Arch Biol Sci. 2016;68(3):509-513 DOI:10.2298/ABS150731040Y

EVALUATION BY HIERARCHICAL CLUSTERING OF MULTIPLE CYTOKINE EXPRESSION AFTER PHYTOHEMAGGLUTININ STIMULATION

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Received: July 31, 2015; **Revised**: September 29, 2015; **Accepted**: October 6, 2015; **Published online**: April 22, 2016

Abstract: The hierarchical clustering method has been used for exploration of gene expression and proteomic profiles; however, little research into its application in the examination of expression of multiple cytokine/chemokine responses to stimuli has been reported. Thus, little progress has been made on how phytohemagglutinin (PHA) affects cytokine expression profiling on a large scale in the human hematological system. To investigate the characteristic expression pattern under PHA stimulation, Luminex, a multiplex bead-based suspension array, was performed. The dataset collected from human peripheral blood mononuclear cells (PBMC) was analyzed using the hierarchical clustering method. It was revealed that two specific chemokines (CCL3 and CCL4) underwent significantly greater quantitative changes during induction of expression than other tested cytokines/chemokines after PHA stimulation. This result indicates that hierarchical clustering is a useful tool for detecting fine patterns during exploration of biological data, and that it can play an important role in comparative studies.

Key words: hierarchical clustering; cytokine expression; phytohemagglutinin; peripheral blood mononuclear cells; Luminex

INTRODUCTION

Phytohemagglutinin (PHA) is a lectin highly concentrated in red kidney beans [1], and commonly used in medical research. For example, it has been used as a molecular tracer in neuroscience [2], and it has been demonstrated that PHA is capable of inhibiting lymphoid tumor spread and influencing the increase in proliferation of cytokine-induced killer (CIK) cells [3,4], as well as stimulating gallbladder contraction by cholecystokinin (CCK) [5].

PHA helps mature lymphocytes that have lost the potency of cell division after mitogenetic stimulation by binding to carbohydrate moieties on the surface of cells, provoking small lymphocyte transformation into lymphoblast, followed by cell proliferation and lymphokine release [6,7]. PHA has been used as a mitogen triggering T cell division in medical research [8,9], as a stimulant of peripheral blood mononuclear cells (PBMC) in immunology research, and as an inducer of cytokines, including interferon (IFN) and interleukin (IL) [10,11]. However, little progress has been made on how PHA affects the expression of various cytokines, and PHA is usually considered as a general stimulator of the expression of all cytokines. Thus far, only a few studies have been reported in this field; for example, an *in vivo* animal experiment was conducted to study the differential expression of cytokines in PHA-induced skin inflammation in galliform birds [12].

Hierarchical clustering is a powerful approach for the exploration of protein expression data by blindly grouping samples on the basis of chosen parameters [13]. It has been widely used in various fields of biological sciences, including the acquisition of microarray data and tumor classification [14-17]. In this study, hierarchical clustering analysis was implemented on cytokine expression profiles in four different stimulation media quantitated by the Luminex test to explore the characteristic expression of cytokines after PHA stimulation.

MATERIALS AND METHODS

Data

Cytokine expression data were obtained from a previously published study in which the experimental materials and methods are described in detail [18]. Briefly, human peripheral blood mononuclear cells (PBMCs) from one healthy donor were isolated from whole blood by density gradient centrifugation and cultured in four different stimulation media, designated as A: IL-4+IL-6; B: IL-4+IL-6+H_2O_2; C: IL-4+IL-6+PHA; and D: IL-4+IL-6+PHA+H_2O_2. Supernatants were obtained from the same well after 2, 6, 12 and 16 h of stimulation for quantitative analysis of protein. A total of 51 biomolecules were measured by the 51-plex cytokine assay on the Luminex instrument and the corresponding concentrations were calculated.

Hierarchical clustering analysis

Results of average protein concentrations were used for data analysis. IL-4, IL-6 and proteins whose expression levels were out of the measurement range (including FASLG and VCAM1), were removed from further analysis. A data set that included 47 proteins was analyzed by the hierarchical clustering method. Before hierarchical clustering, the concentration data of each protein were normalized using the equation:

$$x_{ij} = (x_{ij} - \bar{x}_j)/s_j$$

where $\bar{x}_j$ is the mean value of protein concentration and s_j is the corresponding standard deviation.

In the first part of the analysis, original protein expression data from four different stimulation media were examined separately in order to obtain clustering results for each stimulated condition. Cytokine distribution in the clustering result was counted and compared between different conditions after the dendrograms were successively divided into three or four branches. Overall cytokine expression data from all four conditions were interpreted by the same clustering method and referenced to the previous analysis.

For the second part of the investigation, the data set was preprocessed using the values of protein concentrations in condition C (D) divided by their corresponding concentrations in condition A (B) at the same measuring time, in order to obtain the ratio by which we implemented the same hierarchical clustering method. Protein distribution in the clustering result was also studied to confirm the investigation regarding the expression of cytokines after PHA stimulation.

The distance matrix in the hierarchical clustering analysis was calculated by Euclidean distance and a dendrogram was generated using Ward's minimum variance clustering method.

RESULTS

Cytokine classification obtained by comparing the expression profiles in four different stimulation media

Expression data of 47 proteins from four different stimulation media were selected for hierarchical clustering analysis. In the clustering results (Fig. 1), the dendrograms were divided into one main and two minor branches, with 42 proteins clustering on the main branch in each condition. Comparison of the protein distribution of the four clustering results revealed that conditions A and B (without PHA stimulation) shared the same pattern, as well as conditions C and D (PHA stimulated). More specifically, 40 of the 42 proteins coexisted on the main branches of all four stimulated conditions. Chemokine (C-C motif) ligands 3 (CCL3) and 4 (CCL4) on the major branch of conditions A and B were absent from the main branch of PHA-stimulated conditions C and D, while other molecules, such as intercellular adhesion molecule 1 (ICAM1) and vascular endothelial growth factor A (VEGF-A) exhibited opposite behavior.

For further analysis, the resulting dendrograms were separated into four branches to explore the changes in protein expression after PHA stimulation. Several proteins were clustered in the same subbranch

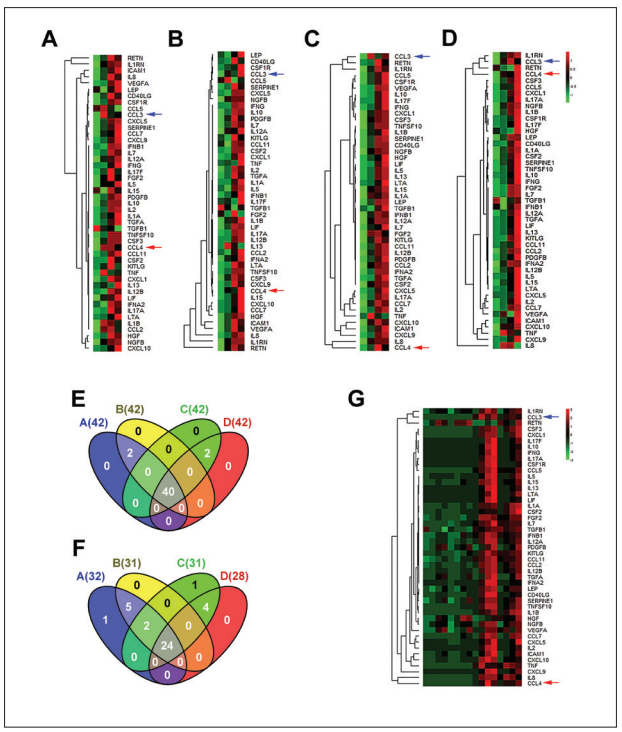

Fig. 1. Cytokine cluster results in different stimulation media. A total of 42 proteins clustered in the main branch in all four groups. CCL3 and CCL4 in the main branch were exclusive to the medium without PHA stimulation (panels A and B); ICAM1 and VEGF-A were in the opposite condition (panel C and D). Venn graphs (Oliveros J. VENNY. An interactive tool for comparing lists with Venn Diagrams. 2007) show protein distribution in the main branches under four conditions (panel E and F). The cluster result was nearly identical with the PHA-stimulated condition where the data from all four conditions were used for hierarchical clustering (panel G). The positions of CCL3 and CCL4 in the dendrogram are indicated with blue and red arrows, respectively.

or two adjoining subbranches. Relatively small clustering distances were noted in conditions A and B, and the same situation was observed in the PHA-stimulated condition. Thirty-two, 31, 31 and 28 proteins clustered on the main branch of conditions A, B, C and D, respectively, after proteins in adjacent subbranches with smaller clustering distances were merged into the same group. A total of 24 proteins co-existed in the main branches of all four stimulated conditions, with colony-stimulating factor 3 (CSF3), chemokine (C-X-C motif) ligand 1 (CXCL1) clustering in conditions A, B and C, 5 proteins: CCL4, CXCL9, IL17A, IL2, tumor necrosis factors (TNF), clustered in conditions A and B; 4 proteins, CD40 ligand (CD40LG), leptin (LEP), nerve growth factor b (NGF-b), endothelial plasminogen activator inhibitor (serpin E1) clustered in conditions C and D, hepatocyte growth factor (HGF) clustered in condition A, and CCL7 clustered in condition C. It is noteworthy that CCL4 was still only present on the main branches of conditions A and B, while CCL3 was excluded from the main branches in all four conditions. The clustering results of all data, regardless of the differences after stimulation, are roughly the same as after PHA-stimulated conditions.

Cytokine classification according to quantitative changes in expression between PHA-stimulated conditions and non-PHA-stimulated conditions

To further confirm the influence of PHA on the heterogeneous expression of cytokines, hierarchical clustering analysis was conducted on the ratio between the PHA-stimulated conditions and non-PHA-stimulated conditions. CCL3 and CCL4 were among nine proteins (CCL3, CCL4, CXCL1, CXCL9, IL17A, IL1B, IL2, IL8 and TNF) that were excluded from the main branch in the dendrogram (Fig. 2A). In addition, four (CXCL9, IL17A, IL2 and TNF) out of the nine proteins only existed in the main branch of conditions A and B when the dendrogram was divided into four branches, indicating that the expression patterns of CCL3 and CCL4 changed. However, proteins such as ICAM-1 and VEGF-A that display different expression patterns when compared to other cytokines in the clustering results of four different stimulated conditions, were not among these nine proteins. CCL3 and CCL4 exhibited more distinct differences in quantity than other cytokines in the unison graph (Fig. 2 B), where TNF was differed even more than CCL3.

DISCUSSION

Changes in protein expression can be inconspicuous during the early stage of a response to stimulation. It is crucial to detect such variations on a small scale in order to monitor their further development. The hierarchical clustering method has been widely ap-

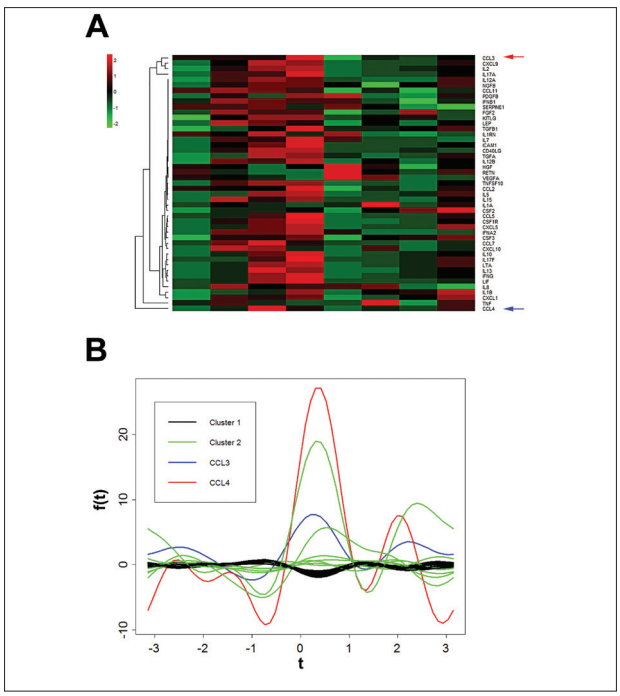

Fig. 2. Cytokine classification according to quantitative changes in expression after PHA stimulation. **A** – nine proteins, including CCL3 and CCL4, were clustered on the main branch in the result presented with the heat map, indicating quantitative changes in expression in comparison to other cytokines. The positions of CCL3 and CCL4 in the dendrogram are indicated with blue and red arrows, respectively; **B** – unison graph of quantitative changes in cytokine expression after PHA stimulation is shown in the panel; blue and red lines indicate changes in CCL3 and CCL4 expression; the green line represents potential cytokines exhibiting similar changes in expression. Significant differences between these proteins and other proteins in the main branch are represented by black twisting lines.

plied in mining gene associations according to expression profiles, based on the similarities of up- or downregulation of various genes. The method also has the capacity to identify subgroups with different expression patterns. By grouping every subject into a specific branch, small variations between samples that may hold biological significance can be detected using this method. In this study, differences in cytokines in different branches obtained by the clustering result are based on small differences in their expression patterns. Although almost all of the examined cytokines were upregulated as a consequence of cell stimulation in the four different media, the fact that different cytokines underwent different quantitative changes could reflect the changes in cytokine expression after PHA stimulation.

It has already been shown that PHA is capable of functioning as a stimulant for T lymphocyte division and lymphokine release, yet the details of cytokine expression after PHA stimulation remain unclear. In this work, certain cytokines, such as CCL3 and CCL4 exhibited different quantitative changes after PHA stimulation, i.e. larger increases in comparison to other cytokines. Such differences may contribute to the elucidation of the mechanism that underlies PHA-induced heterogeneous expression of cytokines, as confirmed for PHA activation of different cytokine genes in $CD4^+$ and $CD8^+$ T cells. Regulation of cytokine expression in T cell subsets is complicated by the diverse kinetics of mRNA induction [19].

Investigation into cytokine expression is restricted by many factors, such as individual differences in cytokine expression. Previous research suggested that two-year-old children do not respond in the same way to PHA stimulation of cytokine expression as their mothers [20]. Research on this subject requires large samples and improved design of experiments. For example, controls have to be properly arranged to exclude the influence of other substances and to provide comparisons of the differences between PHA and other mitogens, and the cross-effects of PHA and other stimulants. An improved or derived hierarchical clustering method can be used to evaluate heterogeneous cytokine expression and provide directions for investigations into T lymphocytes.

The hierarchical clustering method was shown to be capable of detecting small variations between heterogeneous data sets and subtle changes in biological processes. The method is a promising tool for investigating protein expression patterns, especially when combined with effective data processing methods and laboratory experiments.

Acknowledgments: This work was supported by the National Natural Science Foundation of China (No. 81571592, 31371203), Beijing Nova Program of Science and Technology (No. 2007B024), and Program for New Century Excellent Talents at University (No. NECT-12-0773). The authors wish to express their profound

gratitude to Dr. Garrison Fathman, Professor at Stanford University School of Medicine for his professional assistance in data collection.

Authors' contributions: Hongwu Du was responsible for the design of the study and data collection. Chunhe Yang undertook the analysis and prepared the manuscript. All authors contributed to the final version of the manuscript.

Conflict of interest disclosure: The authors declare no conflict of interest.

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