacea may turn on the antioxidant defense system and regulate the rearrangement of cytoskeleton, which in turn may contribute to the positive enhancement of the immunemodulatory activities of DCs.

The present study has identified a pool of known or unknown genes associated with the differential expression of a spectrum of cytokines and chemokines and other immune cell activities in response to treatment with Echinacea compounds in DCs (Tables 1 to 4, and Figure 3). In addition, changes in level of molecules related to cell adhesion, immune-response and antigen presentation were observed in this human DC model, providing candidate target genes/proteins for future cross-talk studies of the biology of human DCs. These differentially expressed genes and proposed candidate signaling pathways/networks obtained via bioinformatics approaches have provided us with useful potential clues and molecular targets for future studies of molecular mechanisms underlying specific immune modulatory effect(s) of important medicinal herb Echinacea purpurea and derived phytocompounds in iDCs.

**Conclusion**

Since dendritic cells are well characterized and recognized as potent antigen presenting cells and have been shown to play critical roles in both innate and adaptive immunities, we have employed human DCs for functional genomics studies to evaluate possible immuno-modulatory effects.
Confirmation of the 2-D gel proteomic results on up-regulation of Mn-SOD protein expression with Western blot analysis. (A) Differential expression of Mn-SOD protein in iDCs treated with [BF/S+L/Ep] (75 μg/ml), cichoric acid (50 μg/ml) or vehicle control, at both 12 and 24 h post-treatment, as revealed by 2-D gel electrophoresis, SYPRO Ruby dye staining, and quantification by PDQuest analysis. The arrows indicate the Mn-SOD protein spots detected in different treatments. The expression ratio compared to control gel is shown in black. (B) Western blot analysis of expression of Mn-SOD and actin in human iDCs treated with phytocompounds. Total cellular proteins were extracted and separated by 10% SDS-PAGE, and Western blot analysis performed with anti-Mn-SOD or anti-actin antibodies. Actin was used as a sample-loading control. Similar results were obtained from three independent experiments.
Figure 8
Confirmation of the 2-D gel proteomic results on down-regulation of cofilin protein expression with Western blot analysis. (A) Differential expression of cofilin in human iDCs treated with [BF/S+L/Ep] (75 μg/ml), cichoric acid (50 μg/ml) or vehicle control, at 12 and 24 h treatment, as revealed by 2-D gel electrophoresis. (B) Western blot analysis of expression of cofilin in human iDCs. Other experimental details are the same as described in Figure 7. Similar trends were observed in three independent experiments.
of specific phytocompound mixtures from a popular medicinal herb extract, *Echinacea purpurea*. Specific and differential gene expression patterns were detected in time-course experiments of iDCs treated with the bioactive phytocompound fraction ([BF/S+L/Ep]) and its major index compound (cichoric acid). Transcriptome analyses showed significant up regulation of specific chemokine and cytokine genes, including CXCL2, CCL5, CCL2, IL-8, IL-1β and IL-18, within 4 h after [BF/S+L/Ep] treatment of iDCs. Bioinformatics studies suggested that a key molecular signaling network involving a number of the detected immune-modifier genes may lead to the activation of a downstream adenylate cyclase 8 activities, contributing to the observed cellular and molecular activities in test cells. Subsequent proteomic studies further showed that expression of specific antioxidant and cytoskeletal proteins, known to play important cellular physiological roles in DCs, are increased in cichoric acid or [BF/S+L/Ep]-treated cells. Based on these results, we conclude from this study that specific phytocompound mixtures present as major or active components in the test traditional herb may confer defined and significant immuno-modulatory effects on specific immune cell types. The candidate target molecules and molecular signaling mechanisms identified from this and previous study [25] provide us with useful information and hypothesis for future studies on characterization of human DCs in response to treatment with medicinal phytocompounds at the transcriptome and proteome levels.

**Methods**

**Plant materials and crude extract preparations**

*E. purpurea* plants at flowering stage were harvested from a reputable organic farm in Puli, Nantou County, Taiwan. Stem and leaf (S+L) tissues of fresh plants were extracted at room temperature by imbibition with 70% aqueous ethanol as previously reported [25]. The total 70% ethanolic extracts were concentrated (1 L) and successively partitioned with ethyl acetate (1 L × 3 times) and ethyl alcohol as previously reported [25]. The total 70% ethanolic extracts were concentrated (1 L) and successively partitioned with ethyl acetate (1 L × 3 times) and ethyl alcohol (1 L × 3 times) to yield three sub-fractions, designated as [BF/S+L/Ep] hereafter, was subjected to chromatography on RP-18 silica gel with the H2O/MeOH solvent system to yield four fractions. Fraction 1 was further purified by filtration though a Sephadex LH-20 (Amersham Pharmacia Biotech) column with a H2O/MeOH gradient (5-20% for 5-30 min, and 20-50% for 30-60 min) for isolation of single compounds. Quantification of the index compounds in the active [BF/S+L/Ep] fraction involved HPLC. The peak areas corresponding to the content of candidate compounds of a known concentration of the active fraction were calculated on the basis of a standard calibration curve of the individual index compounds. The HPLC profile and the relative content of the index compounds were routinely used for quality control.

