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ORIGINAL RESEARCH

Alpha-Tocopherol Alters Transcription Activities that Modulates Tumor Necrosis Factor Alpha (TNF-α) Induced Inflammatory Response in Bovine Cells[1]

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Abstract: To further investigate the potential role of α-tocopherol in maintaining immuno-homeostasis in bovine cells (Madin-Darby bovine kidney epithelial cell line), we undertook in vitro experiments using recombinant TNF-α as an immuno-stimulant to simulate inflammation response in cells with or without α-tocopherol pre-treatment. Using microarray global-profiling and IPA (Ingenuity Pathways Analysis, Ingenuity® Systems, http://www.ingenuity.com) data analysis on TNF-α-induced gene perturbation in those cells, we focused on determining whether α-tocopherol treatment of normal bovine cells in a standard cell culture condition can modify cell's immune response induced by TNF-α challenge. When three datasets were filtered and compared using IPA, there were a total of 1750 genes in all three datasets for comparison, 97 genes were common in all three sets; 615 genes were common in at least two datasets; there were 261 genes unique in TNF-α challenge, 399 genes were unique in α-tocopherol treatment, and 378 genes were unique in the α-tocopherol plus TNF-α treatment. TNF-α challenge induced significant change in gene expression. Many of those genes induced by TNF-α are related to the cells immune and inflammatory responses. The results of IPA data analysis showed that α-tocopherol-pretreatment of cells modulated cell's response to TNF-α challenge. In most of the canonical pathways, α-tocopherol pretreatment showed the antagonistic effect against the TNF-α-induced pro-inflammatory responses. We concluded that α-tocopherol pre-treatment has a significant antagonistic effect that modulates the cell's response to the TNF-α challenge by altering the gene expression activities of some important signaling molecules.

Keywords: alpha-tocopherol, bovine, inflammatory response, transcription, tumor necrosis factor

Introduction

Vitamin E is one of the most commonly used single nutrient supplements. After years of research, the physiological functions of vitamin E still remain mysterious in many ways. Recent data has suggested that α-tocopherol is not only an antioxidant but also a regulator of gene expression through its binding to nuclear receptors.[1,2] Evidence also indicates vitamin E (α-tocopherol) plays a role in cell homeostasis that occurs through the modulation of specific signaling pathways and genes related to cell proliferation, metabolic, inflammatory and antioxidant functions. We recently reported global expression profiling and pathway analysis on α-tocopherol-induced gene perturbation in bovine cells.[3] The data confirmed that α-tocopherol is a potent regulator of gene expression and it possesses novel transcriptional activities that affect essential biological processes including inhibition of cell proliferation. The genes identified fall within a broad range of functional categories and provide the molecular basis for its distinctive effects. The report also provided direct evidence that α-tocopherol is involved in maintaining immuno-homeostasis through targeting the C3 (Complement Component 3) gene. The data revealed that one of the characteristics of α-tocopherol and those genes perturbed by α-tocopherol treatment may play very important roles in biological functions.

Tumor necrosis factor-alpha (TNF-α) is a cytokine involved in inflammation-mediated biological defense functions[4] and is a multifunctional proinflammatory cytokine that belongs to the TNF superfamily. Factors such as viruses, parasites, other cytokines, and endotoxins induce TNF-α production. TNF-α is the principal mediator of the inflammatory response with diverse immune system functions, including antitumor activity, antimicrobial activity and mediation of inflammation. It also regulates a number of physiological functions.[5] Two distinct cell surface receptors, TNFR1 and TNFR2, are involved in TNF-α signal transduction. TNF-α and other cytokines activate NFκB, a transcription regulator that is activated by various intra and extra cellular stimuli. TNF-α induced inflammatory responses in cattle has been well documented.[6,7] To further investigate the potential role of α-tocopherol in maintaining immuno-homeostasis in bovine cells, we undertook in vitro experiments using recombinant TNF-α as an immuno-stimulant to imitate inflammation response in cells with or without α-tocopherol pre-treatment. Using global expression profiling and pathway analysis of TNF-α-induced gene perturbation in those cells, we focused on determining whether α-tocopherol treatment of normal bovine cells in a standard cell culture condition can modify cell's immune response to TNF-α stimulation. Here we report our findings regarding the functional category and pathway analysis of differential expressed genes in MDBK cells (The Madin-Darby bovine kidney epithelial cell line) in response to TNF-α challenge with or without treatment with α-tocopherol.

Materials and Methods
Cell culture, α-tocopherol treatments and TNF-α challenge

The MDBK cells (American Type Culture Collection, Manassas, VA; Catalog No. CCL-22) were cultured in Eagle's minimal essential medium and supplemented with 5% fetal bovine serum (Invitrogen, Carlsbad, CA) in 25 cm² flasks as described in our previous report.[8] At approximately 50% confluence (during the exponential phase), the cells were treated with α-tocopherol (designated concentration from 0 to 80 μM) for 24 h (α-tocopherol acid succinate, Sigma-Aldrich Chemie Gmbh, Steinheim, Germany). For cells prepared for microarray study, treatment of 40 μM of α-tocopherol was selected for our experiments after titration for cell responses. This concentration induces biological effects of cell cycle arrest without inducing significant amounts of cell death. It is also in a range of concentrations that were extensively used in many in vitro experiments.[9,10] Stock solution of 10 mM α-tocopherol (Sigma, T3126) was prepared by dissolving α-tocopherol in 100% alcohol. Lyophilized recombinant bovine TNF-α (Endogen® code: RBOTNFAI, PIETCE, Thermo Scientific) was reconstituted with cell culture medium plus 5% fetal bovine serum to 20 μg/mL. For immune challenge, TNF-α was added at 20 ng/mL concentration 16 hrs after cells were treated with or without α-tocopherol preparation. Four groups (control cells without any treatment, cells treated with α-tocopherol, cell treated with TNF-α, and cells first treated with α-tocopherol for 16 hrs and then challenged with TNF-α) and each

with three replicate flasks of cells for both treatment and control groups (a total of 12 samples) were used for the microarray experiments. The control cells were treated with the same amount of solvent without α-tocopherol.

Oligonucleotide microarray, hybridization, image acquisition, and data analysis

The bovine microarray platform used was described in our earlier paper.[11] A total of 86,191 unique 60-mer oligonucleotides were designed and synthesized in situ using photo deprotection chemistry.[12] Each unique oligonucleotide was repeated 4 times on the array (a total of ~340,000 features). These oligonucleotides represented 45,383 unique bovine sequences/genes, including 40,808 Tentative Consensus sequences (TCs) from the TIGR *Bos taurus* gene index (http://www.tigr.org) and 4,575 singletons. Hybridization, image acquisition, and data analysis was described in previous research.[13,14] The microarrays were scanned with an Axon GenePix 4000B scanner (Molecular Devices Corp., Union City, CA) at 5 µM resolution. The data was extracted from the raw images with NimbleScan software (NimbleGen, Madison, WI).

