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Relation between Early Life Human Herpes Simplex Virus – I Infection and Food Allergy: An Animal Model in Balb/C Mice

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Abstract

Impairment of consecutive immune reactions mediated by Th1 and Th2 lymphocytes cause failure of tolerance and emerged allergic disorders, have revealed hygiene hypothesis. It was aimed to investigate the effects of acute and latent HSV-I infections on allergy development. Balb/C mice, both acute or latent HSV-I infection and without infection, were sensitized with egg white and whey proteins and IL-4, TGF-β and IFN-γ cytokine responses were investigated in allergic and tolerant subjects. Cytokine responses revealed that, in the allergy, group IL-4 level was the highest and TGF- β level was significantly lower than acute infection group. In acute, latent and tolerance groups the cytokine levels were observed to be similar in contrast to allergy group. During acute infection, Th-1 lymphocyte cytokine response raised and Th2 response suppressed. Results were suggested, at early ages receiving HSV-I infection might compensate the disequilibrium between T helper subsets, and suppress allergy development..

Keywords: Balb/C mice, egg allergy, cow milk allergy, Herpes simplex virus-I, hygiene hypothesis

1. Introduction

Food allergies are an important health issue, and like other allergic disorders, food allergies seems to be increasing in both the incidence and prevalence rates. Some common food allergies in Europe include milk, eggs, nuts, gluten, and seafood allergies. However, it should be noted that the prevalence of food allergies may vary according to geographic region, individual genetic predisposition, and feeding regimens (Kimber & Dearman, 2001; Lack, 2008). The observed immunological responses to specific foods are based on the abnormal reactions after food intake. These symptoms are the result of an individual's emerging immune system's response to the natural ingredients in the foods or, subsequently, the added chemicals (Helm & Burks, 2000). In a healthy person, the immune system has the ability to preserve stability between the gut's mucosal immunity and systemic tolerance to ingested foods. This equilibrium is either collapsed or unable to be sustained in cases of food allergies (Chatel, Langella, Adel-Patient, Commissaire, Wal & Corthier, 2001; Aoki-Yoshida et al., 2016).

Food allergies appear in the first or second years of life, and the induction of allergen-specific immune reactions depends most often on the formation of allergen specific immunoglobulin E (IgE). Generally, allergic reactions accompanied by gastrointestinal symptoms are observed only in children, regardless of IgE. However, IgE mediated food allergies, which may cause immune reactions and may be encountered at every age, require treatment (Li et al., 2016; Aoki-Yoshida et al., 2016).

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Although the reaction series is complicated, in the formation of food allergies and tolerance, two types of T helper (Th) cells contribute actively to the immune response. These are called Th1 and Th2, of which Th2 plays an active role in the immune reactions of food allergies. Interleukin (IL)-4, secreted by the Th2 cells, plays a leading role in the immune response. IL-4 secretion provides differentiation of the Th cells into more Th2 cells during the initial immune response, and later on if the IL-12 secretion does not increase, causing allergies. However, the Th1 cells suppress the over activation of Th2 by the expression of Interferon (IFN)-γ, which inhibits IgE production. Therefore, after the ingestion of possible allergen proteins, the activations of both the Th1 and Th2 cells prevent the allergic reaction and cause tolerance (Smit, Boon & Lukacs, 2007; Sicherer et al., 2010).

During progressive tolerance, in addition to the cells mentioned above, the antigen processing at the locations of the Peyer's patches and villi leads to the induction of Th3 lymphocytes, which produce the transforming growth factor (TGF)-β. Both the TGF-β secretion and the activation of Th1 induce tolerance, but these mechanisms still have some significantly unclear points. Inflammatory diseases of the gut and mucosal infections could impair the body's tolerance to antigens (Prioult & Nagler-Anderson, 2005). Especially in developed countries, the impairment of tolerance and increased prevalence rates of allergic disorders has led to the development of the hygiene hypothesis. In the hygiene hypothesis, it has been suggested that the deterioration of the Th2 response, decreased or missing Th1 response, or lost equilibrium between the Th1 and Th2 cell responses may be the key factors in allergies. It has been suggested that this balance is lost due to a reduced exposure to bacterial or viral infections in childhood. Additionally, it has been hypothesized that shifting the antigen specific Th2 cell immune response towards Th1 may prevent food allergies (Schaub, Lauener & von Mutius, 2006; Lack, 2008; Palomares, Yaman, Azkur, Akkoc, Akdis M & Akdis CA, 2010).

In viral infections, the first step for the innate immunity toward viral antigens is formed by macrophages secreting cytokines, such as TNF-α, IL-1, and IL-12. IFN-γ expression from the T lymphocytes and natural killer cells is stimulated by IL-12 secretion, which leads to the activation of the effector cells of the cellular immune response, as well as the Th1 and T cytotoxic cells (Fecek, Rezende, Busch, Hassing, Pieters & Cuff, 2010).It was suggested that mucosal transmitted viruses have high infection rates in population and probably might have an active role in regulation of hypersensitivity due to Th1 response. In addition, herpes virus immunity has been reported to be conversely correlated with atopic disorders (Janson et al., 2007). In the present study, the hygiene hypothesis allergy model was created using Balb/C mice, with or without human herpes simplex virus type-I (HSV-I) infections, in order to investigate the relevance between allergies and the induced cellular immune response.

2. Materials and Methods

2.1 Mice

Female Balb/C mice, at 6-8 weeks of age, were obtained from the experimental animal research center of the Ondokuz Mayıs University in Samsun, Turkey. The mice were maintained under normal living conditions, in where drinking water and food were provided ad-libitum. The experiments were performed in accordance with the ethical standards of Declaration of Helsinki and approved by the Ondokuz Mayis University Animal Experiment Committee.

2.2 Herpes Simplex Virus Type-I and virus titration

The HSV-I isolate was provided by Prof. Erturk (originating at Sheffield University) and stored at -80°C. The virus titration assay was performed in Vero cell lines (obtained from the Turkish Public Health Institute, Ankara) growing in 2× the minimum essential medium (MEM) (Sigma), supplemented with 5% fetal calf serum (FCS) for three days (Landry, Smith &Waner, 2003).

2.3 Infection of mice with HSV-I

Five groups of mice (n=7 per group), including a control group, were designed for determining the infective dose of HSV-I. After the mice were given 10 mg/kg of ketamine intraperitoneally for anesthesia, scrapings were generated on the backs of their left ears. Following scarification, 10 microliters of the HSV-I virus suspension was seeded in each group of mice, with a dose of 10^4 - 10^7pfu/ml.

The mice were followed up for 25 days for the clinical prognosis of infection, and 10^5pfu/ml was found to be the adequate infective dose for the HSV-I. Clinical symptoms such as, rough hair coat, lethargy, encrusted lesion site, asymmetric hind-limb paralysis and diarrhea were considered to be signs of HSV-I infection, as appeared. After 25 days of infection, the mice were killed by cervical dislocation, and their trigeminal ganglions were removed and tested for positive latent infections.

2.4 Extraction of milk and egg white proteins

Egg white (EW) (Croguennec, Nau, Pezennec& Brule, 2000) and whey proteins (Neyestani, Djalali & Pezeshki, 2003) were given to the mice. They were prepared as described previously (Kim H, Kwack, Kim J & Ji, 2005), and were sterilized with a 0.45 micrometer pore filter (Millipore). The extracted proteins were separated by fast protein liquid chromatography (FPLC) (Pharmacia Biotech, Sweeden) using diethylaminoethyl (DEAE) sepharose CL-6B (Pharmacia Biotech). Beta-lactoglobulin (Blg) and ovalbumin (Ova) were eluted from the FPCL, and filter concentrated according to the manufacturer's instructions (Sigma). The protein concentrations were determined using the Lowry method, and confirmed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) technique and was carried out 12- 15% gels (Merck).

2.5 Virus infection and antigen challenge of mice

The assay was performed in 5 groups, each consisting of 7 mice, and lasted for 25 days after the exposure to the virus. The study groups were designed as follows:

Group I: Oral tolerance group. Initially, full (undiluted) egg white and whey proteins were given by oral gavage, then ad-libitum in their drinking water.

Group II: Allergy group. The Ova and Blg were given twice intraperitoneally in 250 microliters of PBS solution, then 80 micrograms per mouse of the proteins of each and 10% aluminum hydroxide at one week intervals.

Group III: Allergy group with acute HSV-I infection. The virus was challenged in the mice by scarification on the back of the left ear. After the onset of infection at 7-8 days, 250 µl of the antigen suspension was given intraperitoneally. Sensitization was performed twice at one week intervals.

Group IV: Allergy group with latent HSV-I infection. Twenty-one days after infection, 250 µl of the antigen suspension was given twice intraperitoneally at one week intervals. The samples were taken after 6 weeks of infection.

Group V: Control group with no infection or sensitization.

2.6 Obtaining blood and tissue samples

The blood, spleen, and trigeminal ganglions were taken from all study groups, after completing the experimental process. The lymphocytes were isolated from the spleen, according to the method of Bowman and Hold (2001), immediately after the removal of the organ. The serum samples were stored at -80°C, and later used for the testing of the levels of Interferon-γ (eBioscience, Austria), transforming growth factor β (R&D Systems, USA), immunoglobulin E (Biolegend, USA), and Interleukin 4 (R&D Systems, USA) via the ELISA method (BIO-RAD Model 628).

2.7 MTT lymph proliferation assay

The lymphocyte suspension was seeded on 96-well plates, at a 5×10^6 cell/ml concentration. To each group of wells, 1 µg/ml, 25 µg/ml, 100 µg/ml, 200 µg/ml, or 400 µg/ml of EW, whey, Ova, and Blg was added. As a control for the lymph proliferation, 5 µg/ml of phytohemagglutinin (PHA) was added, and a control well without any antigen was designated. After 72 hours of incubation, 10 µl of a 5 mg/ml 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) (Sigma-Aldrich) solution was added to all of the wells, and incubated for 2 hours at 37°C with 5% CO_2. Followed by a washing step, 100 µl of propanol + 1% TritonX 100 was added, and read at 570-640 nm (Organon Teknika, OT-530 Austria).

2.8 Data analyses

All the tests performed in this study were performed duplicate. The SPSS 16.0 statistical program was used to perform the one-way ANOVA statistical testing for the statistical analyses.

3. Results

For adjusting infective dose of HSV-I, existence of infection symptoms and no dead occurrence within 50% and above of subjects were considered as the infective dose. At 10^6 and 10^7pfu/ml of HSV-I particle challenged mice all clinical symptoms and also hind-limb paralysis were observed. After two days of infection, at 10^7pfu/ml HSV-I given mice group five of six were dead. At 10^6pfu/ml challenged group, followed by asymmetric hind-limb paralysis occurrence on the second day of infection, three of six were died in five days. All clinical symptoms except paralysis were detected among 10^5pfu/ml challenged subjects and were survived to the end of the experiment. The HSV-I infection was confirmed by polymerase chain reaction (PCR) to prove HSV-DNA in infected subjects trigeminal ganglion.

Anion exchange chromatography of whey and EW resulted in 5 and 6 fractions those included Blg and Ova respectively. The eluted fractions of each were subjected to SDS-PAGE (fig. 1A-B). Obtained Ova and Blg were then filter concentrated and used in the study.

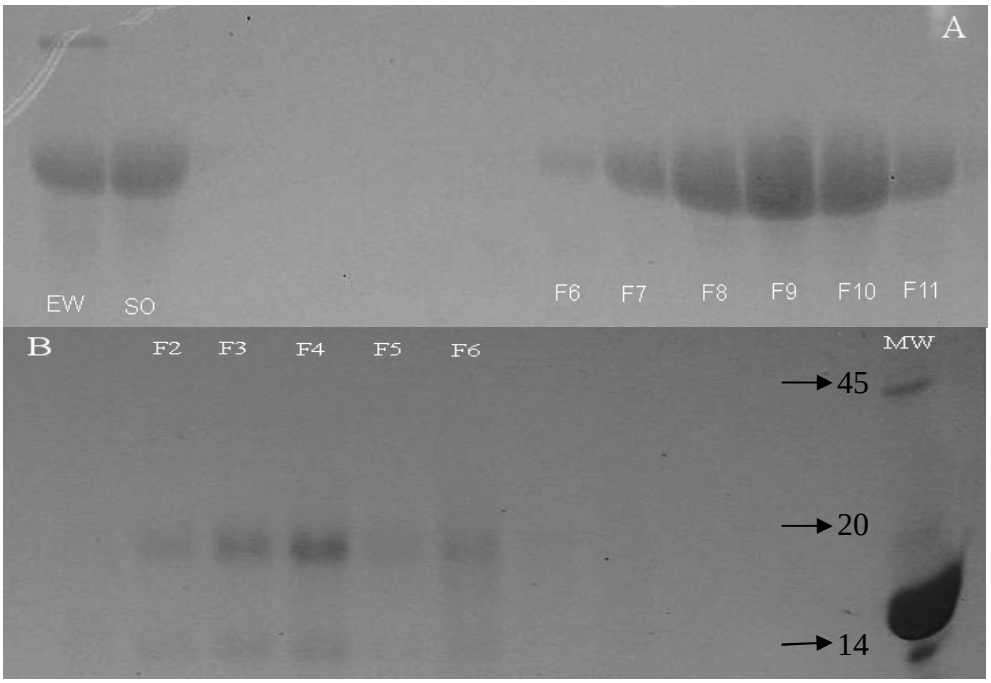

Figure 1: The SDS-PAGE patterns of fractions eluted by anion exchange chromatography of EW and whey. **A)** Ova gained from EW in six fractions 4ml of each. **B)**Blg extracted from whey in five fractions 4 ml of each. **EW:** diluted egg-white; **SO:** Ovalbumin (Sigma); **F:** fractions indicated by numbers; **MW:** molecular weight marker (kDa).

According to the lymphoproliferation assays, the lymphocyte response to the antigen challenge was significantly higher in the allergy group than the control ($p=0.001$), similar to the PHA response (Figure 2). Additionally, the lymphocyte responses of the allergy groups with acute or latent infections produced similar findings to those of the control group ($p>0.05$). The latent infection group responses to the whey and Blg were slightly higher than those of the Ova and EW. According to the whole lymphocyte response, the allergy and tolerance were successfully achieved. Within the groups, all of the given proteins generated similar lymphocyte responses.

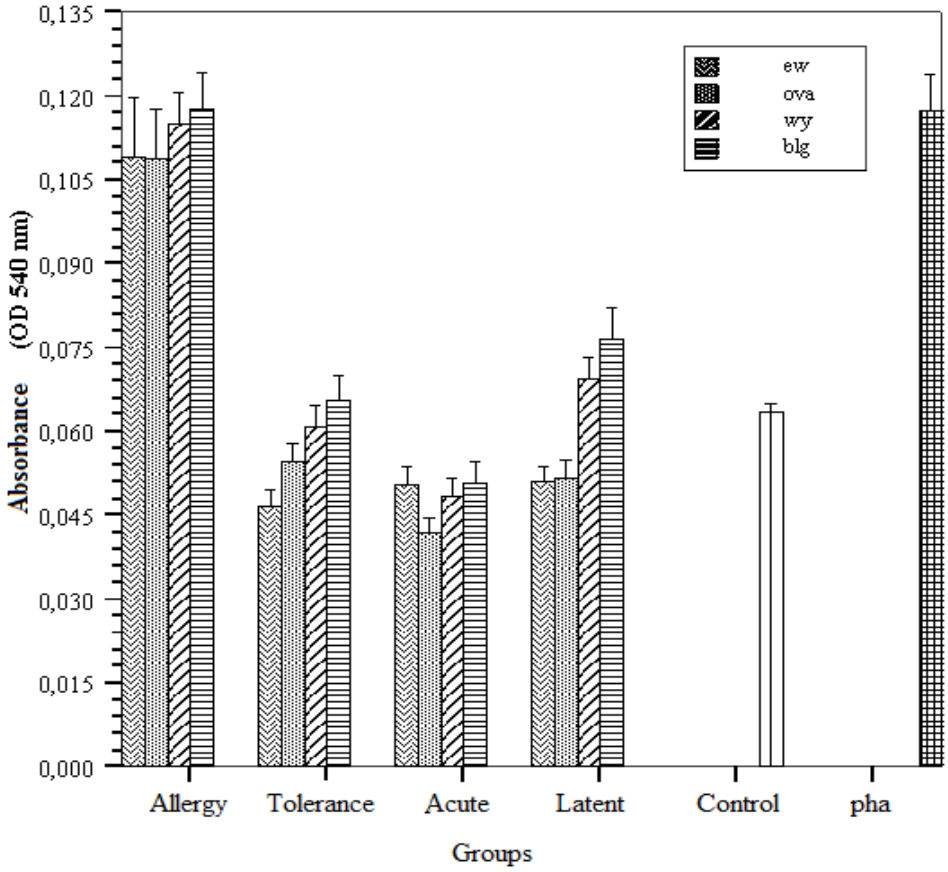

Figure 2: MTT – Lymphoproliferation assay results. Allergen proteins were challenged to lymphocytes, obtained from Balb/C mice spleen, of five different study groups summarized as: **Allergy:** allergy group without HSV-I infection; **Tolerance:** tolerance group without HSV-I infection; **Acute:** allergy group with acute HSV-I infection; **Latent:** allergy group with latent HSV-I infection; **Control:** without allergen; **pha:** phytohemagglutinin (lymphocytes taken from control mice); **ew:** egg white; **ova:** ovalbumin; **wy:** whey; **blg:** β-lactoglobulin.The serum samples were diluted 1:3000 times (manufacturer's recommendation is nearly 3:1000) and the measured IgE levels of the allergy group samples was so high that the absorbance values were detected as > 14.00 ng/ml those were out of the measurement range. In the other research group subjects, the average IgE amounts were detected as: 7.56±1.54 ng/ml, 11.78±0.76 ng/ml, 10.39±2.5 ng/ml, and 6.95±0.6 ng/ml for the control, acute, latent, and tolerance groups, respectively.

In this study, the observed IFN-γ, IL-4, and TGF-β levels in response to allergen proteins in the absence or presence of HSV-I infections were evaluated using the ELISA technique, and summarized in Figure 3. The IL-4 secretions from the Th2 cells cause raised IgE levels in allergies, while the IFN-γ expression from the Th1 cells suppresses the IL-4 and IgE expressions. TGF-β plays an important role in regulating tolerance development, and it is likely to have synergistic effects with IFN-γ. In the allergy group, the IL-4 response was found to be the highest ($p=0.000$), while there was statistical significance between the other subjects for the acute, latent, and tolerance groups with "p" values of 0.000, 0.004, and 0.002, respectively. The IL-4 levels of the tolerance, acute, and latent groups were found to be similar to those of the control group ($p=0.320$, 0.999, and 0.175, respectively). Apart from these, the acute infection group's IFN-γ profile was the highest, and for the control the significance was $p=0.018$; however, for the other assay groups the difference was not statistically significant ($p>0.05$).

In the regulatory cytokines expressed by the T regulator cells, the TGF-β level was found to be high in the acute infection, when compared to the allergy group (p=0.020). Additionally, in the non-allergy groups (latent and tolerance), the TGF-β was found to be high when compared with the allergy groups, but this was not statistically significant (p>0.05).

In the acute infection group, the IL-4 level was found to be similar to that of the non-allergen control group. The IFN-γ levels of the control subjects had similar levels to those of the tolerance and latent groups (p>0.05). These findings suggest that the latent infections of the HSV-I could not sufficiently effect immune responses as acute HSV-I infections, but they raised the TGF-beta expressions.