**Flow cytometry**

At 24 h after treatment with *E. purpurea* crude extract, the S-L extract, or the three subfractions, iDCs were harvested and analyzed by immunoflourescence staining. Monoclonal antibodies labeled with fluorescent dyes i.e., HLADR-FITC, CD14-PE, CD32-PE, and CD83-FITC from Immunotech (Fullerton, CA) and CD86-FITC from Pharmingen (Fullerton, CA) were used. After incubation with specific antibodies at 4 °C for 30 min, cells were washed twice with PBS and fixed with 1% paraformaldehyde, and subjected to analysis using a Coulter EPICS XL flow cytometer (Beckman/Coulter, Durham, NC).

**Cell viability assay**

Human iDCs (4 × 10⁵ cells/ml) were incubated with 0.1% DMSO (vehicle control), test plant extract or derived phytochemicals in basal medium in 96-well plates for 24 h in a 5% CO₂ incubator. All treatments were performed in triplicate. Viability of iDCs was determined by MTT assay, and percentage cell viability was calculated as previously described [25]. The derived plant extracts from *E. purpurea* were examined by a Limulus Amebocyte Lysate (LAL) test (E-TOXATEâ Kits, Sigma, MO, USA) for detection of possible endotoxin (LPS) contamination before bioactivity assays.

**Phytocompound isolation**

The BuOH fraction of the S+L tissues of *E. purpurea*, designated as [BF/S+L/Ep] hereafter, was subjected to chromatography on RP-18 silica gel with the H2O/MeOH solvent system to yield four fractions. Fraction 1 was further purified by filtration though a Sephadex LH-20 (Amersham Pharmacia Biotech) column with a H2O/MeOH gradient solvent system to yield three sub-fractions. RP-18 high-performance liquid chromatography (HPLC) column (Phenomenex Luna, 5 µm C18, 250 × 10 mm) with a solvent system, 0.05% TFA/H₂O (solvent A) and 0.05% TFA/MeOH (solvent B) and gradient profile (5-5% for 0 to 5 min, 5-20% for 5-30 min, and 20-50% for 30-60 min) was used for isolation of single compounds. Quantification of the index compounds in the active [BF/S+L/Ep] fraction involved HPLC. The peak areas corresponding to the content of candidate compounds of a known concentration of the active fraction were calculated on the basis of a standard calibration curve of the individual index compounds. The HPLC profile and the relative content of the index compounds were routinely used for quality control.

**Chemical fingerprinting of the active [BF/S+L/Ep] fraction using LC/MS**

LC/MS was employed to analyze the chemical fingerprint of the [BF/S+L/Ep] fraction. LC/MS was carried out using a ThermoFinnigan LCQ Advantage ion trap mass spectrometer equipped with an Agilent 1100 series liquid
chromatographer, at the Metabolomics Core Facility of the Agricultural Biotechnology Research Center, Academia Sinica, Taiwan. Aliquot of a 5 μl sample of [BF/S+L/Ep] (10 mg/ml) was injected and analyzed at a flow rate of 0.2 ml/min. HPLC profiling involved an RP-18 column (Phenomenex Luna, 3 μm C18, 150 × 2 mm) and eluted compounds detected by UV absorbance at 254 and 330 nm before MS analysis. The solvent gradient for HPLC was 0.05% TFA/H2O (solvent A) and 0.05% TFA/MeOH (solvent B): 5–5% for 0 to 5 min, 5–20% for 5–30 min, 20–45% for 30–40 min, and 45–50% for 40–50 min. Ionization involved the positive ion mode for all analyses.

**DNA microarray analysis of differential gene expression patterns**

Total RNA was isolated by use of TRIzol® Reagent (Invitrogen) according to the manufacturer's instructions to generate cRNA targets. A total of 7 μg of RNA from each sample was used to synthesize the first-strand cDNA with T7-Oligo (dT) primer and T7 RNA polymerase by in vitro transcription reaction. The biotinylated cRNA products were then cleaned according to the Affymetrix protocol. Aliquots of 15 μg of total cRNA per sample were then hybridized to the Affymetrix gene chip HG-U133A containing approximately 22,283 probe sets following the manufacturer's instructions. Images were obtained by use of standard Affymetrix scanners. The cell intensities and detection from the nine hybridized oligonucleotide microarrays were derived by use of the Affymetrix Microarray Suite 5 (MAS 5.0) with default settings. Normalization was by dChip with the PM-MM model-based approach to obtain the expression indices for each probe set [46]. The pool of the replicate arrays, CM-00h-1 and CM-00h-2, was used as a baseline for normalization and was applied to the calculation of log2 ratios, M, for the test arrays. Comparative expression analysis of the two arrays by the MAS 5.0 system reported a level of “no change in gene expression pattern” of 98.0% (with a correlation of 0.9916). This high reproducibility of results indicates very high reliability and very low variation of our experimental data obtained from the in-house Affymetrix system. The gap statistic and K means analysis were used to distinguish gene clusters as described previously [47,48]. The putative signal transduction pathways were analyzed using TRANSPATH Professional 7.1 (BIOBASE Biological Databases, Germany). The microarray data obtained from this study have been deposited to the Gene Expression Omnibus database at NCBI (GEO; http://www.ncbi.nlm.nih.gov/projects/geo/) under the accession number GSE12259.

**2-D gel electrophoresis and image analysis**

Total cellular proteins of iDCs were extracted and prepared in 0.3 ml of lysis buffer [7 M urea, 2 M thiourea, 4% 3,3-cholamidopropyl-dimethylammonio-1-propanesulfonate (CHAPS), 10% 1,4-dithiothreitol (DTT), 2% Pharmalyte pH3-10] by vortexing for 1 h, and protein supernatants were collected after ultracentrifugation at 55,000 rpm for 1 h. Protein concentrations were determined using Bio-Rad BCA protein assay kit (Bio-Rad, Hercules, CA). 2-D gel electrophoresis involved the PROTEAN isoelectric focusing and electrophoresis system (Bio-Rad, Hercules, CA) with modification [49]. Briefly, 550 μg of protein was diluted in 300 μl of rehydration buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 2% 1,4-dithioerythritol, 0.13% BioIyte 5–8 (Bio-Rad) and 0.07% BioIyte 8–10 (Bio-Rad). Protein samples were loaded onto IPG strips (Bio-Rad; 17 cm, pH 5–8) through passive rehydration overnight. Proteins underwent electrophoresis focusing analysis in slow-ramping mode with a final voltage of 8,000 V for a total of 25,000 Vh. Following a two-step equilibration, IPG strips were processed by 10–16% gradient SDS-PAGE and electrophoretic separation at 200 V. Gels were then fixed and stained with SYPRO-Ruby fluorescent stain (Molecular Probes; Eugene, OR) according to the manufacturer's instructions. Stained gels were visualized by a Typhoon scanner system (Amersham). Digitalized gel images were analyzed by 2-D PDQuest, V.T.O. (Bio-Rad). Protein spots on gels were automatically detected with use of the PDQuest system. The 2-D gel images of iDCs proteins obtained with IPG strips (pH 5–8) were matched separately, with each match set containing 9 images, with 3 images for control replicates. The image with the highest number of spots was selected as the master gel. Automatically detected images of protein spots in test gels were then manually edited to include the low intensity spots and corrected for spot artifacts. For the match set containing images from pH 5–8, the spot volume (intensity integrated over the spot area) was normalized to the total volume of spots in the gel. Data were then exported to Microsoft Excel.

**Protein identification by MALDI-TOF-MS**

Protein extraction was performed as previously described [50]. Briefly, each gel spot was cut into small pieces with a scalpel, washed with 700 μl of double distilled H2O, and subjected to reduction reaction by DTT. Alkylation reaction then carried out by adding 55 mM iodoacetamide for 1 h at room temperature in the dark. Gel spots were washed with ammonium carbonate then acetonitrile. Gel pieces were dried and then immersed in trypsin solution. The in-gel digestion was performed at 37°C overnight. Resulting tryptic peptides were extracted twice from the test gel into 70% acetonitrile/5% HCOOH by sonication then centrifugation. The combined supernatant was dried under a Savant Speed Vac, and 6 μl of 1% HCOOH was added to each test sample. Protein identification by MALDI-Q-TOF-MS [51] was performed at the Proteomics Core Facility of the Institute of Biological Chemistry,
Western blot analysis
Total protein was isolated from test iDCs with ice-cold lysis buffer (150 mM NaCl, 0.5% Triton X-100, 50 mM Tris-HCl (pH 7.4), 20 mM EGTA, 1 mM dithiothreitol (DTT), and protease inhibitor cocktail tablets) for 10 min and centrifuged at 12,000 x g for 20 min. Protein samples were subjected to western blotting as described previously [52]. Briefly, the proteins were detected after overnight incubation at 4°C with 1:1000 dilution of Mn-SOD or cofilin polyclonal antibodies (Millipore, Bedford, MA, USA). Equal protein loading was assessed using mouse β-actin (Sigma Chemical Co., St. Louis, U.S.A). The proteins were visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham Pharma- cia Biotech, Buckinghamshire, UK).

Authors’ contributions
CYW served as key experimenter and author of the draft manuscript. VS improved the clarity by critical revision and reorganization of the manuscript, and incorporated some parts of the discussion section. MTC designed, optimized and completed the 2D gel/proteome experiments. CCH performed metabolite profiling and compound identification. HMW, KCY, CHC & PH performed bioinformatics analyses on DNA microarray data as a team. TNW introduced and built the proteome study system of the lab. LFS is Co-PI and Co-correspondent author of this project/manuscript. NSY is PI and principal author of the manuscript. All authors read and approved the final manuscript.

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