Isolation of total RNA

Total RNA was extracted with TRIzol® Plus RNA Purification Kit (Invitrogen) by following the manufacturer's recommendations. Trace genomic DNA in the crude total RNA samples was removed by incubation with 4–10 units DNase I per 100 µg total RNA (Ambion, Austin, TX) at 37 °C for 30 min. Total RNA was further purified with an RNeasy Mini kit (Qiagen, Valencia, CA). The concentration of the total RNA was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE) and RNA integrity was verified with a Bioanalyzer 1000 (Agilent, Palo Alto, CA).

Generation of biotin-labeled cRNA

Biotin-labeled cRNA was generated with a modified procedure of the Superscript Choice System (Invitrogen) for double-strand (ds) cDNA synthesis followed by in vitro transcription. Briefly, the 1st strand cDNA was synthesized from 4.0 µg total RNA with 1.0 unit SuperScript II reverse transcriptase (Invitrogen) in the presence of 100 pmoles T7 promoter Oligo dT primer.

After 2nd strand synthesis, the DNA was purified with a DNA Clean & Concentrator-5 kit (Zymo Research, Orange, CA) and eluted with 8 to 16 µl of deionized (dd) H_2O. The recovered ds cDNA was further concentrated down to 3 µl by a speed vacuum device. The cRNA was synthesized with a MEGAscript in vitro Transcription kit (Ambion). The in vitro transcription reaction was carried out in a total volume of 23.0 µl, which consisted of 3.0 µl ds cDNA, 2.3 µl 10X Ambion reaction buffer, 2.3 µl 10X Ambion T7 enzyme mix, and 15.4 µl NTP labeling mix (7.5 mM ATP, 7.5 mM GTP, 5.625 mM UTP, 5.625 mM CTP, and 1.875 mM biotin-16-UTP and 1.875 mM biotin-11 CTP). The in vitro transcription reaction was incubated at 37 °C for 16 hours in a thermocycler. The cRNA was purified with an RNeasy mini-kit (Qiagen). Generally, 40 to 60 µg of cRNA can be obtained from 4.0 µg of total input RNA. The size range of the cRNA, expected to be between 300 to 3000 bp with the maximum intensity centered at least at 1000 bp, was verified using a Bioanalyzer 1000. The biotinylated cRNA was fragmented into 50 to 200 bp pieces by heating the cRNA in a buffer consisting of 40 mM Tris-acetate, pH 8.0, 100 mM potassium acetate, and 30 mM magnesium acetate at 95 °C for 35 min.

Oligonucleotide microarray, hybridization, image acquisition and data analysis

The bovine microarray platform used was described previously with the detailed information about hybridization, image acquisition and data analysis of microarray.[3,13,14] Briefly, relative signal intensities ($\log_2$) for each feature were generated using the Robust Multi-Array Average (RMA) algorithm.[15] The data were processed based on quantile normalization method[16] using the R package (http://www.bioconductor.org). This normalization method aims to make the distribution of intensities for each array in a set of arrays the same. The method assumes that a quantile-quantile plot of two data vectors with the same distribution will have a straight diagonal line. The method performed better in dealing with bias and reducing variability across arrays comparing to other methods.[16] The background-adjusted, normalized, and log transformed intensity values were then analyzed using Significance Analysis of Microarrays method[17] with two-class unpaired design (SAM version 2.20

from http://www-stat.stanford.edu/~tibs/clickwrap/sam/academic/). SAM is the most popular method for microarray analysis.[18] SAM ranks genes based on a modified t-test statistic. The unique features of SAM are implementing permutation testing and ability to estimate a global false discovery rate (FDR, an expected percentage of false positives among the claimed positives) and a gene error chance (q-value). A sequence was declared to be significant when it met a stringent median false discovery rate (FDR) cutoff at 5%. Since there is no database for the bovine gene expression available, we set the threshold at P-value lower than 0.05 for genes that were up- or down-regulated from our microarray experiments as positively perturbered.

Functional and pathways analyses

To understand the molecular processes, the molecular functions and genetic networks following TNF-α challenge with or without α-tocopherol pre-treatment, the expression data were input into Ingenuity Pathways Analysis (IPA, Ingenuity® Systems, http://www.ingenuity.com), a web-delivered application that enables the discovery, visualization and exploration of molecular interaction networks in gene expression data. Ingenuity Pathways Analysis is a software application that enables biologists and bioinformaticians to identify the biological mechanisms, pathways and functions most relevant to their experimental datasets or genes of interest.[19–23]

Canonical pathway analysis of data sets

Analysis of canonical pathways identified the pathways from the IPA library of canonical pathways that were most significant to the data set. Genes from the data set that were associated with a canonical pathway in the Ingenuity Pathways Knowledge Base were considered for the analysis. The significance of the association between the data set and the canonical pathway was measured in two ways: (1) a ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway is displayed. (2) Fischer's exact test was used to calculate a P-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

Functional analysis of data sets

The Functional Analysis identified the biological functions and/or diseases that were most significant to the data set. Genes from the datasets that were associated with biological functions and/or diseases in the Ingenuity Pathways Knowledge Base were considered for the analysis. Fischer's exact test was used to calculate a P-value determining the probability that each biological function and/or disease assigned to that data set was due to chance alone.

Pathways analysis and network generation

A data set containing gene identifiers and corresponding expression values was uploaded into the application. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. These genes, called Focus Genes, were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these Focus Genes were then algorithmically generated based on their connectivity.

Functional analysis of a network

The Functional Analysis of a network identified the biological functions and/or diseases that were most significant to the genes in the network. The network genes associated with biological functions and/or diseases in the Ingenuity Pathways Knowledge Base were considered for the analysis. Fischer's exact test was used to calculate a P-value determining the probability that each biological function and/or disease assigned to that network was due to chance alone.

Network/pathways graphical representation

A network Pathway is a graphical representation of the molecular relationships between genes/gene products. Genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least one reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge Base. The intensity of the node color indicates the degree of up- (red) or down- (green) regulation.

Results

Microarray

We previously reported that after applying two differential layers of filtering to the normalized data, we identified 910 induced sequences that were enhanced greater than 2.0 fold and 273 sequences repressed greater than 1.5 fold in three biological replicates by α-tocopherol. Some redundant sequences on the microarray are apparent. Out of the genes surveyed (45,383), 1183 (910 + 273) were affected by α-tocopherol. This is approximately 2.60% (1183/45383) of the total genes surveyed. We also verified the microarray findings using Western blotting and RT-PCR.[3] Following the same approach, we challenged the cells with 20 ng/ml of recombinant bovine TNF-α for 24 hrs with or without 40 μM α-tocopherol pre-treatment for 16 hrs. Cells were collected and total RNA was prepared for microarray. Out of the genes surveyed (45,383), 788 (1.90%) were affected when cells were challenged with TNF-α without α-tocopherol treatment, while 1333 (2.67%) genes were affected when cells were challenged with TNF-α after α-tocopherol pre-treatment and 1197 (2.63%) genes were affected when cells were treated with α-tocopherol alone (Electronic Supplementary Material Tables S1, S2 and S3). Since there is no available database for bovine gene expression, genes that were up- or down-regulated with P-value < 0.05 from our microarray experiments, especially those genes homologous to their respective human gene counterparts approved by the HUGO Gene Nomenclature Committee (http://www.gene.ucl.ac.uk/nomenclature) and/or with known functions and pathways, were subject to the Ingenuity Pathway Analysis. We identified 967 genes from α-tocopherol treatment, 986 genes from TNF-α challenge with α-tocopherol pre-treatment, and 606 genes from TNF-α challenge without α-tocopherol pre-treatment. When three datasets were filtered and compared using IPA, there were a total of 1750 genes in all three datasets for comparison; 97 genes were common in all three sets; 615 genes were common in at least two datasets; there were 261 genes unique for TNF-α challenge, 399 genes were unique in α-tocopherol treatment, and 378 genes were unique in the α-tocopherol plus TNF-α treatment group (Fig. 1).
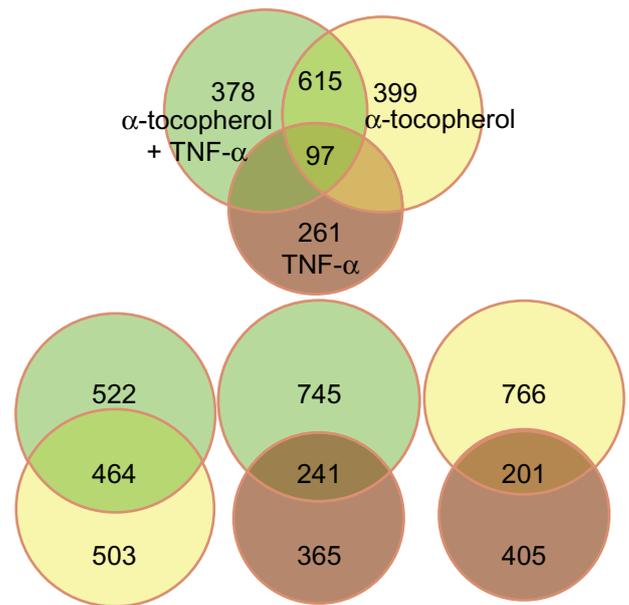

Figure 1. Venn diagrams show the comparison results of three datasets (α-tocopherol treatment, TNF-α challenge, and α-tocopherol pretreatment followed by TNF-α challenge, respectively). On the top is the comparison of all three datasets. In addition to the unique genes that were modified by the respective treatments, there were 97 genes common in all three datasets, and 615 genes that were at least common in two datasets. The lower panel shows three paired dataset comparisons. Green circle: TNF-α challenge with α-tocopherol pre-treatment; yellow circle: α-tocopherol treatment alone and brown circle: TNF-α challenge without pretreatment of α-tocopherol.

Canonical pathways analysis and comparison of datasets with IPA

The analysis of canonical pathways identified the pathways from the IPA library of canonical pathway that were most significant to the datasets. Comparing three datasets using Global Canonical Pathway analysis, the significance calculated for each canonical pathway is a measurement of the likelihood that the pathway is associated with the dysregulated genes by random chance. Interestingly, several pathways were significantly stimulated by TNF-α challenge and pre-treatment with α-tocopherol modified cell's response to the TNF-α challenge. Figure 2 shows the top 10 canonical pathways that were significantly regulated by TNF-α challenge and the comparison of site by site with the α-tocopherol-pre-treated cells, as well as cell's response when treated with both α-tocopherol and TNF-α. TNF-α challenge induced significant change in gene expression. Many of those genes perturbed by TNF-α are related to the cells immune and inflammatory responses (Supplement Table 2). It seems that α-tocopherol pre-treatment has
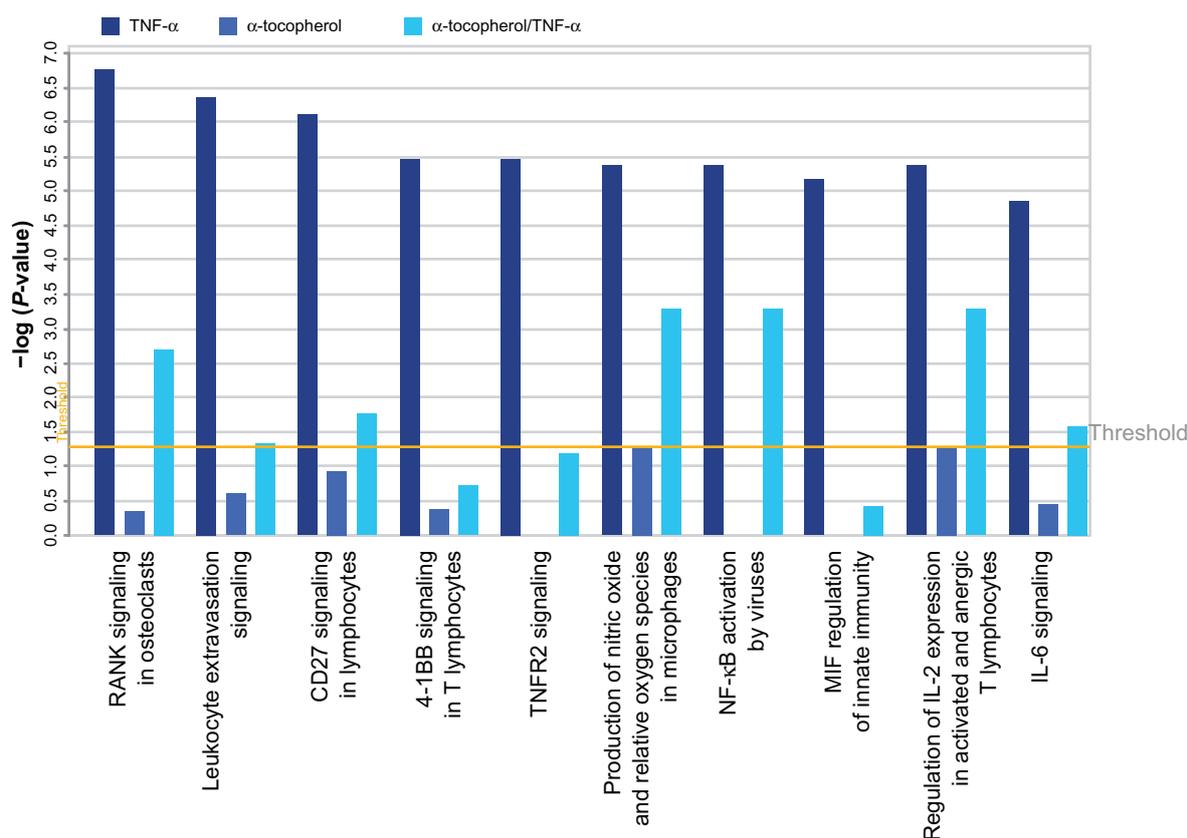
Figure 2. Global Canonical Pathway analysis: Comparison of three datasets (α-tocopherol treatment, TNF-α challenge, and α-tocopherol pretreatment followed by TNF-α challenge, respectively).
Notes: Data sets were analyzed by the Ingenuity Pathways Analysis software (Ingenuity® Systems, http://www.ingenuity.com). The significance is expressed as a *P*-value, which was calculated using the right-tailed Fisher's Exact Test. Figure shows ten major canonical signaling pathways. Threshold: $P < 0.05$.

a significant antagonistical effect that modulates the cells response to the TNF-α challenge.

To explore the details about those canonical pathways that were perturbed by the TNF-α challenge and then modified by pre-treatment of α-tocopherol, we selectively examined eight canonical pathways that are directly related to cell's immune and inflammatory responses (Table 1). TNF family members play important roles in various biological processing including cell proliferation, differentiation, cell death (apoptosis), and modulation of immune responses and induction of inflammation. There are two receptors that are responsible for TNF family member's activity. We observed that when cells were challenged with 20 ng/mL of TNF-α, both TNFR1 and TNFR2 signaling pathways were significantly perturbed (–log [*P*-value] 3.76 and 5.46, respectively; Table 1). In contrary, in cells pre-treated with α-tocopherol, changes in both TNFR1 and TNFR2 signaling pathway were not significant (–log [*P*-value] 1.19 and 1.4, respectively) in response to TNF-α challenge.

These findings indicate that α-tocopherol induced gene regulation activity is involved in maintaining cell immuno-homeostasis through moderating the signaling transduction pathways. Graphical representation of TNFR1 and TNFR2 canonical pathways are shown in Figures 3 and 4 for comparison. In both canonical pathways, the central point that controls the responses to the TNF-α challenge was the nuclear factor kappa B (NF-κB) transcription factors. These factors were significantly up-regulated when cells were challenged with TNF-α. However, when cells were pre-treated with α-tocopherol before the TNF-α challenge, this up-regulation was not detected. In addition to NF-κB, α-tocopherol pre-treatment of cells significantly altered cell response to the TNF-α challenge in terms of gene expression regulation. Table 1 detailed the comparison of the alterations in the gene expression in eight canonical signaling pathways.

Microphage migration inhibitory factor (MIF) is a cytokine that is an integral mediator of the innate immune system. MIF exercise potent pro-inflammatory

Table 1. Comparison of the gene-perturbation in major canonical pathways induced by TNF-α with or without α-tocopherol pre-treatments.

Ingenuity canonical pathways	TNF-α, –log (P-value)	α-tocopherol/TNF-α, –log (P-value)	TNF-α, modified genes	α-tocopherol/TNFα, modified genes
TNFR2 signaling	5.46E00	1.19E+00	RELA, FOS, NFKBIA, IKBKE, NFKB2, NFKBIB, NFKB1, BIRC3	MAP3K14, FOS, NFKBIA, BIRC3
MIF regulation of innate immunity	5.18E00	4.31E-01	PLA2G6, RELA, FOS, NFKBIA, MAPK1, CD74, NFKB2, NFKBIB, NFKB1	PLA2G6, FOS, NFKBIA
LPS-stimulated MAPK signaling	4.54E00	1.41E00	MAP2K6, RELA, FOS, NFKBIA, MAPK1, IKBKE, PIK3CB, LBP, NFKB2, NFKBIB, NFKB1	MAP2K6, MAP3K14, FOS, PRKCQ, NFKBIA, PIK3C3, PIK3CB, LBP
TNFR1 signaling	3.76E00	1.4E00	RELA, FOS, NFKBIA, IKBKE, NFKB2, NFKBIB, NFKB1, BIRC3	MAP3K14, FOS, MADD, NFKBIA, PAK6, BIRC3
PI3K/AKT signaling	3.76E00	5.97E-01	RELA, MAPK1, ITGA2, ITGA5, IKBKE, NFKB2, NFKB1, NFKBIA, PPP2R4, PIK3CB, MAP3K8, NFKBIB, CTNNB1	SHC1, BCL2L1, PPP2R1A, MAPK1, PPP2R4, TYK2, ITGA5, GSK3A
Toll-like receptor signaling	3.7E00	3.69E00	MAP2K6, RELA, FOS, NFKBIA, MAPK1, LBP, NFKB2, NFKB1	MAP2K6, MAP3K14, FOS, TLR1, TOLLIP, NFKBIA, TAB2, LBP, IRAK4, TAB1
ERK/MAPK signaling	2.87E00	9.57E-01	ETS1, PPP1CC, MAPK1, ITGA2, ITGA5, RAPGEF3, CRK, CREB5, BCAR1, PLA2G6, FOS, ARAF, PPP2R4, PIK3CB, ESR1	ETS1, PPP1CC, PAK6, DUSP6, PLCG1, CRK, TLN1, SHC1, PLA2G6, FOS, PPP2R1A, PIK3C3, PRKACA, PIK3CB
Leukocyte extravasation signaling	6.38E00	1.34E00	TIMP3, MAPK1, ACTA2, MMP13, RAPGEF3, CRK, BCAR1, TEC, ITGB2, CLDN8, VAV3, PECAM1, GRLF1, ARHGAP12, PIK3CB, CLDN2, VCL, CTNNB1, MMP9, ACTN1, ACTN3, MSN	MMP7, PRKCQ, ABL1, CTNNA1, PLCG1, MLLT4, CRK, CLDN8, RAP1GAP, VAV3, PIK3C3, CYBB, GRLF1, PIK3CB, CLDN2, VCL

activities. As a cytokine, it plays an important role in septic shock. MIF also regulates innate immune responses to endotoxin and gram-negative bacteria by regulating the TLR4 expression, an important component of the LPS receptor complex. Therefore it is not a surprise that TNF-α challenge activated the MIF signaling pathway (Figs. 1 and 5A and Table 1)

by up-regulating the expression of the CD74, ERK1/2 and cPLA2 genes. Moreover, NF-κB transcription factors and the canonical pathway were modified in response to the TNF-α challenge. In comparison (Fig. 5B), pre-treatment of cells with α-tocopherol before TNF-α challenge significantly altered cell's responses. As the results show, the changes included
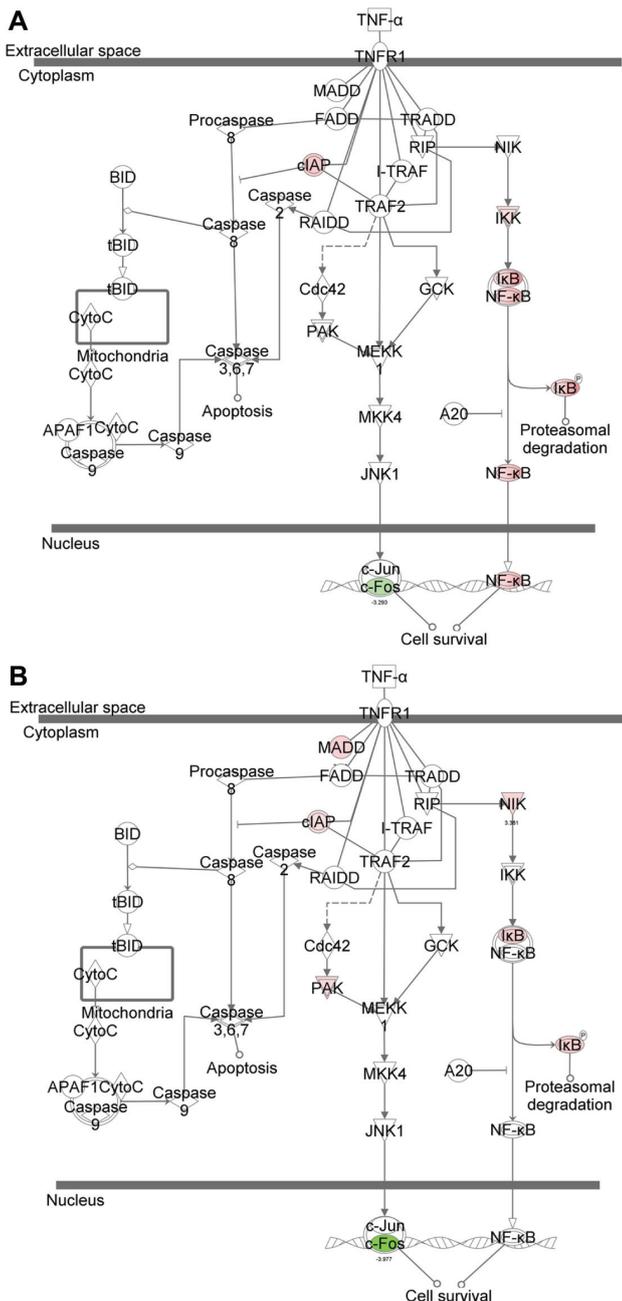
A

B

Figure 3. Graphical representations of TNFR1 signaling pathways and comparison of the gene- perturbation induced by the treatments. (**A**) TNF-α challenge without α-tocopherol pre-treatment; and (**B**) TNF-α challenge with α-tocopherol pre-treatment, respectively.
Notes: Red-notes and green-notes indicate up- and down-regulated genes, respectively. For simplication, α-tocopherol treatment is not shown.

a profound reduction of the expression of CD74, ERK1/2 and reduction of activation of NF-κB activity (Fig. 5B).

Perturbation of gene expression in LPS-stimulated MAPK signaling pathway also demonstrates that the α-tocopherol pre-treatment has antagonistical effects against TNF-α induced pro-inflammation response.

LPS-stimulated MAPK signaling is one of the apoptosis and pathogen-influenced signaling pathways. The key downstream for LPS-induced signaling is the MAPK signaling cascade, such as ERK1/2, p38 and SAPK/JNK. When cells were challenged by TNF-α, ERK1/2 and p38, as well as NF-κB were activated (Fig. 6A). In contrary, when cells were challenged with TNF-α after α-tocopherol pre-treatment, those genes did not up-regulate (Fig. 6B).

Functional analyses comparison of datasets

The functional category and pathway analysis of differentially expressed genes in cells treated with α-tocopherol, TNF-α challenge, and TNF-α challenge with α-tocopherol pre-treatment were explored using the Ingenuity Pathway Knowledge base. Among these differentially expressed genes, a large number were identified as genes relevant to the biological effects of TNF-α and α-tocopherol, as well as modification of the cell response to TNF-α after pre-treatment with α-tocopherol. The core functional analysis of those genes datasets was performed to interpret those datasets in the context of biological processes, pathways and gene networks. The comparison analysis was also performed to analyze changes in biological states across the three datasets and to understand how α-tocopherol can modify the cell response to TNF-α challenge in the biological processes, clinical pathology endpoints, diseases, and pathways. Figure 7 shows the bio-function and tox-function comparisons of three datasets. In all of the top ten bio-functional comparisons, TNF-α challenge induced very significant gene expresson changes in these functional categories. In α-tocopherol treated cells and α-tocopherol pre-treated and TNF-α challenged cells, all these functions were significantly activated. However, the changes were much less in the magnitude of significances in cells challenged with TNF-α and pretreated with α-tocopherol than in cells challenged with TNF-α alone (Fig. 7A).

Tox-functional analyses and comparison of the datasets

TNF-α serves as a mediator in various pathologies because, in cooperation with other cytokines, it initiates the inflammatory response. The IPA-Tox™ analysis and comparison analysis were used to analyze
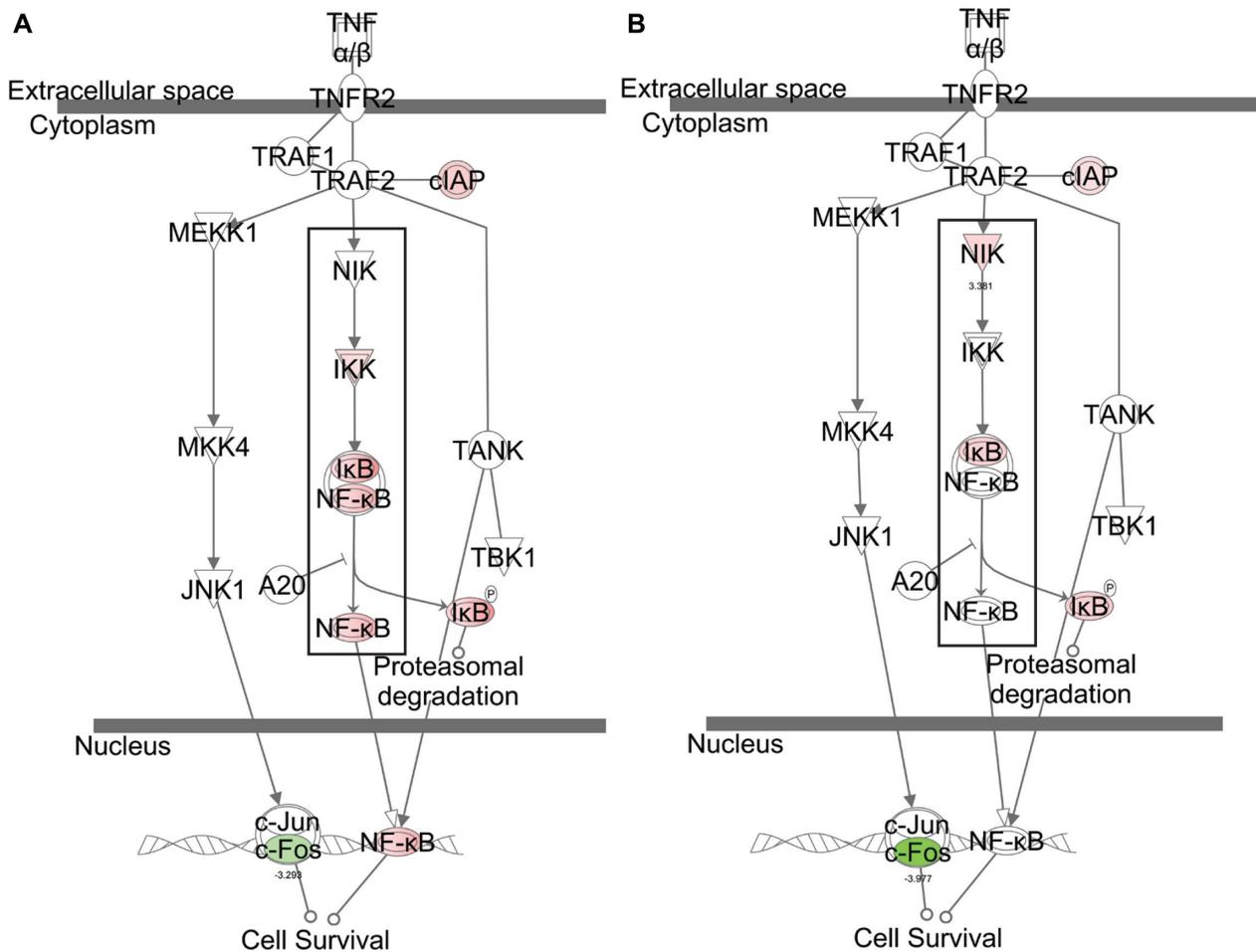
Figure 4. Graphical representations of TNFR2 signaling pathways and comparison of the gene- perturbation induced by the treatments (**A**) TNF-α challenge without α-tocopherol pre-treatment; and (**B**) TNF-α challenge with α-tocopherol pre-treatment, respectively.
Notes: Red-notes and green-notes indicate up- and down-regulated genes, respectively. For simplication, α-tocopherol treatment is not shown.

changes in relevant toxicity phenotypes induced by TNF-α and to compare those phenotypes across all datasets. As shown in Figure 7B, in TNF-α-challenged cells many major functions related to inflammation and diseases were significantly altered. However, α-tocopherol pretreatment before TNF-α challenge significantly decreased the magnitude of those gene expression alterations indicating antagonistical effect of α-tocopherol pretreatment (Fig. 6B).

Discussion

It has been found in recent years that α-tocopherol is more than just a simple fat-soluble antioxidant. The ability of vitamin E to modulate signal transduction and gene expression has been observed in numerous studies.[24,25] Available evidence also suggests a role of vitamin E in cell homeostasis that occurs through the modulation of specific signaling pathways and genes

involved in proliferation, metabolic, inflammatory, and antioxidant functions. In our previous report,[3] using global expression profiling and pathway analysis on α-tocopherol induced gene perturbation in bovine cells, we have generated comprehensive information on the physiological functions of α-tocopherol. Data confirmed that α-tocopherol is a potent regulator of gene expression and controls novel transcriptional activities that affect essential biological processes. The data also provided direct evidence that α-tocopherol is involved in maintaining immune-homeostasis. As the extension of the previous research, this study is focused on the α-tocopherol modification of the cells immune and pro-inflammation response induced by the TNF-α challenge using global gene expression profiling and IPA analysis. The results presented indicate that α-tocopherol pretreatment has significant antagonistical effect against TNF-α induced pro-inflammational response.
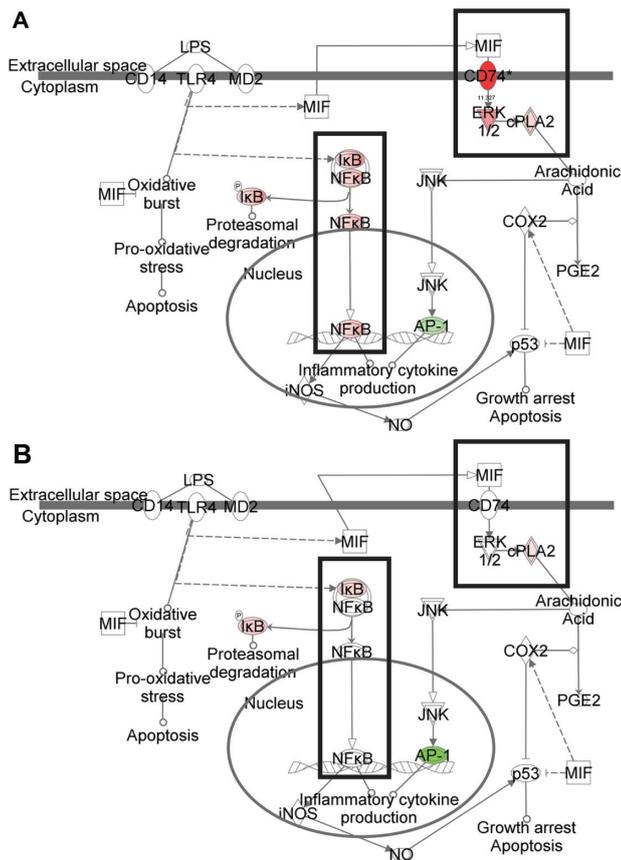
Figure 5. Graphical representations of Microphage migration inhibitory factor (MIF) regulation of innate immunity signaling pathway and comparison of the gene- perturbation induced by the treatments (**A**) TNF-α challenge without α-tocopherol pre-treatment, and (**B**) TNF-α challenge with α-tocopherol pre-treatment respectively.
Notes: Red-notes and green-notes indicate up- and down-regulated genes, respectively. For simplication, α-tocopherol treatment is not shown.

TNF-α is a pleiotropic inflammatory cytokine and serves a variety of functions, many of which are not yet fully understood. Most organs of the body appear to be affected by TNF-α. The primary role of TNF-α is in the regulation of immune cells. The beneficial functions of TNF-α include growth stimulating properties and growth inhibitory processes, its role in the immune response to bacterial, and certain fungal, viral, and parasitic invasions as well as its role in the necrosis of specific tumors. Whereas α-tocopherol can partially suppress the transcriptional changes induced by TNF-α, its effects are context-dependent. In addition to its beneficial functions, TNF-α can also stimulate apoptotic cell death and inflammation. TNF-α is an acute phase protein which initiates a cascade of cytokines and increases vascular permeability, thereby recruiting macrophage and neutrophils to

a site of infection. It acts as a key intermediary factor in the local inflammatory immune response. TNF-α is also one the principal mediators of the lethal effect of endotoxin.[26,27] Pretreatment with α-tocopherol to a certain degree modulates or "buffers" the cells' proinflammatory response (stress) induced by TNF-α and maintaining the magnitude of responses within a range less detrimental to cell survival than frank apoptosis or necrosis. Our results from gene expression profile and analysis are consistent with the typical response of the cells to the TNF-α challenge. Upon TNF-α challenge, cells responded with activation of NF-κB, TNFR1 and TNFR2, as well as ERK/ MAPK canonical signaling pathways. Interestingly, α-tocopherol did not impair these signaling components but rather altered the TNF-α-induced activation of the NF-κB gene. The data also indicated that NF-κB is the center of several canonical signaling pathways. Members of the NF-κB family of dimeric transcription factors regulate expression of a large number of genes involved in immune responses, inflammation, cell survival, and cancer.[28] NF-κB transcription factors are rapidly activated in response to various stimuli including TNF-α and other cytokines, as well as infectious agents.[28] As the consequence of the activation of NF-κB, the expressions of pro-inflammation cytokines by monocytic cells increase. In both TNFR1 and TNFR2 signaling pathways, the TNF-α-induced activation of the kinase IKK gene, a key regulator of the NF-κB transcription factor, was altered by the α-tocopherol pre-treatment. The IKKs and IKK-related kinase are instrumental for activation of the host defense system.[28] The data presented is consistent with the idea that the transcription factor NF-κB and consequently IKK are central to signal transduction pathways of mammalian cells[29] in immune responses. This pathway is involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation.

In the signaling pathway of MIF (macrophage migration inhibitory factor) regulation of innate immunity, activation of CD74 and ERK1/2 genes induced by TNF-α were also altered by the pre-treatment of α-tocopherol. MIF is a critical mediator of the host defense and is involved in both acute and chronic responses in the lung. MIF regulates innate
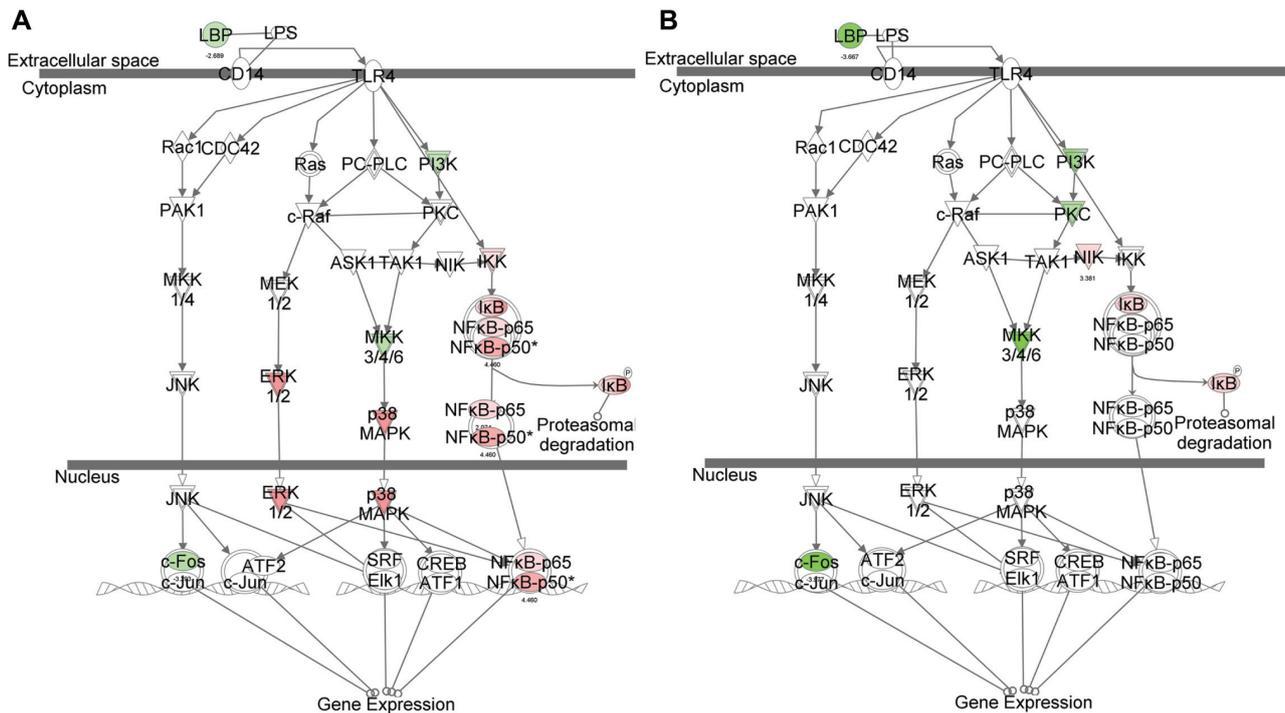
Figure 6. Graphical representations of LPS-stimulated MAPK signaling pathway and comparison of the gene-perturbation induced by the treatments (**A**) TNF-α challenge without α-tocopherol pre-treatment, and (**B**) TNF-α challenge with α-tocopherol pre-treatment respectively.
Notes: Red-notes and green-notes indicate up- and down-regulated genes, respectively. For simplicity, α-tocopherol treatment is not shown.

immune responses to endotoxin and gram-negative bacteria by modulating the expression of TLR4, the signaling-transduction component of the LPS receptor complex. As the results of the activation of MIF, cells increase the production of NF-κB and TNF-α. CD74 contributes to MIF-induced pulmonary inflammation[30] and is also a regulator of Fas-mediated apoptotic signaling.[31]

The results of IPA data analysis showed that α-tocopherol pre-treatment of cells altered cell's response to the TNF-α challenge and illustrated, in most of the canonical pathways, the antagonistical effect of α-tocopherol pretreatment against the TNF-α-induced immune and pro-inflammation responses. However, there was one exception; toll-like receptor signaling pathways did not show differences, at least statistically (*P*-value 2.01E-04 vs. 2.06E-04, Table 1). Toll-like receptors (TLRs) belong to the family of pathogen-associated pattern recognition receptors. Binding of ligand to TLR triggers a cascade of signaling events via the TLR-adaptor complex. P38MAPK, JNK and NF-κB are the downstream signaling molecules. As the results of the activation of these downstream molecules, nuclear transcriptional factors or regulators are activated and leading to the induction of pro-inflammatory cytokines and antibacterial and anti-virus responses. How to consolidate these conflicted results may require more bench side experimentation and the statistic number alone may not be enough to draw conclusions.

This study is the first global expression profiling and pathway analysis of α-tocopherol-induced gene perturbation and the functional genomic study on gene regulation alteration of α-tocopherol pre-treatment in bovine cells in response to the TNF-α challenge. It has generated comprehensive information on the physiological functions of α-tocopherol pre-treatment against TNF-α-induced pro-inflammation responses. Our data confirmed that α-tocopherol is a potent regulator of gene expression and α-tocopherol possesses novel transcriptional activities that can alter the cell's response to TNF-α challenge. These data may also reflect that one of the characteristics of α-tocopherol and those genes perturbed by α-tocopherol treatment may play very important roles in biological functions. Its biological activities have potential to reduce harmful pro-inflammation reaction without compromising the host's immune system. Therefore the potential
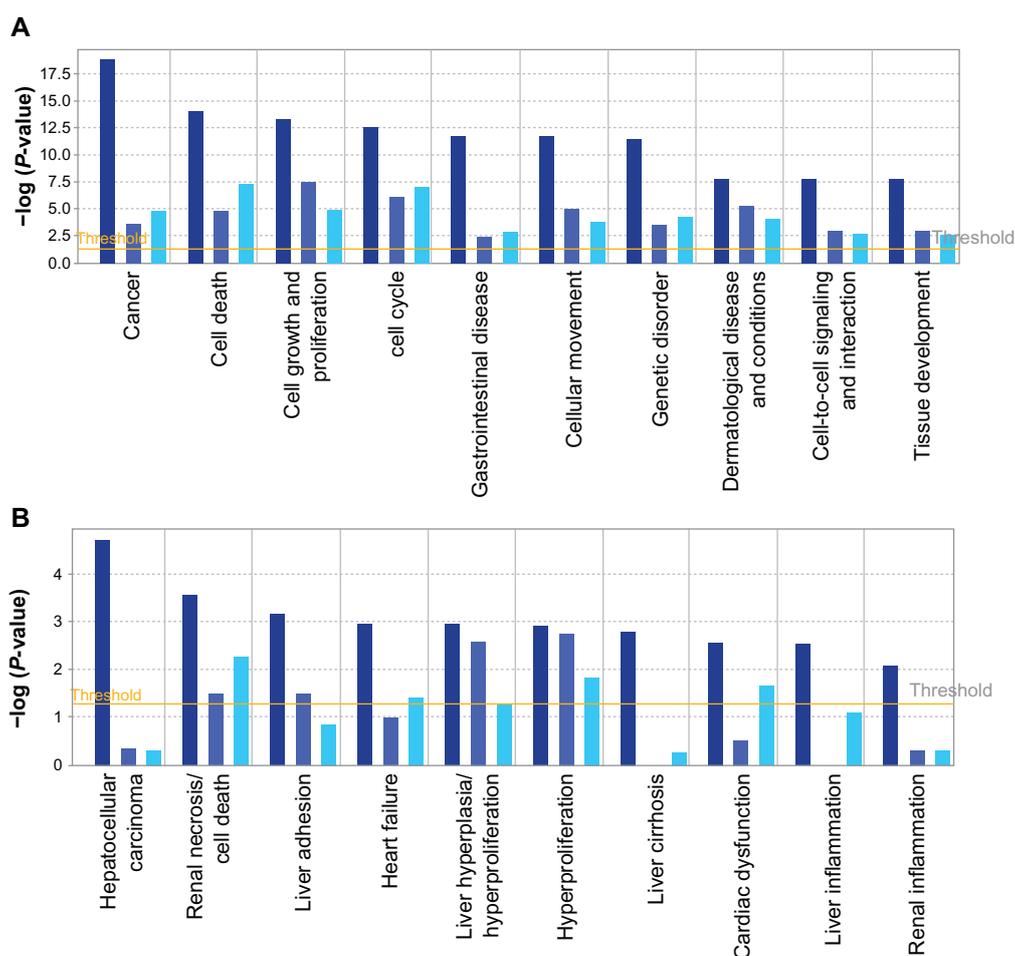
Figure 7. Global functional analysis and comparison. The significance value associated with a function in Global Analysis is a measure of probability that genes from the dataset file under investigation participate in that function. The significance is expressed as a *P*-value, which was calculated using the right-tailed Fisher's Exact Test. (**A**) Bio-function analysis and comparison; (**B**) Tox-function analysis and comparison.

utilization of antagonistical activity of α-tocopherol against the TNF-α induced pro-inflammation response appeals for further exploration.

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Supplementary Tables

Supplementary Tables 1–3
8303 Supplementary tables 1-3.zip