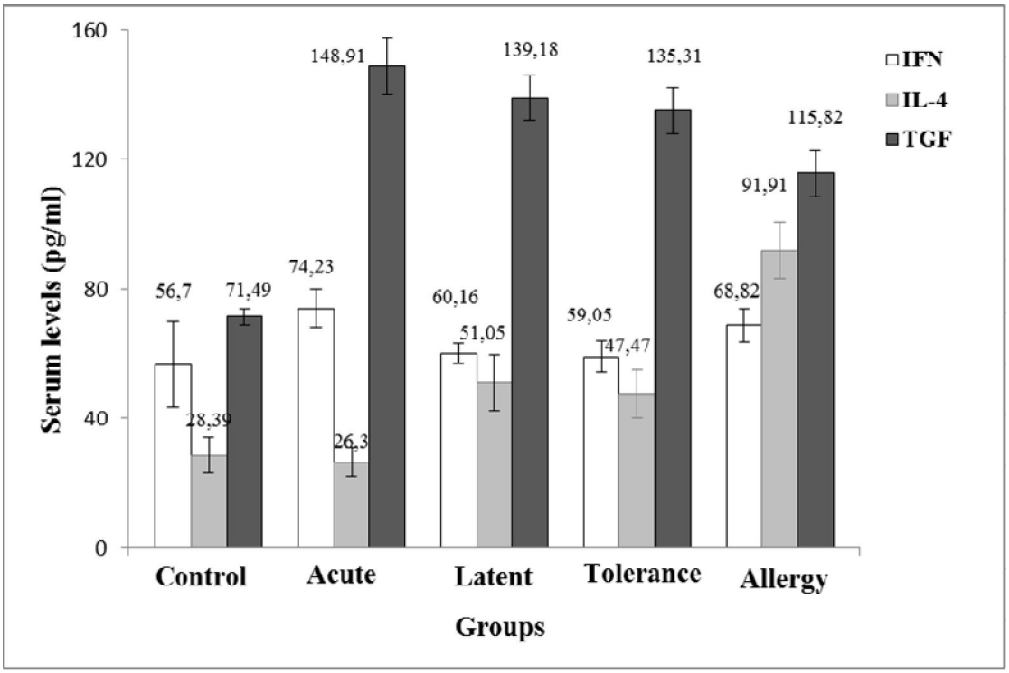

Figure 3: Serum sample cytokine levels of each Balb/C mice assay groups. Allergy induction was performed with egg white and whey proteins. **Allergy:** allergy group without HSV-I infection; **Tolerance:** tolerance group without HSV-I infection; **Acute:** allergy group with acute HSV-I infection; **Latent:** allergy group with latent HSV-I infection; **Control:** without allergen and HSV-I infection; **IFN:** interferon-γ; **IL-4:** interleukin; **TGF:** transforming growth factor-β.

With respect to our findings, in the presence or absence of the HSV-I infection, the allergy and tolerance responses were successfully achieved. This suggests that during the regulation of the allergic response, both the IFN-γ and TGF-β played significant roles in this experiment, and suppressed the Th2 response inducing IL-4 expression. All of the detected cytokine expressions and lympho proliferation assay responses revealed similar results.

4. Discussion

Information about the epidemiological parameters and the causes of food allergies remains limited, but numerous studies have been performed. The prevalence of egg, peanut, and milk allergies in childhood, especially, tends to have been increasing over the last two decades. Additionally, there is increasing interest in the possible relationships between diet variety, living conditions, and allergy generation. Changes in the dietary composition have suggested three theories with regard to allergy development: the dietary fat hypothesis, anti-oxidant hypothesis, and vitamin D hypothesis. Apart from these, the hygiene hypothesis remains the most studied and reposted theory (Lack, 2008). The lymphocyte response of the allergy group showed a definite proliferation of allergen proteins, in contrast to the control and tolerance groups, as expected. However, in both infection groups, the lympho proliferation was suppressed, unlike in the allergy group.

Lymphocyte proliferation was induced in the allergy groups under the induction of the Th1 and Th2 cytokines, in response to the proteins (Flinterman et al., 2010). The allergy group response, likely with PHA, was higher, as opposed to both infection groups. As observed in different studies, the stimulation of various cell lines showed similar results (Smit et al., 2007; Flinterman et al., 2010). In the lympho proliferation assay, the proliferation upon the administration of EW proteins and whey, in the allergy group was raised, when compared with the acute, latent, tolerance, and control groups.

A previous study by Fecek et al. (2010) demonstrated a change in the allergic response due to raised Th1 and IFN-γ after the viral infection, but this study detected no effect on the mucosal IgA. Smit et al. (2007) used an animal model for the RSV infection in respiratory tract hyper-reactivity, and observed both resistance and a reduced allergic response after an allergen challenge. In the acute infection group, as in the previously mentioned studies, the IFN-γ was found to be high ($p>0.05$), but the IL-4 cytokine response decreased ($p=0.000$). In the latent infection group, the cytokine responses were similar to those of the tolerance group. However, the acute and latent infection groups showed high TGF-β responses to the allergen proteins when compared to the control ($p=0.000$) and allergy ($p<0.05$) groups. This activity reflects the regulatory role of TGF-β with IFN-γ for IL-4 suppression. It is interesting that a high level of IFN-γ was detected, contrary to the expectations for the allergy group, as reported by Rowe et al. (2004), suggesting that IFN-γ plays a bipartite role in allergy development . On the other hand, $CD4^+$ and $CD8^+$ T lymphocytes seem to play key role in defensing HSV infection. $CD4^+$ lymphocytes produce IFN-γ, which has been shown to specifically facilitate the clearance of viral infection from HSV infected mice (Wagner & Bloom, 1997).

The IL-4 level was raised and lymphocyte hyper-reactivity was increased in the allergy group, after the allergen protein challenge; additionally, the TGF-β response was observed to be low. However, the TGF-β was high in the course of accompanying the development of the tolerance process. During acute infection, a high level of IFN-γ was expressed, with regard to the allergic response; conversely, it decreased the IL-4, suggesting that viral stimulation reduces the allergic response. In a previous study, Smit et al. achieved IFN-γ expression by using anti-IFN-γ, obtaining similar supportive findings of it being a likely suppressor of IL-4 expression (Smit et al., 2007).

The disequilibrium between the Th1 and Th2 immune responses has been attributed to the hygiene hypothesis, and exposure to infectious agents at early ages was the basis of this hypothesis (Schaub et al., 2006). The immune environment and the individual exposure to specific agents had effects on this process, as well as on the experimental conditions; therefore, discrepancies between the studies were present (Kemp &Björkstén, 2003; Smit et al., 2007; Aoki-Yoshida et al., 2016). In this assay, while the IFN-γ levels were expected to be low in the allergic subjects, in whom the Th2 response could not be suppressed, with high IL-4 levels no significant reduction, was observed. However, in the acute infection group, the IL-4 level was decreased, but the IFN-γ level was raised. In a previous report, Flinterman et al. (2010) observed high IFN-γ levels, as opposed to the expectations of the peanut allergen challenged subjects.

There was statistical significance in the TGF-β ($p<0.05$) and IL-4 ($p=0.000$) levels between the allergy and the other groups, with the exception of the IFN-γ group. In the tolerance group, the IFN-γ response was lower than in the acute infection group ($p>0.05$). This result suggests that the HSV-I infection possesses a protective effect in early life, which might be because of the polyclonal stimulatory induction on the immune system. Via this possible mechanism, the IFN-γ might decrease the IgE expression due to the suppression of IL-4 in inhibiting the allergic response (Saghafian-Hedengren, Sverrenark-Ekström, Linde, Lilja & Nilsson, 2010). It should be kept in mind that while the TGF-β expression was high in the non-allergic groups (acute, latent, and tolerance), the Th2 cytokine IL-4 level was decreased according to the allergy group. These findings may be proof of the regulatory function of TGF-β in the aspect of the allergy group cytokine responses, as previous reports have indicated (Palomeres et al., 2010). Increased IL-4, decreased TGF-β, and decreased IFN-γ levels in the allergy group, when compared with the acute infection group, can be supportive of the observed results for the hygiene hypothesis. However, the tolerance and latent infection groups show an increased production of IL-4 to whey and EW proteins, when compared to the control group. The IL-4 level (the induced Th2 product) underscores that these tolerant subjects have whey and EW specific IgE.

In the allergy mechanism, during the sensitization phase, IL-4 and IL-13 are the main cytokines for inducing B-cells to produce antigen specific IgE (Kimber & Dearman, 2001; Kemp & Björkstén, 2003; Palomeres et al., 2010).

In a study performed in the USA, HSV-I seropositivity was reported to be as 35% and 18% of five year old black and white children respectively. By late puberty in the lower socioeconomic populations, seroprevalence was raised 70 to 80%. On the other hand, in higher socioeconomic conditions, it was detected as 40 to 60% (Fatahzadeh & Schwartz, 2007). It was reported that, HSV-I seropositivity was found to be as 85.1% in Turkish population (Dogan, 2013). In industrialized population when mucosal viral infection like HSV-I seroprevalence is less, the atopic disease development possibility was seem to be high (Janson et al., 2007; Lack, 2008; Palomeres et al., 2010). In the aspect of the results and previous suggestions, inverse correlation between herpes virus immunity and atopic disorders might have supportive value for interpreting hygiene hypothesis.

In summary, HSV-I infected Balb/C mice were used as an experimental animal model to obtain supporting evidence for the hygiene hypothesis. The obtained results suggest that, at early ages, the contraction of infections has induction effects on Th1 responses, which might affect the Th1/Th2 balance and suppress allergy development. The functional activity of IL-4 in the allergy pathway, and TGF-β and IFN-γ in the tolerance response can be evaluated in the aspects of this study. However, because of discrepancies between the experimental models, used methodologies, and assessment protocols, there still exists missing unidentified mechanisms to consider with regard to the relationship between allergies and the hygiene hypothesis. In further analyses, the selective induction of T cells for allergen proteins should be achieved to identify the immune mechanisms which may act in this complex system.

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