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Oral tolerance: Recent advances on mechanisms and potential applications

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Abstract

Oral tolerance is a type of immune hypo-responsiveness induced by oral administration of food or harmless gastrointestinal antigens. It is evident that the induction of oral tolerance can protect our body from enteric problems, such as food allergies and colitis caused by autoimmunity. Here we review the immunological mechanisms of oral tolerance, the role of T cell cytokines in generating tolerance and the impact of Peyer's patches and mesenteric lymph nodes, and discuss the part played by commensal microflora in the regulation and maintenance of the intestinal barrier. The potential clinical applications of oral tolerance in human disease therapy are also included in this review. Understanding the mechanisms of oral tolerance may lead to the development of alternative strategies for preventing or suppressing the symptoms of autoimmune diseases and allergies.

Key words: Oral tolerance; immune system; mucosal tissues; microflora; clinical trial

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Introduction

The immune response functions to prevent invasion of pathogens and also includes suppression tolerance mechanisms to prevent targeting self-antigens and the development of autoimmunity. Tolerance can occur both in developing and mature lymphocytes (central and peripheral immune tolerance, respectively).

In central immune tolerance, immature or developing T and B cells that react strongly to self-antigens are eliminated by clonal deletion.[1] However, clonal deletion is not perfect because some self-reactive T cells may not react strongly enough to induce deletion signals and antigens must be present in the thymus to induce central tolerance. Peripheral tolerance occurs outside of primary lymphoid organs (the thymus and bone marrow). Mechanisms of peripheral tolerance include clonal anergy (lymphocytes fail to respond), deletion of chronically activated T cells (activation induced cell death) and suppression by regulatory immune cells.[2,3]

The most common methods to induce tolerance to non-harmful antigens in humans and animals involve introducing proteins or peptides without adjuvants by intravenous injection, portal vein injection, intranasal administration, oral mucosal or sublingual administration or gastrointestinal mucosal administration or skin administration.[4,5] However, oral tolerance remains the most rigorously investigated form of tolerance because the oral administration of proteins or peptides can lead to the induction of systemic and local unresponsiveness to innocuous antigens. A benefit of induced mucosal tolerance (orally or nasally) is that it is non-invasive. However, when inducing mucosal tolerance with normal food antigens or synthetic antigens a high amount of antigen is needed, as compared with systemic (intradermal) induction and an adjuvant may be needed in some situations.

The capability to induce oral tolerance has been recognized for decades. Many studies of induced oral tolerance have been performed using animal models and several have been performed using human subjects.[6] The aims of most oral tolerance studies have been to prevent and treat diseases, particularly allergic and autoimmune diseases (ADs), and even to prevent transplantation rejection.[7] The aim of this review was to focus on the key issues of oral tolerance, particularly the mechanisms of induced oral tolerance, the roles of the immune system, mucosal tissues and commensal microflora and the application of oral tolerance in humans. Furthermore, a comprehensive conclusion on oral tolerance with an emphasis on future research directions is provided.

Mechanisms of oral tolerance induction

Many experiments aimed to explain the mechanism of oral tolerance induction have been performed using animals with varying doses of antigens, mostly ovalbumin, in the feed. Oral tolerance occurs after either administration of a single high dose of antigen (> 20 mg) or repeated exposure to lower doses (100 ng–1 mg).[8] These two forms of tolerance, now termed high- and low-dose tolerance, are mediated by distinct mechanisms. The proposed mechanisms of oral tolerance involve clonal anergy, deletion (apoptosis) of antigen-specific T cells, active immune suppression, bystander suppression, and antibody response (**Figure 1**). Both CD4+ T helper (Th) and CD8+ cytotoxic T cells are important for oral tolerance induction. A subset of Th cells, including Th1, Th2 and regulatory T cells (Tregs), is most often associated with oral tolerance. However, the exact mechanisms of oral tolerance induction by Th9, Th17 and Th22 cells remain unknown.[9]

Feeding of mice with a high dose of antigen results in anergy of antigen-specific T cells in the Peyer's patches (PPs), mesenteric lymph nodes (MLNs), spleen and peripheral lymph nodes (LNs). This strategy also induces apoptosis of antigen -specific T cells in PPs. However, transferring T cells from mice fed a high dose of antigen to naïve mice failed to transfer tolerance.[2]

In contrast, feeding a lower dose of antigen induced proliferation of antigen-specific T cells and subsequent secretion of cytokines, particularly interleukin (IL)-4, IL-10 and transforming growth factor-beta (TGF-β), resulting in suppression of the Th1 immune response.[10] CD4+ and CD8+ T cells isolated from mice fed a low antigen dose were able to transfer immunity against further immunization with the same antigen when administered to naïve mice.[11] In contrast, feeding either a high or low dose of ovalbumin antigen to rats with ovalbumin-induced arthritis resulted in T cell anergy that could not be transferred to naïve rats.[12]

In IL-4-deficient mice, feeding a high dose of ovalbumin suppressed both Th1 and Th2 responses, and induced tolerance. Likewise, tolerance was induced in both Th1- and Th2-defective mice by feeding a high dose of ovalbumin. These results indicate that oral tolerance can be induced independently of Th1 or Th2 cytokines.[13] Moreover, feeding mice a high or low Ag dose was associated with increased TGF-β production by stimulated splenocytes. Furthermore, feeding mice a high Ag dose reportedly induced proliferation of a specific T- cell subset with an activated phenotype (increased CD69 and CTLA-4 with decreased CD45RB and CD62L expression) and secretion of the cytokines interferon (IFN)-γ, IL-4 and IL-10 in the PPs or MLNs soon after feeding and prior to anergy or apoptosis.[2]

Oral antigen administration can induce systemic immune tolerance via bystander suppression, which occurs when tolerance is developed against one antigen, such as by oral administration, and then that antigen is administered in combination with a second antigen, resulting in tolerance to both. Bystander suppression can be induced by a specific antigen and then suppressed in an antigen-nonspecific manner.

The mechanism of bystander suppression may best be explained by the microenvironment at the priming site where a tolerized antigen might induce hyporesponsiveness to a second antigen. Bystander suppression does not reflect clonal deletion or reduced clonal expansion of T cells specific to the bystander antigen.[14] Upon serum transfer, CD25+ Tregs play a role in the suppression of T cell proliferation.[15] Bystander suppression may be used to induce tolerance when the immunogenic antigen is unknown. Repeated low-dose ingestion of an antigen leads to induction of antigen-specific Tregs that have suppressor activity. Various Treg populations may be induced, including TGF-β-producing CD4+ Th cells and gut-derived antigen-specific CD4+/CD25+/Foxp3+ T cells, also known as induced Tregs (iTregs).[16,17] While various T-cell subsets with regulatory activity have been shown to confer tolerance in transfer experiments, gut-derived iTregs are critical for oral tolerance induction as deletion of iTregs in a DEREG (DEpletion of REGulatory T cells) mouse model resulted in loss of tolerance.[18] In contrast, naturally occurring thymus-derived Tregs do not appear to be required for successful oral tolerance induction.[19]

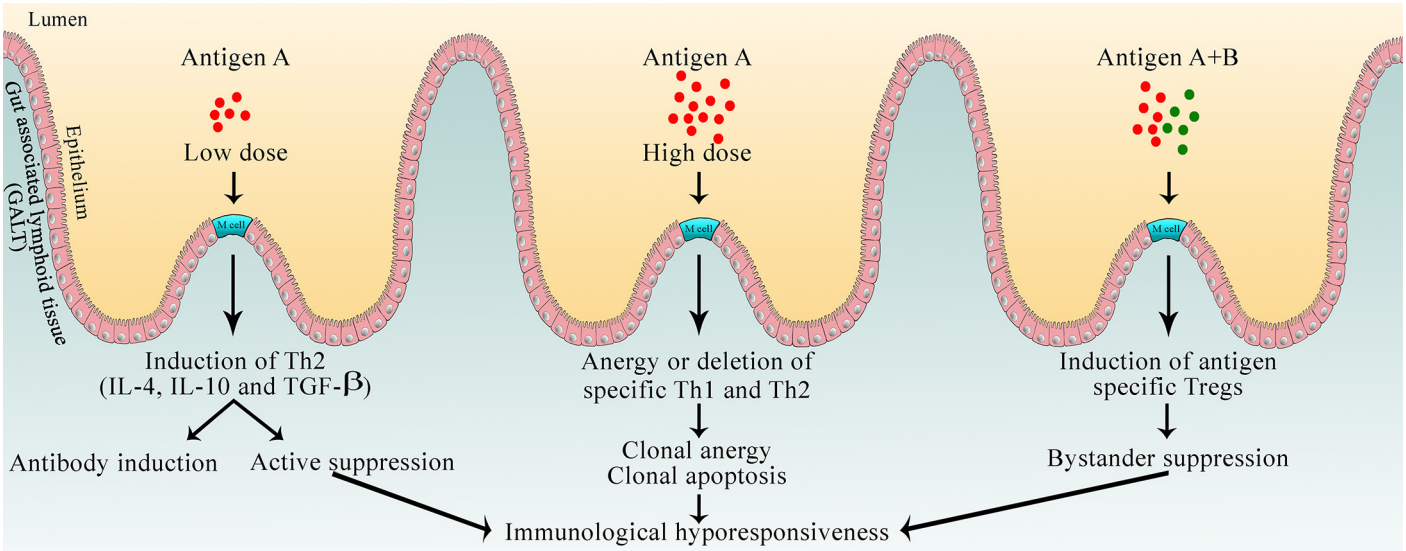

Figure 1. Mechanisms of immunological hyporesponsiveness include active immune suppression, clonal anergy, cell deletion (apoptosis), bystander suppression and antibody induction.

In human adults, the oral antigen uptake which can persuade systemic immune response are poorly understood. The tolerance to food antigens (bovine gamma globulin, ovalbumin and soybean) is dose specific and usually mediated by T cell anergy. A study of repeated low dose and single high dose of keyhole limpet hemocyanin (KLH) has been documented in healthy volunteers. Repeated low dose oral KLH induced antigen specific CD4+ T cells for the gut homing receptor integrin b7 and the cytokines IL-2 and TNF-α. Oral feeding of KLH increased a subsequent parenterally induced systemic CD4+ T-cell response and cytokine production of IL-4 and IL-10, whereas IFN-c, IL-2 and TNF-α-producing cells were decrease. In contrast, a single high dose oral KLH had less effect on antigen-specific immune responses comparing with repeated low dose oral KLH.[20] Foxp3+ Tregs play a key role in controlling the magnitude of immune responses to the antigens. One study reported that a low dose antigen promotes induction of Foxp3+ in human CD4+ cells.[21] Besides, these Tregs not only suppress Ag-specific responses, but also mediate bystander suppression. Bystander suppression of T cells specific to the tetanus toxoid was detected after tolerance induced by bovine gamma globulin or ovalbumin with heterogeneity in the responses between individuals and types of food antigens.[22]

Table 1 summarizes the studies of high and low dose tolerance in this context. Overall, these studies of antigenic exposure in both mice and human suggest that the different mechanisms of oral tolerance are determined by the dose of fed antigen. These mechanisms are mutually inclusive manner, which more than one mechanism may be functional with the same antigen.

Table 1. Dose tolerance studies performed in Animals and human

Animal Studies	
High Dose Tolerance	**Low Dose Tolerance**
1. Anergy of antigen-specific T cells in PPs, MLNs, spleen and peripheral LNs.[2] 2. Apoptosis of antigen-specific T cells in PPs.[2] 3. Suppression of Th1 and Th2 responses in IL-4-deficient mice.[13] 4. Induced proliferation of a specific T- cell subset with an activated phenotype and secretion of the cytokines such as IFN-γ, IL-4 and IL-10 in the PPs or MLNs.[2]	1. Induced proliferation of antigen-specific T cells and subsequent secretion of cytokines, particularly IL-4, IL-10 and TGF-β.[10] 2. Immunity transferred via CD4+ and CD8+ T cells from immunized mice to naïve mice.[11] 3. Induction of iTregs.[16,17]
Human Studies	
High Dose Tolerance	**Low Dose Tolerance**
Induction of CD4+ T cell periferation without antigen-specific response.[20]	1. Induction of antigen-specific CD4+ T cells, antigen-specific IgG1, IgG3 and IgG4, IL-2, IL-4 and TNF-α.[20] 2. Induction of Tregs by suppressing Ag-specific responses, together with bystander suppression.[21]

Another mechanism of oral tolerance is through induction of antibodies. Tolerance to antigens can be transferred using serum from antigen-fed mice.[23,24] Administration of IgG antibodies to mice can also suppress IgE-mediated hypersensitivity by mast cells.[25] In human, early introduction of peanut in children resulted in increased peanut-specific IgG4 that conferring protection against peanut allergy.[26] Perezabad *et al.* reported that the oral food desensitization in children involves decreased reactivity of mast cells and basophils, increased food-specific IgG4 antibodies, and eventually decreased food-specific IgE antibodies.[27] Thus, the role of antibodies appear to be crucial for oral tolerance induction, but the mechanisms underlying this effect remains to be determined.

Role of cytokines in oral tolerance

Although no single mechanism is solely responsible for tolerance, cytokines play important roles in oral tolerance induction. Over the past several years, much attention has focused on the function of various T cell-secreted cytokines in tolerance induction.

T cell-produced cytokines are classified into six groups: Th1 cytokines, such as IL-2 IL-12 and IFN-γ, which stimulate cell-mediated immune responses, Th2 cytokines, such as IL-4, IL-5, IL-10 and IL-13, which stimulate antibody production during an allergic reaction that counteract Th1 cytokines, Th17 cytokines, such as IL-17 and IL-17F, which are involved in the host defense against extracellular pathogens, Th22 cytokines, such as IL-22, which induces human skin-homing memory T cells and functions in host defense at mucosal surface as well as in tissue repair, Th9 cytokines, such as IL-9, which are associated with the immunopathology of asthma, and regulatory T (Treg) cytokines, such as IL-10 and TGF-β, which suppress T cell proliferation and regulate the functions of macrophages, as well as Th1 and Th2 effector T cells. A dominance of Th1 cytokines plays a negative role in oral tolerance and causes a loss of tolerance, whereas a dominance of Th2 and Treg cytokines plays a positive role and promotes oral tolerance by suppressing the Th1 response (**Table 2**).[29,33,35,36,38-40,72,73] Food and non-harmful antigens normally induce the Th2 or regulatory cytokine response.[28] For example; TGF-β -producing cells are increased in the colonic tissue of mice in response to oral tolerance to haptenized colonic proteins, and in the brain of mice with tolerance to myelin basic protein (MBP). The PPs of mice fed three times with low doses of interphotoreceptor retinoid-binding protein and systemically administered IL-2 had secreted more TGF-β, IL-4 and IL-10 after antigen stimulation. In addition, the splenocytes of mice fed with a low dose of MBP secreted higher levels of IL-4, IL-10 and TGF-β after stimulation.

Table 2. Role of cytokine in oral tolerance

Cytokines with positive roles	Cytokines with negative roles
1. Th2 cytokines: IL-4,[29,32,33,36] IL-13[9,32,33] 2. Treg cytokines: IL-10,[29,32,36] TGF-β[29,31,35] 3. Others: macrophage-derived IL-1β,[72] ILC3-derived GM-CSF,[72] ILC3-derived IL-22[73]	Th1 cytokines: IL-12,[35] IFN-γ[38-40]

Furthermore, the peripheral LNs from mice fed hen egg white lysozyme had higher IL-4 and TGF-β production after antigen stimulation. Another study demonstrated that oral administration of staphylococcal enterotoxin A enhanced the immune tolerance to MBP in the gut mucosa by increasing IL-10 and TGF-β levels.[29] Recently, TGF-β present in the milk also enhanced the generation of tolerance to antigens carried in the breastmilk.[30]

T-cell proliferation was decreased in TGF-β knockout mice fed a high dose of antigen. However, feeding TGF-β knockout mice a low dose of antigen also resulted in decreased T cell proliferation, but to a lesser degree than in normal mice. TGF-β knockout mice and normal mice fed a low dose of antigen have decreased cytokine production (IL-10, IL-4 and IFN-γ), suggesting that TGF-β might not be the exclusive mechanism for tolerance, particularly in this mouse model for which tolerance was induced by feeding a low antigen dose rather than inhibition of cytokine production. Thus, tolerance might be induced by clonal anergy.[11] These findings indicate that cytokines play important roles in the regulation of mucosal immune responses. Other studies have indicated that TGF-β can convert peripheral CD25−/CD4+ T cells to CD25+/CD4+ T cells that have a phenotype and function resembling those of natural Tregs, which may be caused by TGF-β-induced expression of Foxp3 and CTLA-4, and induction of anergy of CD25−/CD4+ T cells in a contact-dependent manner, leading to reduced secretion of cytokines by Th1 and Th2 cells. In this study, IL-10 had no role in the conversion of CD25− T cells to CD25+ T cells.[31]

Numerous studies have demonstrated that the administration of specific cytokines can induce oral tolerance. For example, feeding IL-10 or IL-4 together with an oral antigen enhanced the development of oral tolerance compared with feeding of the antigen alone. The administration of IL-4 intraperitoneally together with MBP feeding also reduced the severity of experimental autoimmune encephalomyelitis (EAE), while injection of IL-4 or feeding MBP alone did not. This study also found that IL-4 can induce TGF-β secretion by T cells *in vitro*, while culture with IL-4 and IL-13 induced the generation of peripheral CD25+ Tregs with an anergic phenotype that suppressed the proliferation of CD25−T cells *in vitro*.[32] IL-4 and IL-13 can downregulate expression of toll-like receptors (TLR)-3 and -4, and secretion of IL-8 in human intestinal epithelial cell lines, suggesting that signalling of IL-4 and -13 is decreased through TLR-3 and -4.[33] Also, TLR2 activators were recently found to modulate oral tolerance in mice[34] however; the role of TLR2-mediated cytokines in oral tolerance remains unclear.

In an experimental granulomatous colitis mouse model, the systemic administration of anti-TGF-β or IL-12 abrogated oral tolerance,[35] indicating that TGF-β and IL-12 have opposite effects in the regulation of the mouse mucosal immune response.

IL-4 knockout mice developed abnormal PPs that lack germinal centres but had intact MLNs. These mice also had defective gut antibody and T cell responses after immunization with ovalbumin and keyhole limpet hemocyanin, but a normal systemic response to intravenous immunization.[36]

Although IL-4 and IL-10 have been associated with oral tolerance, IL-4 or IL-10 knockout mice had normal oral tolerance. In an autoimmune uveitis mouse model, IL-4 or IL-10 knockout mice had defective oral tolerance development in response to oral antigens at doses that induce cytokine production in naïve mice. Reconstitution of IL-10 in IL-10-depleted mice restored the ability to develop oral tolerance; however, reconstitution with IL-4 did not. In an EAE model, IL-10 knockout reduced oral tolerance development, but to a lesser degree than normal mice, while IL-10 knockout mice seemed to develop more severe disease.[37]

Despite many studies showing that IFN-γ levels are reduced concomitantly with a decrease in T cell proliferation *in vitro* and with the severity of IFN-γ-mediated autoimmunity, feeding of an antigen at either a high dose[38] or low dose[39] increased IFN-γ levels in PPs, MLNs and the spleen *ex vivo* or after culture with antigen. In IFN-γ receptor knockout mice and IL-12 deficient mice, oral tolerance resulted in a normal decrease in delayed-type hypersensitivity (DTH) and antibody response. IFN-γ or IFN-γ receptor knockout mice still had a decreased Th2 response, indicating that oral tolerance in these mice was not the result of the deviation to Th2 cytokines.

Although the outcomes of blocking IFN-γ were controversial, IFN-γ was shown to decrease the number of antigen -specific T cells at effector sites during oral tolerance, and induces effector cell death after stimulation without co-stimulatory cells *in vitro*. IFN-γ knockout mice had a greater T cell response (increased DTH and proliferation) and altered cytokine production compared with normal mice,[40] suggesting that IFN-γ may play a role in the regulation of T cell responses.

The role of PPs and MLNs in oral tolerance

The intestinal immune tissue is a primary site of sensitization to oral antigens. Gut-associated lymphoid tissue (GALT) plays an important role in oral tolerance induction. GALT, which is composed of PPs and MLNs, is organised in the intestinal lymphoid follicles. This section describes the involvement of PPs and MLNs in oral tolerance induction.[41]

MLNs are clearly essential for oral tolerance induction; however, the necessity of PPs remains controversial. Fujihashi *et al.* demonstrated that mice with normal MLNs, but lacking PPs, failed to develop oral tolerance after administration of high-dose ovalbumin,[42] which was demonstrated by DTH development, induction of an antibody response and T cell proliferation. However, oral tolerance was induced in response to hapten but not a hapten-ovalbumin conjugate. Conversely, other studies reported that normal oral tolerance can be induced in the absence of PPs. For instance, Spahn *et al.* found that mice deficient of PPs could develop oral tolerance to ovalbumin, resulting in decreased DTH and IFN-γ production and higher TGF-β secretion, similar to normal mice,[43] while oral tolerance induction failed in mice lacking both PPs and MLNs. Worbs *et al.* verified that MLN lymphadenectomized mice and C-C chemokine receptor 7 [a homing receptor for T cells and dendritic cells (DCs) to LNs] knockout mice failed to develop oral tolerance.[44]

Cellular components of GALT have been shown to be crucial for oral tolerance induction. The role of microfold (M) cells in oral tolerance induction was investigated by Suzuki *et al.*,

who demonstrated that a delivery system targeting antigens against M cell proteins facilitated oral tolerance induction due to a reduction in Ag-specific CD4+T cells and increased levels of TGF-β1- and IL-10-producing CD25+/CD4+ Tregs in both systemic and mucosal lymphoid tissues.[45] Moreover, much evidence indicates that B cells are also important for oral tolerance induction. B cell-deficient mice having defective PP and M cell development fed antigens have defective production of the regulatory cytokines IL-10 and TGF-β. The EAE mice retained a state of anergy after being fed antigens with more severe encephalomyelitis than normal mice. Other studies have demonstrated that B cell-deficient mice have the same responses as normal mice to oral ovalbumin administration, which decreased the extent of T cell proliferation and IFN-γ secretion, while retaining a decreased DTH response and increased secretion of IL-4, IL-10 and TGF-β *in vitro*.[46] Furthermore, DCs also play an important role in determining the induction of immunity or tolerance. Gastrointestinal DCs reside in the lamina propria, PPs and MLNs and migrate to the intestinal lymph ducts and thoracic ducts. Viney *et al.* showed that mice fed the haematopoietic growth factor FMS-like tyrosine kinase-3 (FLT3) ligand, which increases the number of mature DCs in peripheral tissues, had an increased number of DCs in the spleen and mucosal sites. Mice treated with the FLT3 ligand developed greater tolerance to orally administered ovalbumin than control mice,[47] suggesting that DCs also have an important function in oral tolerance development.

Gastrointestinal DCs are divided into subtypes according to the expression of specific surface molecules and have different locations, as well as T cell stimulatory, cytokine production and migratory activities. It is evident that intestinal and hepatic lymphoid DCs have at least three subsets based on a high or low expression of signal regulatory protein-α (SIRP-α),[48] and the presence or absence of CD103, CD11b, and CX3CR1.[49,50] Low SIRP-α-expressing DCs are found in the T cell areas of PPs and MLNs, while high SIRP-α-expressing DCs are found outside of these areas. Intravenous lipopolysaccharide administration induced the migration of high SIRP-α-expressing DCs to the T cell areas and enhanced lamina propria DC migration to the draining LNs of the intestines.

In pigs, DCs in the lamina propria are mainly CD11b+/SIRP-α+, while those in the subepithelial dome of PPs are mainly CD11b-/SIRP-α+ and those in the interfollicular region of PPs are CD11b−/SIRP-α−. In MLNs, the DCs are mainly CD11b+/SIRP-α−, while those in the intestinal LNs (after removal of MLNs) are CD11b+ and SIRP-α positive or negative.[51] These findings indicate that DCs that migrate to the MLNs are mainly from the lamina propria and those in the intestinal LNs have mature phenotypes but are poor T cell stimulators.

In mice, CD8α+ plasmacytoid DCs in the spleen and MLNs secrete IFN-α after stimulation,[52] but are less effective at inducing T cell proliferation and produce more IL-10 and less IFN-γ during culture with ovalbumin specific T cells, as compared with CD8α+ non- plasmacytoid DCs. MLN CD8α+ plasmacytoid DCs drive naïve CD25− T cells into a regulatory phenotype that can suppress proliferation of other T cells and produce greater amounts of IL-10 and IL-4 with some IFN-γ.

In mice PPs, there are three distinct populations of CD11c+DCs: CD11b−/CD8α+, CD11b+/CD8α− and CD11b−/CD8α−, respectively. CD8α+DCs are derived from lymphoid precursors and found predominantly in the interfollicular T cell areas, whereas CD11b+DCs are derived from myeloid precursors and found predominantly in the subepithelial dome. CD11b−/CD8α− DCs are found in the subepithelial dome,[52] interfollicular T cell areas, and intraepithelium.[53] After stimulation with an antigen, CD11b+ cells mature and move to interfollicular T cell areas to present the acquired antigens to T cells. An *in vitro* study showed that CD11b+ cells from PPs produced higher levels of IL-10 after stimulation with a CD40 ligand trimer and that these DCs stimulated naïve T cells into Th2 cells, whereas PP CD11b+ DCs in the MLNs and peripheral LNs did not secrete IL-10. CD8α+ DCs and CD11b−/CD8α− DCs produce IL-12 after stimulation.[54] In a mouse model of collagen-induced arthritis, mice with oral tolerance had increased numbers CD11b+ DCs and CD25+/CD4+ Tregs in the PPs. The CD11b+DCs produced more IL-10, induced a higher number of CD25+/CD4+ Tregs *in vitro* and also induced T cells to produce more IL-10 and TGF-β.[55]

The local microenvironment is important for the function of DC subsets. The tolerogenic features of DCs can be influenced by various factors produced by intestinal epithelial cells, such as TGF-β and retinoic acid.[56,57] Another factor is mucin secretion of goblet cells can increase the uptake by CD103+ DCs, which favour the induction of Tregs and promote the development of tolerance response.[58] In addition, intestinal commensal microbes also play critical roles in shaping the function of DCs and promoting tolerance. For example, DCs cultured in the presence of intestinal epithelial cells and Gram-positive commensal bacteria differentiate into IL-10-producing tolerogenic DCs.[59] Taken together, these findings establish a key role of the local microenvironment in tolerance regulation of DCs.

The role of commensal flora in oral tolerance

The intestinal tract is colonized after birth with a variety of ingested environmental and maternal commensal microflora. Previously, it was believed that epithelial cells and microflora do not interact because the microflora would not be able to access pattern recognition receptors on the epithelial cells that recognize common microbial molecules. However, it has been reported that the presence of microflora and their metabolites in the gut is important for the development and maintenance of intestinal immune homeostasis.[60] For example, commensal bacteria and their metabolites, such as short-chain fatty acids (SCFAs), including acetate, butyrate and propionate, play a role in homeostasis.[61,62] SCFAs derived from microbiota-mediated digestion of diet fibres prevent inflammation. Recently, it was found that the exposure of monocyte-derived DCs to SCFAs inhibited the release of proinflammatory cytokine induced by incubation with lipopolysaccharides,[63] which might be due to the effect of lipid mediators that activate anti-inflammatory peroxisome proliferator-activated receptors.[64]

In addition, normal microbial colonization of the intestine is important for the development of tolerance to foods and plays vital roles in the regulation and maintenance of the intestinal barrier. Murine models have been used to demonstrate that bacteria and their components can affect the induction of oral tolerance. For instance, Rodriguez *et al.* reported that germ-free mice colonized with microbiota from a healthy human infant

and sensitized with whey protein exhibited milder allergic symptoms following challenge with β-lactoglobulin than their germ-free counterparts.[65] In another study, Gaboriau-Routhiau and Moreau demonstrated that feeding mice cholera toxin or labile toxin with ovalbumin prevented the development of oral tolerance; however, as compared with germ-free mice, the presence of gut microflora shortened this effect and facilitated recovery of oral tolerance.[66]

Moreover, Lotz *et al.* reported that recognition of microbes by TLRs induced tolerance immediately after birth by exposure to exogenous endotoxins to facilitate microbial colonization and the development of intestinal host-microbe homeostasis.[67] TLR2 has been identified as a regulator of oral tolerance in the gastrointestinal tract. It was recently demonstrated that the probiotic *Bifidobacterium breve* develops regulatory IL-10 secreting T cells via TLR2 stimulation by CD103+ DCs, thus reducing inflammation in the large intestine.[68] Indeed, it is evident that microbiota have the ability to induce the development of Foxp3+Tregs. In a mouse model, protection against allergic sensitization to food conferred by Clostridia-containing microbiota was associated with an increase in the content of Foxp3+ Tregs in the colonic laminar propria and an increase in the concentration of IgA in faeces. Furthermore, *Clostridium spp*.[69] as well as *Bacteroides fragilis*[70] is potent inducers of Foxp3+Treg differentiation. Another study revealed that bacterial capsular polysaccharide A can alter the migratory capacity of CD39+/Foxp3+/CD4+ Tregs.[71] Polysaccharide A-treated mice had increased numbers of CD39+/Foxp3+/CD4+ Tregs homing to the inflamed central nervous system in EAE, which delayed the onset and reduced the severity of EAE, suggesting that bacteria-specific Foxp3+Tregs may also direct anti-inflammatory responses in the gut. Recent studies showed that microbiota can regulate host intestinal immunity by triggering intestinal macrophages to secrete IL-1β.[72,73] This IL-1β supported GM-CSF and IL-22 release by local type 3 innate lymphoid cells (ILC3s). ILC3-derived Il-22 has important function in mucosal defence by strengthening the epithelial barrier.[73] Meanwhile, ILC3-derived GM-CSF functions in DC and macrophage secretion of retinoic acid and IL-10, which were found to maintain the homeostasis of mucosal Tregs.[72] In this process, Tregs plays a major role in promoting B cell class switching to produce IgA response, inducing T cell anergy of effector cells, and inhibiting the inflammatory process. Together, these data support a role of microbiota in establishment of oral tolerance. It is possible that intestinal microbiota may be important for the development of T-cell tolerance. We proposed a mechanism in which an intestinal microbiota and their products induce oral tolerance by interacting with the intestinal epithelial and immune cells in the mucosal system (**Figure 2**).

The role of diet in oral tolerance

Some nutrients, such as retinoic acid (vitamin A) and tryptophan, are known to affect the immune system by conditioning mucosal DCs, thus providing the anti-inflammatory microenvironment needed in the mucosa.[74,75] Vitamin A upregulates DC enzymes, such as retinal dehydrogenase 2 (RALDH2), which catalyse the synthesis of biologically active retinoic acid to maintain mucosal immunity by generating the homing regulator T and B cells. Besides, vitamin A from diet is essential for the generation of Tregs.[76] In vitamin A–deficient mice, it was clearly shown the loss of IgA secreting B cells and T cells in the intestine.[77] This indicates the role of vitamin A in inducing oral tolerance.

Vitamin D has been proposed to play a part in oral tolerance as it can affect B and T cell migration, and Th17 cell maturation.[78] Moreover, vitamin D is required for the development of subset of intraepithelial CD8aa-expressing T-lymphocytes from the intestinal mucosa. Nevertheless, there was evident showing the involvement of vitamin D–deficiency with increased risk of shrimp and peanut allergy.[79] While one study found a feasible chance of food allergy after obtained vitamin D supplementation.[80] However, future investigation of clinical correlation is needed to support the effect of vitamin D on development of oral tolerance.

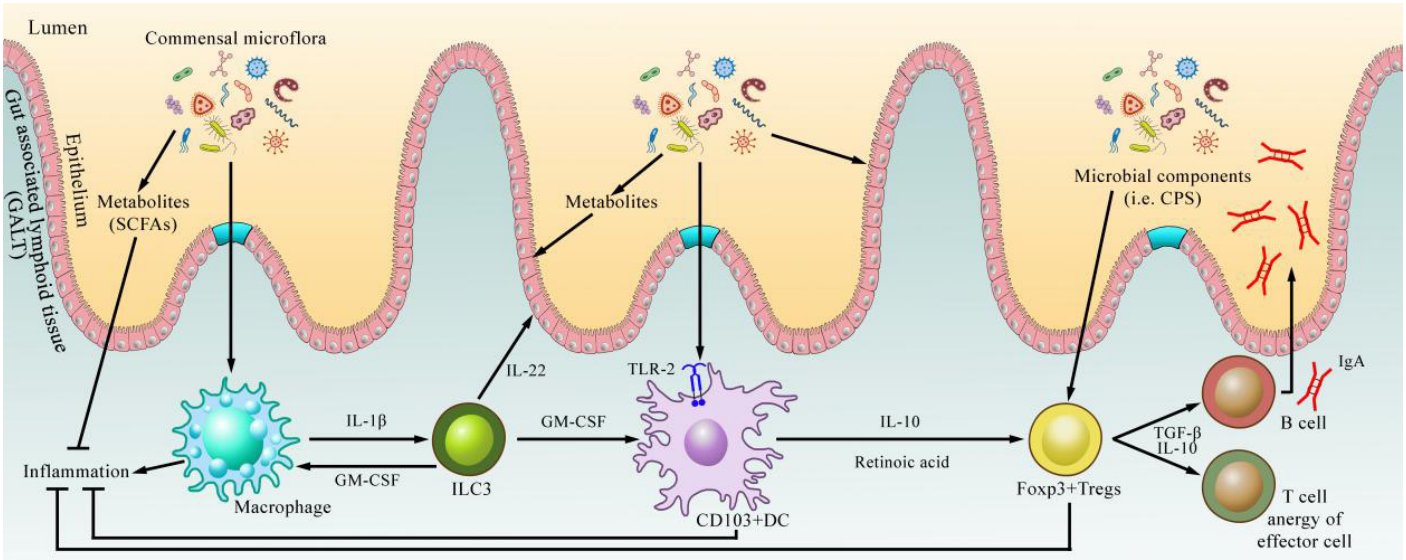

Figure 2. Mechanisms of commensal flora in oral tolerance are multifactorial process involving with the maintenance of the intestinal barrier and interaction with immune cells to support induction of oral tolerance.

Table 3. Human trials of oral immunotherapies.

Diseases	Oral antigens	Country	Phase	Type of study	Age	Sample size	Duration	Description	Efficacy	Lab support
Milk allergy[82]	Cow milk (very low amount)	Japan	N/A	open-label trial (A single, case control study)	≥ 5 yr	12	January 2013 to March 2015	Adverse allergic reactions were rare and most symptoms were mild	Yes	Yes
Autoimmune uveitis[87]	HLA-derived peptide mimicking retinal autoantigen	Germany	First result	Open study without placebo control group	N/A	5	N/A	Intraocular inflammation was improved and systemic corticosteroids were reduced in seven patients	N/A	N/A
Multiple sclerosis[88]	Bovine myelin basic proteins	US	III	A double-blind clinical trials	27–42 yr	34	N/A	Antigen-specific T cells from peripheral blood increased TGF-β1 secretion	N/A	Yes
Rheumatoid arthritis[89]	Bovine type 2 collagen, chicken type 2 collagen	China	III	A double-blind, randomized, methotrexate (MTX)-controlled study	18–65 yr	503	October 2004 to December 2005	Production of IFN-γ was decreased and production of TGF-β3 was increased	Yes	Yes
Crohn's disease[90]	Autologous colonic protein antigen	US	II	A randomized, double-blind, placebo-controlled, one-center trial	≥ 18 yr	31	N/A	The remission rate and quality of life (Inflammatory Bowel Disease Questionnaire score) was improved	N/A	Yes
Type 1 diabetes[91]	Insulin	US and Canada	Trial	placebo control	3–45 yr	103, 391	September 1996 to October 2002	The proportion of developed diabetes decreased in patients' relatives with insulin autoantibody (IAA) levels confirmed ≥ 80 nU/ml	N/A	Yes

N/A: not available

Trials of oral immunotherapies for human diseases

Although there have been many oral immunotherapy experiments using rodents, oral tolerance can also be induced in humans, which has led to improved treatment for several human diseases, particularly food allergies and ADs. **Table 3** is an attempt to summarize a wide range of clinical trials done using oral tolerance induction.

An oral immunotherapy for food allergies was first demonstrated in 1908 in a patient with egg anaphylaxis. Furthermore, oral desensitizing treatment with different types of food allergies resulted in successful desensitiszation in 83.3% of 59 patients.[81] Additionally, a high-risk population reacting to very low amounts of milk were treated with low-dose-induction oral immunotherapy and became subsequently unresponsive to cow's milk.[82] Recently, oral and sublingual immunotherapy trials for peanut, egg and milk allergies were summarized by Hamad and Burks in 2017.[83] Most trials showed that most patients achieve desensitization but only a small percentage achieve sustained unresponsiveness.

However, it became evident in clinical trials for food allergy that desensitization to a food allergen resulted in loss of clinical reactivity to the allergen. This loss of reactivity was frequently short-lived once exposure to the allergen was discontinued (2–24 weeks). Thus, Sampson *et al.* suggested this temporary state of nonresponsiveness off therapy as a "remission".[84] In addition, most of food allergens have been characterised regarding their sequence, structure and immunological properties. This knowledge provides a clue for development of novel strategy for oral immunotherapy for food allergy. Recently, new innovative forms of allergen molecules such as recombinant allergens and their derivatives have already been shown to be effective in clinical trials.[85,86] Probably, new molecular forms of oral immunotherapy may be available for the treatment of allergic patients in the near future.

Oral administration of autoantigens has also been tested for the treatment of human ADs. Currently, clinical trials of a variety of ADs are being conducted, including uveitis, multiple sclerosis, arthritis, Crohn's disease and type 1 diabetes mellitus. For example, a human leukocyte antigen-derived peptide that mimics the retinal autoantigen was used to induce oral tolerance for the treatment of chronic autoimmune uveitis, which improved intraocular inflammation and enabled a reduced dose of systemic corticosteroids in seven patients.[87] In multiple sclerosis patients, oral treatment with bovine MBPs daily for a minimum of 2 years increased TGF-β1-secreting antigen-specific T cells isolated from peripheral blood.[88] Indeed, using human heat shock proteins

linked to cholera toxin B to develop oral tolerance in patients with Bechet's disease and uveitis enabled the withdrawal of immunosuppressive drugs with no relapse in five of eight patients, and three were relapse-free for 10 months without treatment. In addition, oral administration of bovine type 2 collagen for 3 months resulted in a clinical improvement in eight of 11 juvenile arthritis patients. Six of these patients had decreased IFN-γ and increased TGF-β3 production by peripheral blood mononuclear cells. Likewise, chicken type 2 collagen has been effective in the treatment of rheumatoid arthritis and is safe for human consumption.[89] Moreover, in a clinical trial, patients with Crohn's disease given autologous colonic protein antigen by oral administration had a higher remission rate, as compared to the untreated group, although the difference was not statistically significant.[90] Oral insulin treatment was used to prevent the development of type 1 diabetes mellitus in the relatives of type 1 diabetes mellitus patients.[91] The oral insulin-treated group had a lower incidence of diabetes among those who had some degree of autoimmunity (insulin autoantibody ≥ 80 nU/ml), but no benefit was achieved in patients without insulin autoantibodies.

Results from the above studies suggest that patients with ADs are heterogeneous in their responses to oral tolerance induction with the responding group having immunologic profiles that differ from the unresponsive group. Multiple factors are likely involved in individuals with ADs who are more refractory to developing immune tolerance. The observed heterogeneity of responses may be caused by different stages of disease, drug treatments, genetic factors and/or environmental factors among patients.

Conclusions and future perspectives

Oral tolerance is a type of immune hyporesponsiveness to gastrointestinal antigens that can be acquired through oral administration. This review discusses a number of mechanisms of immunological hyporesponsiveness and highlights possible methods to facilitate the prevention or therapy of ADs and allergy. Although the mechanisms underlying oral tolerance are complex and involve multiple cellular and molecular processes, we have reviewed four mechanisms of immunological hyporesponsiveness including (1) anergy or the suppression of specific T cell subsets and Th1 and/or Th2 cytokines, (2) suppression by Th2 or regulatory cytokines, (3) bystander suppression by Tregs, (4) and induction of antibody response. Although most T cell subsets (Th1, Th2 and Tregs) and their cytokines are clearly involved in the development of oral tolerance, little is known about the underlying signaling pathways that regulate the immune responses responsible for oral tolerance. A better understanding of these processes is needed in order to identify new targets for modulating tolerance.

There is much evidence that PPs, MLNs and DCs actively participate in maintaining intestinal immune homeostasis and play important roles during oral tolerance induction. Likewise, there is increasing interest in the possible role of dietary factors in maintenance of mucosal immunity and development of oral tolerance. Interestingly, microflora in the gut also contributes to oral tolerance induction by interacting with intestinal epithelial cells and delivering tolerogenic signals that are transmitted to the immune system. Microflora and their metabolites, such as SCFAs, including acetate, butyrate and propionate, also play a role in homeostasis and immune system development in the gut. When these factors are disrupted, the system may be biased towards the Th2 phenotype and interfere with Treg development. There is a considerable interest in investigating the role of the intestinal microflora and metabolites in oral tolerance induction. Perhaps, probiotics may be useful materials for oral tolerance induction.

At present, clinical trials of oral tolerance are being conducted in a variety of human diseases, including allergy and ADs. However, the success of oral administration to induce hyporesponsiveness to gastrointestinal antigens in humans is limited, which might be due to the fact that most studies of oral tolerance induction have been conducted in non-human subjects, such as rodents and larger mammals (i.e. rabbits, pigs, sheep and non-human primates). It is possible that these animals may not respond to oral immunity similarly to humans. Moreover, in regard to the physiology of human intestinal absorption, a sufficient dose for humans may differ from those needed for other animals. Furthermore, there are individual genetic differences and genetic disorders in humans that have not yet been identified, which may explain why trials of oral tolerance in patients are more difficult than in animal models and why there is heterogeneity in the response to oral antigens. Indeed, additional studies on the variations and formulations of antigens to induce oral tolerance are required to determine the most effective method for humans. Lastly, we need to pay more attention to the short- and long-term safety of oral tolerance. In summary, while there is still a long way to go in better understanding of oral tolerance induction, the continued progress in this field has brought us closer to effective clinical development of oral tolerance for the treatment of human diseases.

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Conflicts of interest

All contributing authors declare no conflicts of interest.

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ORIGINAL ARTICLE

Efficacy of air purifier therapy in allergic rhinitis

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Abstract

Background: With the rising prevalence of allergic rhinitis, the utility of indoor environmental management deserves increasing scrutiny. This research aims at evaluating the ability of air purifiers to be a therapy of allergic rhinitis.

Methods: 32 subjects (25 ± 13.5 years old) diagnosed with allergic rhinitis were selected and HEPA air purifiers placed in their bedrooms for 4 months. Before the intervention and each month, dust samples were collected with a vacuum cleaner and the dust collector assessed for allergen content. Additionally, static dust collectors were left in place all month to collect dust by sedimentation. Particulate matter (PM) was assessed in terms of $PM_{indoor/outdoor}$ ratios. The Rhinitis Quality of Life Questionnaire (RQLQ) was used to assess symptoms.

Results: Der p 1 (78 (30, 82) ng/g) was the dominant dust mite allergen in air samples of patients' bedroom as well as static collections. Der f1 (444 (345, 667) ng/g) was the dominant allergen in bedding. Der f1 levels in both air and bed sampling significantly decreased after initiation of HEPA air purifiers ($P < 0.05$). $PM1.0_{indoor/outdoor}$, $PM2.5_{indoor/outdoor}$, $PM10_{indoor/outdoor}$ all decreased ($P < 0.001$) with the HEPA filtration intervention. According to RQLQ data, HEPA filtration was associated with improvements in activity limitation, non-nasal-eye symptoms, practical problems, and nasal symptoms ($P < 0.001$).

Conclusion: HEPA air purifiers can effectively reduce PM and HDM allergen concentration in the indoor air, and thereby improve clinical manifestations of patients with AR.

Key words: Allergic rhinitis, Air purifier, House dust mite, Rhinitis Quality of Life Questionnaire, PM

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Introduction

Allergic rhinitis is the most common type of rhinitis and its burden is increasing globally.[1,2] It affects quality of life and can be expensive. Wang et al[3] illustrated increasing prevalence of allergic rhinitis in the major cities in China. The total prevalence has increased from 11.1% in 2005 to 17.7% in 2011 while Guangzhou's prevalence has increased from 13.2% to 17.4%. Genetic variance cannot cause this pace of change; either some environmental factor is causative, or perhaps a disease classification factor is relevant (diagnostic shift from non-specific rhinitis, or increased awareness of allergic disease by the population). Importantly, environment allergens have well-elucidated impacts on allergic rhinitis.[2]

Ambient particulate air pollution (often represented as particulate matter, PM) is a risk factor for allergic disease.[2] Outdoor PM may contain various metallic elements that initiate or augment pediatric allergic rhinitis.[4] But the exposure to indoor ambient allergens is of primary importance.[5] For allergic patients, therapeutic interventions are often undertaken to help minimize relevant allergen exposure.[6]

Based on prior research, it is assumed that these environmental modification efforts should be effective at improving the quality of life of allergic rhinitis patients, by means of decreasing the ambient concentration of PM and relevant allergens. Although HEPA air purifiers are presented as tools to filter PM and allergens from air that passes through the device, the clinical utility of these units remain uncertain. We performed an intervention with air purifiers to determine the effect on improving the indoor environment, while simultaneously evaluating the intervention's effect as an adjuvant therapy.

Methods

Ethical Statement

The study protocol was approved by the Ethics Committee of First Affiliated Hospital, Guangzhou Medical University (No. GYFYY-2015-47). Written informed consent was obtained from

parents or guardians of all children before they participated in this study.

Rhinitis Quality of Life Questionnaires

The Rhinitis Quality of Life Questionnaire (RQLQ) was designed by Juniper and others.[7] The RQLQ used in this study was based on Juniper's RQLQ with slight modifications. This RQLQ evaluates limitation of activity, sleep (lack of a good night's sleep, waking during the night, difficulty getting to sleep), non-nasal symptoms (tiredness, fatigue, worn out, reduced productivity, poor concentration, thirst), practical problems related to allergies (need to blow nose repeatedly, need to rub/eyes, inconvenience of having to carry tissues or handkerchief), nasal symptoms (stuffy/blocked, sneezing, runny, itchy), eye symptoms (itchy, watery, swollen, sore), emotions (irritable, frustrated, impatient, embarrassed by nose/eye symptoms, restless). There are 28 questions requiring answers on a 0–6 points scale. Higher scores in RQLQ indicate more adverse effects on the quality of life.

Screening Method

Subjects diagnosed with allergic rhinitis who presented to the First Affiliated Hospital of Guangzhou Medical University were recruited. Our diagnoses were based on a 2015 allergic rhinitis clinical practice guideline.[8] The patients underwent allergen skin prick testing. Eligibility for the study required positive skin prick test positivity to at least one of the house dust mite (HDM) primary allergens, Der p 1 or Der f1, or alternatively, positive ImmunoCAP testing of serum for Der p 1 specific IgE and/or Der f1 specific IgE (defined as ≥ 0.35 kU/L). The subjects were not able to change their accommodations during the study. Furthermore, study participants needed to be able to properly cooperate with our research (including correct and persistent use of the supplied air purifier, performing the questionnaire investigations, completing follow up visits, cooperating with sample collecting). The details of the research were provided and all subjects signed informed consent.

Intervention

This study provided a new HEPA air purifier (BA1030/1045, BRI Air Purifier, Xiamen, China, CADR: 200-400m^3/h) for use from October, 2015 to February, 2016. Our team taught subjects or their parents how to correctly use the air purifier and confirmed correct usage during each return visit.

The Use of Air Purifiers

Air purifiers were set near the head of the bed while avoiding blockage by foreign matter (such as clothes). No concurrent humidifiers were allowed and it was recommend that door and windows should be kept closed when patients were utilizing the air purifier.

Review Methods

Trained investigators performed phone interviews to investigate the subjects' compliance with air purifier use each month. This involved ascertaining whether they correctly used the air purifier, how many times they used it in a week, and how long they used it each time. In the event incorrect usage was identified, education was provided.

Sample Collection

A trained investigator was assigned to collect the sample and data from each subject's room, using consistent technique throughout the study.

Air dust sample collection

Air dust samples were collected every month in the subjects' homes. A vacuum dust sampler (FCD-50 double dust sampler, China Galaxy Science Co. Ltd, Yancheng, Jiangsu, China) was set in the middle of the subject's room. The dust sampler utilized glass fiber filtering (A/E glass fiber filter, America PALL company, ply: 1 μm, diameter: 47 mm). Airflow volume was adjusted to 20 l/min. The dust sampling occurred for 4 hours, during which time doors and windows remained sealed closed.

Bedding dust sample collecting

Simultaneously, bedding dust samples were collected by means of filter paper glass fiber and a vacuum cleaner (Hazier ZW1401B). An elastic band was used to hold the filter on the top of the vacuum hose. Bedding dust samples were collected over 15 min.

Static dust sample collection

A glass fiber filter was placed within a 60 mm round open culture plate in each subject's bedroom to collect HDM by natural sedimentation. Each sample collection occurred for one month, during which time the sampling material was not moved or covered.

PM data collection

Indoor and outdoor PM(1.0, 2.5, 10) concentration data were collected by a DT-9881 Air Quality Detector (CEM) once a month. Indoor collections were performed at five locations (four corners of the bedroom and one point in the middle of the room). Outdoor collections were performed in triplicate on the balcony or out of the window.

RQLQ data collection

Each subject provided RQLQ questionnaire responses each month.

Sample Extraction

Dust samples were weighed immediately after collection. The glass fiber filter was shredded manually and placed into a 10 ml flask to which was added 1 ml of 1% BSA and 0.05% Tween-20 PBST at 4°C and shaken overnight. The fluid was then extracted by centrifugation at 4°C, 3000 g at 30 min, and supernatants stored at -20°C.

Assay of HDM Allergen

HDM allergens Der p 1 and Der f1 were assayed by double -antibody sandwich ELISA kits according to the manufacturers' instructions (Indoor Biotechnologies, Charlottesville, VA, USA). The mass of Der p 1 and Der f1 in each gram of dust was calculated.

Statistical Analysis

SPSS 19.0 statistical software (SPSS Inc, Chicago, IL, USA) was applied to report and analyze our data, including descriptive statistics for the subjects' basic characteristic (gender, age and sIgE), the duration of use of the HEPA air purifier, the concentrations of HDM allergens, PM and RQLQ results. The concentration of HDM was assessed by the Kolmogorov-Smirnov test. If normally distributed, independent sample t testing was employed. Abnormally distributed data were assessed by independent sample nonparametric tests. $PM_{indoor/outdoor}$ ratios were compared to the baseline ratio prior to intervention. Repetitive measure analysis of variance (ANOVA) was used to compare the monthly data (concentration of HDM allergen, PM, RQLQ). $P<0.05$ was considered statistically significant.

Results

Baseline Characteristics of the Subjects

32 subjects were enrolled (4–61 yrs old) (**Table 1**).

Table 1. Baseline characteristics of the subjects

		n	%	$\bar{x} \pm s$
Sex	male	12	37.5	
	female	20	62.5	
Age				25 ± 13.5
	< 18	12	37.5	
	≥ 18	20	62.5	
Der p1 sIgE/KU/L		32		52 ± 38.8
Der f1 sIgE/KU/L		32		50 ± 38.5

Duration of HEPA Air Purifier Use

The mean duration of daily HEPA air purifier use was 9.6 ± 3.3 hours and fairly consistent during the course of the study. (**Figure 1**)

Baseline HDM Allergen Concentration in Bedroom

Bedroom air and static collections found mainly Der p 1, while bedding samples were dominated mainly by Der f1. The bed's Der f1 and Der p 1 concentrations were substantially higher than air's Der f1 and Der p 1 (see **Table 2**).

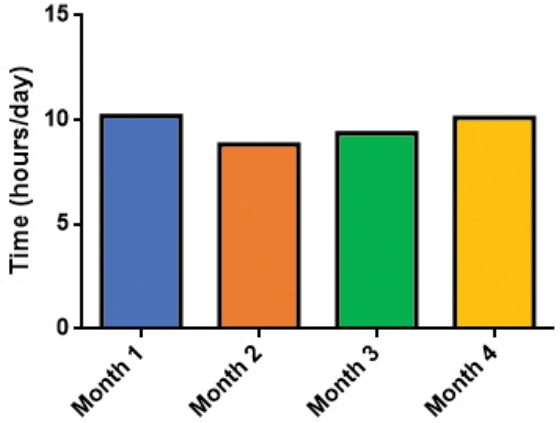

Figure 1. HEPA air purifier use by month.

Table2. Dust mite allergen levels before intervention

	Der p1 (P_{50} (P_{25}, P_{75}) ng/g dust)	Der f1 (P_{50} (P_{25}, P_{75}) ng/g dust)
air	78 (30, 82)*	30 (29, 31)*
bedding	139 (35, 426)*	444 (345, 667)*
static	158 (60, 175)*	60 (57, 80)*

*$P<0.05$

Efficacy of Air Purifiers to Remove HDM Allergen

Initiation of air purification was associated with significant declines in Der p 1 and Der f1 concentrations in static and bedding dust samples as shown in **Figure 2**. The concentration of house dust mite (HDM) in the air tended to decrease slightly with air purifier use, but the difference was not statistically significant. (**Figure 2**)

Efficacy of Air Purifier on PM in Bedrooms

$PM1.0_{indoor/outdoor}$, $PM2.5_{indoor/outdoor}$, $PM10_{indoor/outdoor}$ were found to be 0.94, 0.89, 0.91, respectively, at baseline (prior to intervention). These ratios declined after initiation of the HEPA intervention, indicating relatively lower $PM_{indoors}$ after initiating HEPA filtration (**Figure 3**). The changes in $PM_{outdoor}$ were also shown in **Figure 3**.

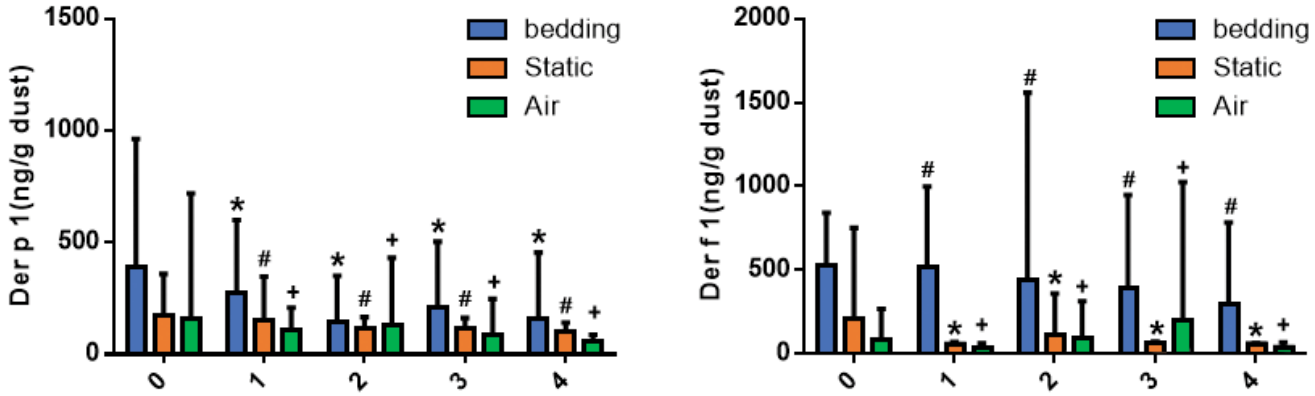

Figure 2. Changes in Der p 1 and Der f 1 concentrations.
0: prior to intervention with HEPA filtration; 1: after 1 month; 2: after 2 months; 3: after 3 months; 4: after 4 months. +: $P > 0.05$, *: $P < 0.01$; #: $P < 0.001$. All compared with baseline.

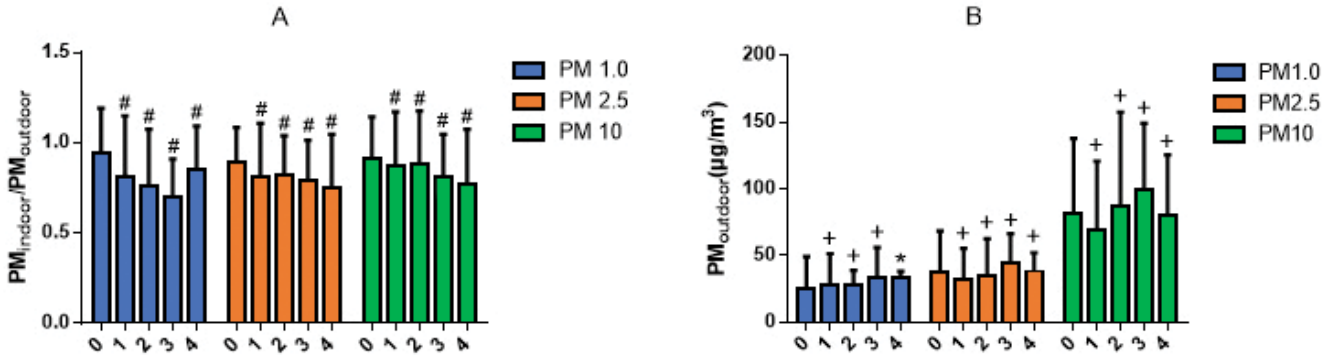

Figure 3. (A) Changes in $PM_{indoor/PMoutdoor}$; (B) Changes in $PM_{outdoor}$.
0: prior to intervention with HEPA filtration; 1: after 1 month; 2: after 2 months; 3: after 3 months; 4: after 4 months. +: $P > 0.05$, *: $P < 0.05$, #: $P < 0.001$. All compared with baseline.

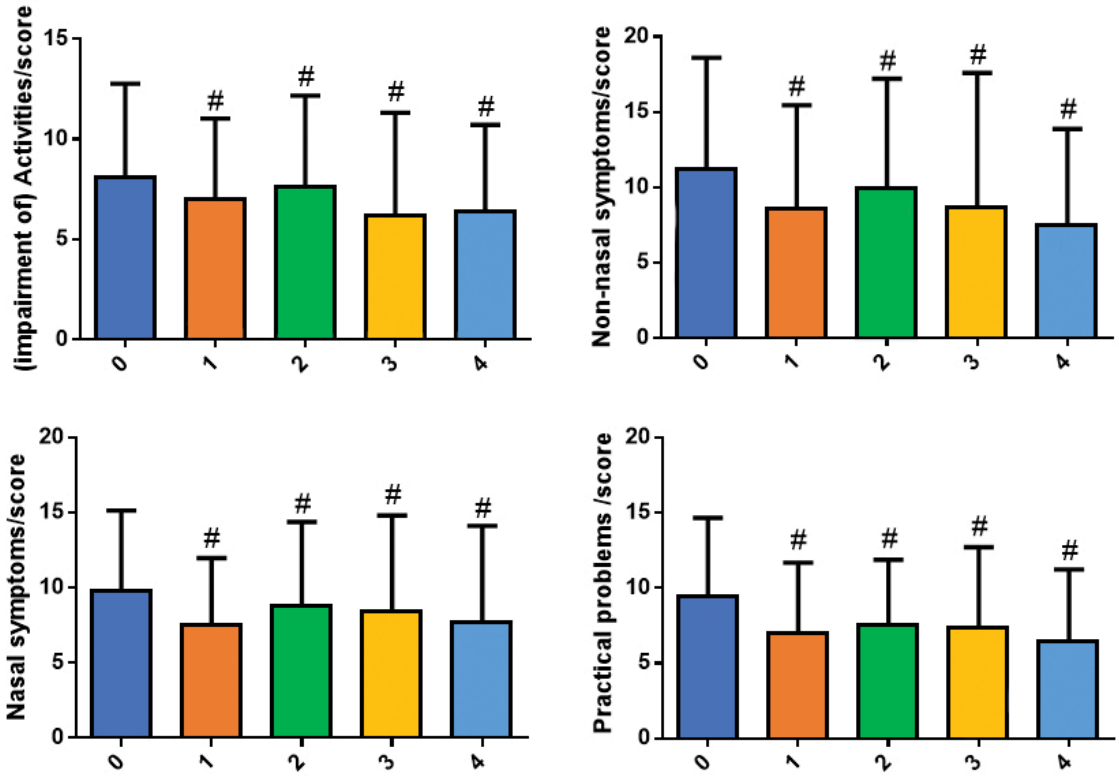

Figure 4. RQLQ score (Activity limitation, non-nasal symptoms, nasal symptoms, practical problems).
0: prior to intervention with HEPA filtration; 1: after 1 month; 2: after 2 months; 3: after 3 months; 4: after 4 months. #: $P < 0.001$. All compared with baseline.

Clinical Efficacy of Air Purifiers

According to RQLQ questionnaire results, scores for limitation of activity, non hay-fever symptoms, practical problems, and nasal symptoms were significantly decreased after initiation of the air purifier (**Figure 4**). The scores from three other items (eye symptoms, emotional, and sleep) also tended to decrease, although not statistically significantly.

Discussion

HDM is an important cause of allergy symptoms,[9] and PM serves as an allergic rhinitis risk factor.[10] Interventions to decrease indoor HDM allergens and PM have been studied internationally.[5] Studies incorporating air purifiers as an intervention remain few, and the evaluation of intervention effects on levels of HDM, PM, and patients' subjective symptoms are rarely reported in the literature.

According to our results, the concentration of Der f1 in bedding is significantly higher than that of Der p 1 in our region during the seasons of this study. This result is consistent with the data of Wang et al[11] The concentration of Der p 1 in samples collected from air was relatively higher than the concentration in the bed sheets.

The concentrations of Der p 1 and Der f1 collected with the static method (constant collection over a full month) significantly decreased after initiating the air purifier. This is consistent with the data of Agrawal et al,[12] which reveal that removal of dust mite from the air will diminish surface allergen by assuring that allergen sedimentation rate is decreased. It should be

noted that our research used HEPA air filtration, while the one conducted by Agrawal et al[12] used electrostatic air cleaners. Our research also found that the concentration of Der p 1 and Der f1 decreased in bedding. Surprisingly, the concentration of HDM allergen in air, as collected by ambient air dust sampler, did not decrease. It is possible that the airflow of the air sampling equipment stirred up static dust and that this affected the concentration of HDM allergen in the collected samples sufficiently to dampen the effect size of the air purifier on this variable. Overall, air purifiers as used in our study may effectively lessen indoor levels of HDM allergen.

Before utilizing the HEPA air purifier, both $PM2.5_{indoor/outdoor}$ and $PM10_{indoor/outdoor}$ was significantly lower than 1. Our data support the contention that indoor air is better than outdoor air in general. After the air purifier was used, the $PM1.0_{indoor/outdoor}$, $PM2.5_{indoor/outdoor}$, and $PM10_{indoor/outdoor}$ all decreased, while there was no significant changes in the outdoor PM levels during the study period, which showed that the indoor PM levels were significantly decreased. This result is similar to Kajbafzadeh et al's results,[13] which compared indoor PM to evaluate air purifier efficacy. One reason why indoor air quality could have been affected during the intervention period is in the case of substantial change in outdoor air quality. There were only modest changes in outdoor PM. We used ratios to help us control for the effects of differing outdoor PM over time.

Inactivity scores, non-hay fever symptoms, practical problems, and nasal symptoms all decreased in the RQLQ during the intervention period, suggesting that HEPA air purifiers might have a positive impact on life quality.

A limitation in this study is that there was no control group. It is conceivable that the concentration of HDM allergen decreased not because of the HEPA air purifier intervention but because of the change of seasonal climate. However, Wang et al's study[11] demonstrated that HDM allergen will not change with season. Zhang et al's study[14] of this matter, specific to Guangzhou, did show that the concentration of Der f1 allergen in summer was higher than in winter, however, Der p 1 did not exhibit significant seasonal difference. Our current study demonstrated that the concentration of Der p 1 decreased after utilizing HEPA air purification, an effect not expected to be seen with seasonal changes in our province. We are not able to evaluate any potential placebo effect of the air purifier on subjective quality of life data.

In summary, HEPA air purifiers seemed to provide a favorable factor in reducing PM's concentration and HDM in the bedrooms of our subjects. Air purifiers also may effectively improve allergic rhinitis patients' quality of life.

Acknowledgement

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ORIGINAL ARTICLE

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Effects of aerobic exercise and vitamin C supplementation on rhinitis symptoms in allergic rhinitis patients

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Abstract

Introduction: Exercise training and vitamin C supplementation have both been recommended as an effective adjuvant treatment in the management of symptoms in patients with many diseases. However, its effects on rhinitis symptoms remain unclear. The aim of the present study was to determine the effects of exercise training alone, and in combination with vitamin C supplementation, on rhinitis symptoms in allergic rhinitis patients.

Methods: Twenty-seven rhinitis patients were randomized into 3 groups: control (CON; n = 8), exercise (EX; n = 9), and exercise combined with vitamin C (EX + Vit.C; n = 10). The exercise training protocol consisted of walking and/or running on a treadmill at 65-70% heart rate reserve for 30 min per session, 3 times per week for 8 weeks. The EX + Vit.C group ingested 2,000 mg vitamin C per day.

Results: After 8 weeks, both EX and EX + Vit.C groups increased peak aerobic capacity and peak nasal inspiratory flow (PNIF) and exhibited significantly decreased rhinitis symptoms, nasal blood flow (NBF) and malondialdehylde levels compared to pre-test. Rhinitis symptoms and NBF after nasal challenge with house dust mite decreased significantly in the EX and EX + Vit.C groups. The EX and EX + Vit.C groups had significantly lower nasal secretion interleukin (IL)-4, but higher nasal secretion IL-2 levels, than the CON group.

Conclusions: This study clearly confirms that aerobic exercise training significantly improved clinical of allergic rhinitis and cytokine profiles. Nonetheless, with the limited power of small sample size, whether adding vitamin C is any beneficial is not shown. A larger randomized controlled trial is thus warranted.

Key words: Nasal inspiratory flow, Nasal blood flow, Cytokine levels, Malondialdehylde, Nasal challenge

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Introduction

Allergic rhinitis is a prevalent disease caused by a malfunction of the immune system in response to a hypersensitive reaction to allergic allergens in the nasal mucosa, which is characterized by itching, nasal congestion, sneezing, and rhinorrhea.[1] In the nose, allergens are targeted by allergen -specific immunoglobulin E (IgE) which bind to IgE receptors on mast cells and basophils and release chemical mediators such as histamine, leukotrienes, and cytokines which can cause symptoms of allergic rhinitis to develop.[2] A number of recent studies have shown an increase of IL-4 level in allergic rhinitis patients. Alternatively, IL-2 induces macrophage activation, which is very effective in controlling infection along with intracellular pathogens. Patients suffering from allergic rhinitis have to cope with the discomfort, the cost of nasal and oral medications and their associated side-effects, and a worsening quality of life.[3]

Aerobic exercise has been recommended as an effective adjuvant treatment in the management of symptoms in patients with a variety of disease states.[4-7] However, previous studies have demonstrated that acute high-intensity exercise can cause a worsening of rhinitis symptoms.[8] Moreover, high-intensity exercise has been shown to decrease forced expiratory volume

in 1 second (FEV_1),[9] and increase IgE levels[10] in patients with allergic rhinitis. Recently, Tongtako et al.[11] reported that both acute exhaustive and moderate-intensity exercise reduced allergic rhinitis symptoms. However, a significantly enhanced IL-2/IL-4 ratio was found following acute moderate exercise. Since IL-2 is critical for supporting T cell activation, preventing autoimmunity and controlling infection along with intracellular pathogens effectively while IL-4 act as a coordinator of airway inflammatory processes in allergic disorders. In addition, Silva et al.[12] have reported that aerobic exercise training increased plasma IgE and reduced eosinophils, IL-4, IL-5, IL-13, airway remodeling, mucus synthesis, the thickness of smooth muscle and nasal resistance in a chronic murine model of allergic airway disease. Therefore, based on the available evidence aerobic exercise training may have a beneficial effect in terms of controlling rhinitis symptoms.[11-12]

Vitamin C (ascorbic acid) is an important antioxidant in the body and has been used to prevent and treat various diseases.[13-15] It has been suggested that vitamin C deficiency causes immunosuppression, and may boost the immune system and act as an anti-inflammatory agent by inhibiting cytokine secretion.[16]

However, the effects of vitamin C supplementation on rhinitis symptoms in allergic rhinitis patients are still controversial. Some studies have reported that vitamin C may prove beneficial for allergic rhinitis sufferers. Helms and Miller reported that vitamin C sprayed into the nose reduced symptoms by reducing fluids that stimulate congestion and swelling in the nasal cavity.[17] It has been shown that supplementing with at least 2 grams per day of vitamin C prevents the release of histamine from white blood cells, and therefore may represent a promising non-pharmacological treatment therapy for allergic rhinitis patients.[18] In contrast, a number of studies have reported that vitamin C has no effect on allergic sensitization[19] and allergic rhinitis.[20]

Since aerobic moderate exercise training has been shown to be effective for improving the health status of allergic rhinitis patients,[11-12] and data are equivocal as to whether vitamin C supplementation has positive effects on allergic rhinitis, an important question to answer is whether combining moderate aerobic exercise training with vitamin C supplementation will confer a greater protective effect in attenuating proinflammatory cytokine and allergic rhinitis symptoms compared to aerobic exercise training alone. Therefore, the primary aim of the present study was to evaluate the effects of moderate aerobic exercise training alone, and combined with vitamin C supplementation, on rhinitis symptoms, nasal cytokine secretion, nasal blood flow (NBF), and peak nasal inspiratory flow (PNIF). It was hypothesized that combining moderate aerobic exercise training with vitamin C supplementation would be more effective than moderate aerobic exercise training alone in reducing the allergic response in rhinitis patients.

Methods

Study Design and Procedure

The sample size calculation were performed by using G* power program at power = 0.9 and effect size = 0.4, the total sample size of 24 patients would be required. Rhinitis patients subjects were randomly assigned by the investigators allocated into 3 groups using a computerized random number generator: sedentary control, aerobic exercise training group, and aerobic exercise training combined with vitamin C supplementation group. Any steps concealed the sequence until interventions were assigned. During the study trial (8 weeks) the EX group underwent aerobic exercise training and received placebo supplementation, while the Ex + Vit. C group underwent aerobic exercise training and received Vitamin C supplementation. The participants knew they were in the exercise or no-exercise but they did not know vitamin C or placebo. The control group did not engage in the aerobic exercise training protocol or receive any form of supplementation during the study trial. At pre - and post-study trial (8 weeks) body height, body weight, body fat, BMI, lung function, resting heart rate, blood pressure, VO2peak, total IgE, specific IgE, plasma Vit. C and malondialdehylde were measured. In addition, pre- and post-study trial rhinitis symptoms, peak nasal inspiratory flow, nasal blood flow and nasal secretions for cytokines analysis were evaluated as prior to and following a nasal challenge by house dust mite. The research assistants and medical laboratory scientists who assessed the outcomes and analyzed blood biochemistry were blinded to the interventions.

Participants

Thirty-three patients with allergic rhinitis, aged 18 to 45 years old, were recruited to this study from the Chulalongkorn university health service center. All allergic rhinitis subjects presented with clinical symptoms of persistent rhinitis (nasal congestion, sneeze, nasal itching, and running nose) for more than 4 days per week, and presented with a positive skin prick test (wheal diameter > 3 mm.) to house dust mite (*D. pteronyssinus*) (ALK, Hørsholm, Denmark) and using normal saline as the negative control. Subjects with known asthma, chronic rhinosinusitis, hypertension or cardiovascular diseases, and a smoking habit, were excluded from participating in this study. Moreover, anterior rhinoscopy was performed to exclude anatomical abnormalities. Subjects were asked to refrain from taking antihistamine medication for at least 3 days prior to testing, and to abstain from using oral steroids and nasal steroids for at least 2 week prior to the start of the study. In addition, the subjects discontinued using leukotriene receptor antagonists for at least 1 week prior to testing. The subjects were not to have participated in a regular exercise program for at least 6 months prior to the start of the study, and to avoid taking any form of dietary supplement during the course of the study.

All subjects gave written informed consent prior to participation in the study. Medical and activity history were obtained via questionnaires. The study was approved by Institutional Review Board, Faculty of Medicine, Chulalongkorn University, COA No. 481/2011. This study was registered as a clinical trial with clinical trials.gov (study # NCT 02123914).

Exercise training protocol

Subjects underwent an exercise training protocol for 30 minutes per session three times a week for 8 weeks at the Faculty of Sports Science, Chulalongkorn University, under the supervision of the primary investigators. Heart rate was continually monitored (Polar, Finland). The exercise training

regimen consisted of 5 minutes of warm up and stretching, followed by walking and/or running on a treadmill (Landice, USA) at an intensity of 65-70% heart rate reserve for approximately 40 min, followed by a cool down for 5 min.

Dietary supplementation

The subjects ingested vitamin C tablets (The Government Pharmaceutical Organization, Thailand) 2 times/day (one pill of 1,000 mg in the morning and one in the evening) for 2 months. The placebo and vitamin C tablets were identical in size and appearance to each other. The placebo tablets were manufactured by the Faculty of Sports Science, Chulalongkorn University.

General physiological characteristics

Heart rate (HR) and blood pressure were taken after a 10-min rest period using digital sphygmomanometer (GE Dinamap CARESCAPE , V100 , USA.). Body composition was performed using a bioelectrical impedance analyzer (InBody 220, Biospace, Seoul, