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Quantitative Association of Humoral or Cellular Immunologic Markers with the Prediction of Skewed Adaptive Immunity in Agricultural Workers

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ABSTRACT

The paradigm of immune homeostasis between type-1 helper T cells (Th1) and type-2 helper T cells (Th2) has been extensively examined with respect to infectious diseases, autoimmune diseases, or tumor progression. Such studies have mostly relied on the profiling of cytokines/chemokines using animal studies or cell culture-based *in vitro* tests. No quantitative analyses have yet been systematically performed to determine the relationship between the various immune parameters in *in vivo* investigations. Using peripheral blood from 55 chicken husbandry workers or grapevine orchard workers, we obtained data for various immunologic markers as follows: proportion of major immune cell subpopulations, concentrations of plasma immunoglobulin subclasses, and levels of cytokines produced from activated peripheral blood mononuclear cells. Correlational analyses were carried out to examine the association between these parameters. As the IFN-γ:IL-13 ratio was strongly associated with the production levels of IFN-γ, TNF-α, and to the IFN-γ:IL-4 ratio, the IFN-γ:IL-13 ratio could prove to be a valuable monitoring index to examine Th1 or Th2 predominance. IL-17 levels correlated well with those of IFN-γ or TNF-α. Plasma levels of IgG4, a typical IgG subclass expressed upon the occurrence of allergic diseases, strongly correlated with IgG1 levels, suggesting the implication of IgG1 in allergic hyperreactivity. The proportion of cytotoxic T cells correlated significantly with levels of IFN-γ, and the proportion of natural killer T cells correlated well with IL-13 levels. These two cell types are sources for the production of IFN-γ and IL-3 respectively; hence, results of this study might provide potential monitoring markers for cytotoxic T cells or natural killer T cell-mediated immunoreactivity. Overall, the present study provides several putative candidates for the surveillance or prognosis of the *in vivo* alteration of humoral or cell-mediated immunity.

Key words : Th1 or Th2 response, Human adaptive immunity, Immunotoxicological biomarkers, Cytokines, Immune cell subpopulation

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Introduction

Balance between type-1 helper T cell (Th1) versus type-2 helper T cell (Th2) has been claimed to play a pivotal role for alleviation or aggravation of infectious diseases, autoimmune diseases, tumor progression, or ill health conditions [1-3]. The Th1/Th2 paradigm has been extensively discussed to certain microbial diseases such as tuberculosis, Acquired Immune Deficiency Syndrome, or leishmaniasis, to autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, or glomerulonephritis, and to various cancer occurrence including hepatocellular carcinoma, lung cancer, or laryngeal carcinoma [4-6]. Furthermore, The Th1/Th2 hypothesis has been also applied to evaluate immune modulations driven by toxic materials exposed from occupational or environmental settings [7,8].

Organic dusts are composed of various hazardous agents such as dust, microorganisms, toxic gases, or endotoxin from animal husbandry environment [9-12]. Farmers' respiratory illness has been investigated focusing its association with exposure to the organic dusts. Organic dust-mediated respiratory allergy has been reported to exhibit immune alterations characterized with predominance of Th2 response in livestock husbandry workers, which was demonstrated through evaluating the plasma level of immunoglobulin subclass, profiling of cytokines produced from activated peripheral blood mononuclear cells (PBMCs) stimulated *ex vivo*, or phenotying of peripheral immune cell subpopulation [10,12]. The assessment of Th1/Th2 balance is considered important for predicting potential occurrence of respiratory illness, applying therapeutic strategy, or preventing exacerbation of existing allergic diseases. This consideration has been implied with various immune alterations demonstrated at diverse scopes of ill health mentioned above.

The Th1/Th2 paradigm has mostly relied on the cytokine/chemokine profiles obtained from laboratory animal studies, *in vitro* cell culture, or specific diseases [1-6]. Therein, when predicting an *in vivo* immune disturbance between Th1 and Th2 response for human subjects exposed to hazardous substances with no apparent illness, it will be necessary to assess as many as possible parameters reflecting innate, humoral, and cell-mediated immunity. These parameters may include plasma immunoglobulin subclasses (IgG subclass, IgE, and IgA), cytokines (interleukin (IL)-4, IL-5, IL-13, interferon (IFN)-γ, IL-12, tumor necrosis factor (TNF)-α, and etc.) secreted from activated T lymphocytes or in plasma, and immune cell proportion in peripheral blood (helper T lymphocyte, cytotoxic T lymphocyte, natural killer (NK) cell, B lymphocyte, or granulocyte) [7,8,10-12]. In addition, combination of appropriate parameters well related with the Th1 or Th2 response could endow the better insight on imbalance between the Th1 and Th2 immune reactivity.

Table 1. Demographic characteristics of study subjects

	Male	Female	Total
Chicken farmer			
Number tested	23	13	36
Age (year: mean ± SD)	61.3 ± 10.8	59.9 ± 10.4	
Grapevine orchard farmers			
Number tested	15	4	19
Age (year: mean ± SD)	67.3 ± 7.1	56.5 ± 3.5	
Total	63.0 ± 9.7	59.1 ± 9.2	55

The present study was conducted to delineate which parameters addressed above are fairly correlated or clustered to predict potential inclination to Th1 or Th2 response. Livestock husbandry workers and grapevine orchard workers, who reported no apparent health problems, became the study subjects for donating peripheral blood to determine the levels of immunologic parameters above. Since no systemic quantitative analysis on the relationship among those parameters has been reported so far, the correlational matrix obtained from the present study could contribute to choose appropriate parameters for determining the Th1 or Th2 predominance in healthy agricultural workers.

Materials and Methods

1. Study subjects

Farmers (n = 36) working at broiler chicken farms in 4 areas (Jinan-gun, Iksan-city, Jeongeup city of Jeonbuk province, Korea) and farmers (n = 19) working at grapevine orchard in Damyang-gun of Jeonnam province were asked to participate in this study. Their demographic characteristics are shown at Table 1. No significant difference was found on the distribution of sex and the average age (chicken farmers: 60.8 ± 10.5, orchard farmers: 65.1 ± 7.9). Collection of blood (10 mL) from the participants was approved by the Institutional Review Board of the Daegu Catholic University (approval# 2019-0005).

2. Measurement of immune function

Each blood sample was collected into EDTA vacutainer tubes and the number and absolute number or proportion (%) of white

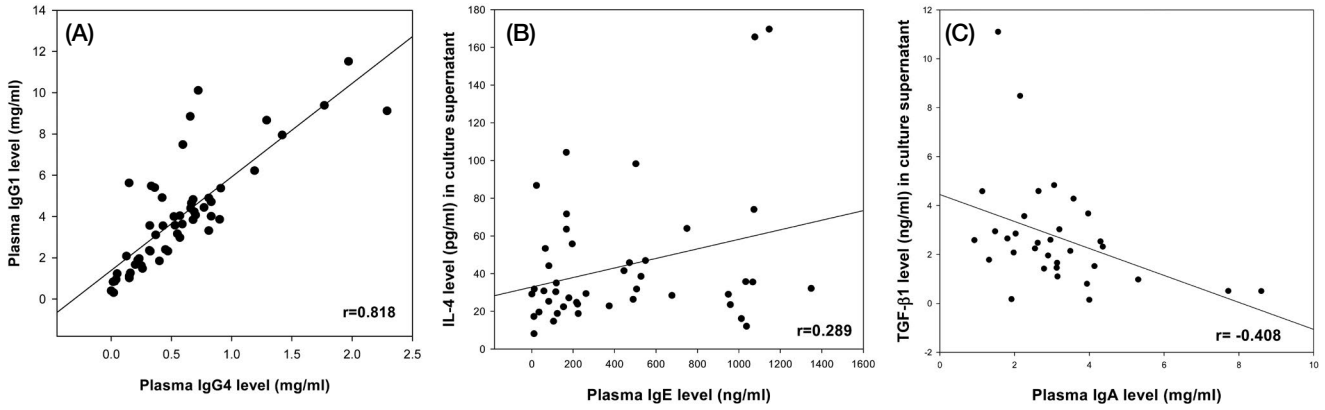

Fig. 1. Correlations between the plasma IgG1 and IgG4 level (A), between the level of IL-4 produced from activated peripheral blood mononuclear cells and the plasma IgE level (B), and between the level of TGF-β1 produced from activated peripheral blood mononuclear cells and the plasma IgA level (C). The correlation coefficients are statistically significant at $p < 0.05$.

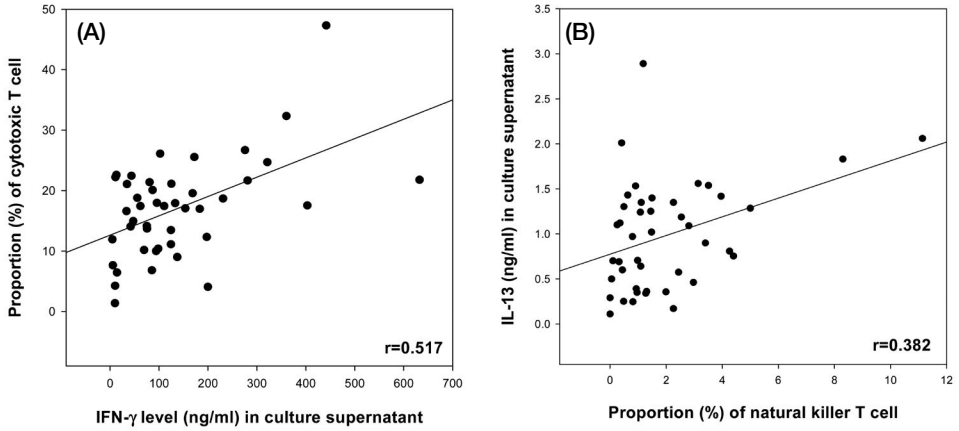

Fig. 2. Correlations between the proportion of cytotoxic T cell and the level of IFN-γ produced from activated peripheral blood mononuclear cells (A) and between the level of IL-13 produced from activated peripheral blood mononuclear cells and the proportion of natural killer T cell (B). The correlation coefficients are statistically significant at $p < 0.05$.

blood cells (WBC), red blood cells (RBC), platelets, lymphocytes, monocytes, and granulocytes was then determined using an automatic blood analyzer (ADVIA 2120, Siemens, Munich, Germany). Thereafter, each sample was processed to generate plasma. Total IgE titres in plasma were measured using a Total IgE ELISA Kit (IBL International GmbH, Hamburg, Germany). Plasma levels of IgG sub-classes (IgG_1, IgG_2, IgG_3, IgG_4) and IgA were measured in a sandwich ELISA as described in elsewhere [13,14].

PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation (Ficoll-Paque Plus, GE Healthcare Life Sciences, Uppsala, Sweden). After counting and assessing viability, cells were then placed in 24-well plates at 10^6 cells/mL/well in complete RPMI medium containing 1 mM non-essential amino acids, 1 mM sodium pyruvate, 0.075% bicarbonate, 2 mM glutamine, 50 mM 2-mercaptoethanol, and 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), as well as 5 ng phorbol 12-myristate 13-acetate (PMA), 500 ng ionomycin (Sigma, St. Louis, MO, USA), and 10 U recombinant IL-2 (Roche, Mannheim, Germany), and cultured for 72 hr at 37ºC in a 5% CO_2 incubator. At the end of this period, culture supernatants were collected and stored in a −80ºC freezer until analyzed for IL-4, IL-13, IL-17, IFN-γ, TNF-α, and transforming growth factor (TGF)-β1 using sandwich ELISAs as described previously [13,14]. Regarding on flow cytometric analyses, Four-color flow cytometry (BD Accuri™ C6 Plus, BD Biosciences, Franklin Lakes, NJ, USA) was used for analysis of peripheral immune cell subpopulation. Anti-CD3-FITC Ab and anti-CD19-PE

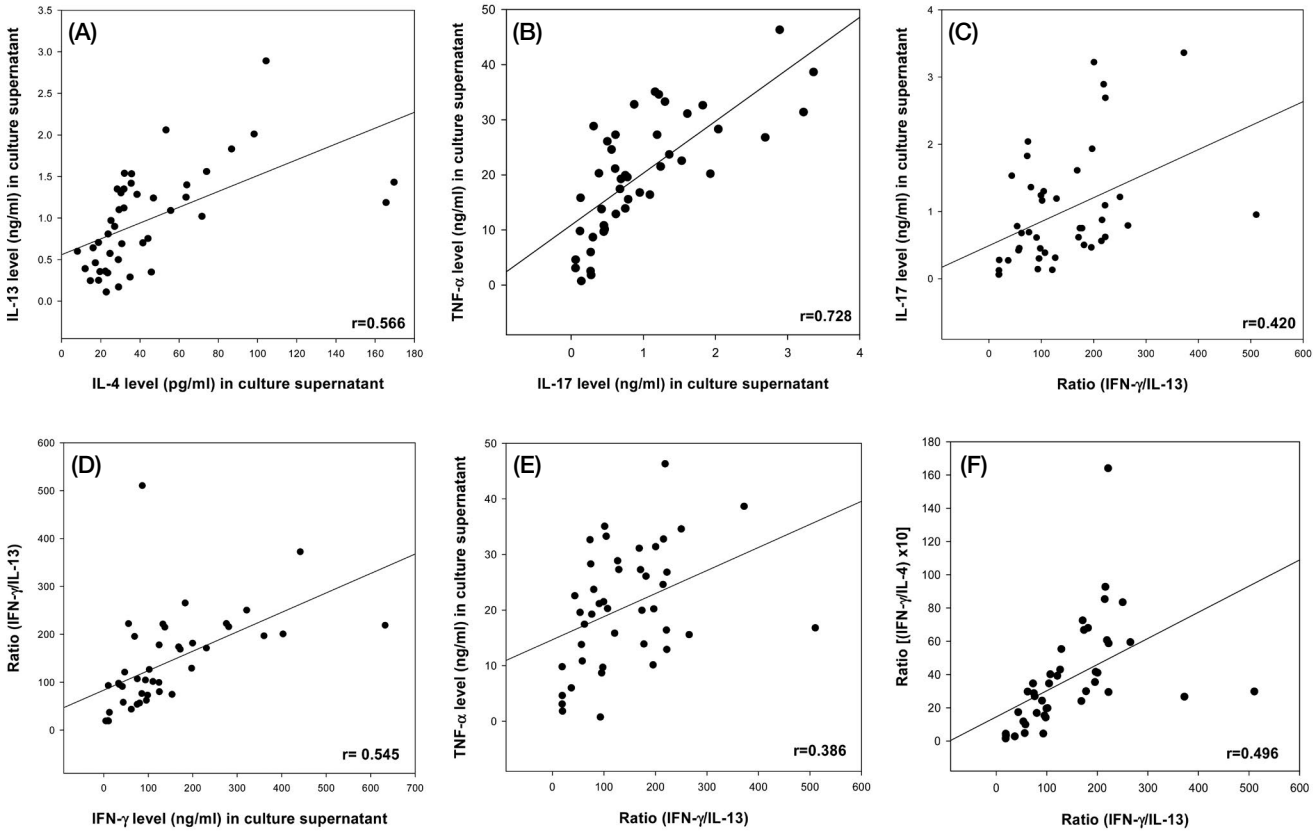

Fig. 3. Correlations between the level of IL-4 and IL-13, which were produced from activated peripheral blood mononuclear cells (A), between the level of IL-17 and TNF-α (B), between the IFN-γ:IL-13 ratio and the IL-17 (C), between the level of IFN-γ and the IFN-γ:IL-13 ratio (D), between the IFN-γ:IL-13 ratio and TNF-α (E), and between the IFN-γ:IL-13 ratio and the IFN-γ:IL-4 ratio (F). The correlation coefficients are statistically significant at $p<0.05$.

Ab (BD) were used for identifying T cell and B cell population. Anti-CD4-APC Ab and anti-CD8-PE Ab were used for sorting helper T cell and cytotoxic T cell, respectively. NK cell was identified through gating on $CD3^{-}CD56^{+}CD16^{+}$ population, and NKT cell was defined through gating on $CD16^{+}CD56^{+}$ population [15]. Each fluorescence conjugated isotype controls were used for subtracting a non-specific background binding of fluorescent Abs.

3. Statistical analyses

SigmaPlot 14 (Systat Software, San Jose, CA, USA) was used for statistical analyses. Pearson Product Moment correlation or Spearman Rank Order correlation test was performed for correlational analyses among the parameters including plasma level of IgG1, IgG2, IgG3, IgG4, IgE, IgA, proportion of immune cell subpopulation (helper T lymphocyte, cytotoxic T lymphocyte, B lymphocyte, NK cell, NKT cell), and cytokine production level (IL-4, IL-13, IL-17, IFN-γ, TNF-α, TGF-β1) The criterion for statistical significance was set up at $p<0.05$.

Results

1. Association of immunoglobulin subclass level with cell-mediated immunologic parameters

A significant correlation ($r=0.818$) was observed between the plasma level of IgG1 and the level of IgG4 (Fig. 1A). The plasma concentration of IgE was significantly correlated ($r=0.289$) with the level of IL-4 secreted from *ex vivo* activated PBMCs (Fig. 1B). In addition, a significant negative association ($r=-0.408$) was observed between the plasma IgA level and the TGF-β1 produced from *ex vivo* activated PBMCs (Fig. 1C).

2. Associations among the cell-mediated immunologic parameters

The proportion of peripheral cytotoxic T lymphocyte was

significantly associated (r=0.517) with the level of IFN-γ produced from activated T lymphocytes (Fig. 2A). The peripheral percentage of NKT cell was also significantly correlated (r=0.382) with IL-13 concentration in the culture supernatants (Fig. 2B).

A skewing of PBMC towards Th1 or Th2 immune reactivity is often determined by measuring IFN-γ along with IL-4 and/or IL-13 production from activated PBMCs, and then calculating the IFN-γ:IL-4 or IFN-γ:IL-13 ratio [10,14]. The ratios are calculated by dividing the amount of IFN-γ by the amount of IL-4 followed by multiplication of 10 or by the amount of IL-13 in the same culture supernatant. The production level of IL-4 was significantly correlated (r=0.566) with that of IL-13 (Fig. 3A), and a significant correlation (r=0.728) was also observed between the levels of IL-17 and TNF-α (Fig. 3B). The magnitude of IFN-γ:IL-13 ratio demonstrated significantly positive associations with various cell-mediated parameters including the level of IL-17 (r=0.420, Fig. 3C), IFN-γ (r=0.545, Fig. 3D), TNF-α (r=0.386, Fig. 3E), and IFN-γ:IL-4 ratio (r=0.496, Fig. 3F).

Discussion

The present study revealed that IFN-γ:IL-13 ratio could be a valuable prognostic indicator for predicting the *in vivo* predominance of Th1 immune reactivity in apparently healthy human subjects, since the ratio was strongly correlated with the production level of IFN-γ or TNF-α, representative cytokines secreted from Th1 lymphocyte [1-3,10-14]. In addition, the IFN-γ:IL-13 ratio was also well correlated with the IFN-γ:IL-4 ratio. The IFN-γ:IL-4 ratio has been traditionally cited as a valuable tool for assessing the immune imbalance toward Th1 or Th2 response in various immunologic investigations including chemical-mediated immune dysregulation, progression of inflammatory diseases, or clinical outcomes of cancer [4-8,16, 17]. However, according to the present study, the IFN-γ:IL-4 ratio may not be a better index than the IFN-γ:IL-13 ratio to define the Th1/Th2 imbalance since no significant association was resulted between the IFN-γ:IL-4 ratio and the other parameters examined. The present result may have limitations to have a direct comparison with those previous studies due to presumably not enough number of study subjects or aged population with single occupational category as agricultural farmers. Nevertheless, the value of IFN-γ:IL-13 ratio for reflection of Th1/Th2 disturbance is preferred for further confirmation. Statistical significances were obtained for the quantitative association of IL-17 with IFN-γ or TNF-α production. Type-17 helper T cell (Th17) is known as the major cell producing IL-17, but Th1 lymphocyte was also reported to produce IL-17 in minor quantity compare to Th17 [18]. Interaction between IL-17 and IFN-γ or TNF-α has been reported mostly as synergistic but some investigations also reported their antagonistic role [19-21]. The present result could be a supportive evidence to demonstrate positive relationship between the cytokines.

IgG is a mediator of humoral immunity against microbial infection, and normal levels usually reflect normal function of B lymphocyte. Abnormal levels of serum or plasma IgG subclass have been reported in various diseases including allergic diseases or autoimmune diseases [22-24]. Upregulation of serum or plasma IgG4 or IgE level has been reported from subjects with respiratory allergic diseases including farmers' lung disease [10,12-14]. IgG1 is known to play a major role in humoral immune response through complement fixation, neutralization or Fc receptor mediated phagocytosis, which could enhance a protective ability against infection or tumor progression [23]. Therefore, propensity of IgG1 toward Th2 response is still debating whether it functions similarly as IgG4 or IgE. The present study demonstrated a significantly strong correlation between the plasma level of IgG1 and IgG4, which implies certain role of IgG1 for immune reactivities associated with predominance of Th2 response and further evaluation on this phenomena seems worthy. Meanwhile, the plasma IgA level was negatively associated with the level of TGF-β1 produced from activated PBMCs. This observation seems conflicting with the facts, in which the importance of TGF-β1 and IL-10 has been emphasized in the regulation of B cell class switching to IgA [25-27]. TGF-β1's contribution to IgA secretion in mucosal sites are carried following antigenic stimulation, on the other hand, we measured the level of TGF-β1 produced from *ex vivo* polyclonally activated PBMCs and determined the total IgA concentration in plasma regardless of antigenic specificity. These discrepancy could be a possible reason for the present result conflicted with the existing knowledge.

IFN-γ is an important mediator for anti-viral or anti-tumor effects of cytotoxic T lymphocyte [28,29]. Th1 lymphocyte is a major immune cell for IFN-γ production in adaptive immune responses and NK cell in innate immune reaction. Cytotoxic T lymphocyte also produces IFN-γ and plays an autologous feedback to activation of cytotoxic T lymphocyte. Therefore, the present result, which is showing the positive correlation between peripheral proportion of cytotoxic T lymphocyte and

the level of IFN-γ produced from activated PBMCs, is well agreed with the existing knowledge. The present study also showed that peripheral percentage of NKT cell significantly correlated with IL-13 concentration in the culture supernatants. This observation is also congruent with previous reports, in that both IL-4 and IL-13 could orchestrate the induction or progression of respiratory allergic diseases [30,31].

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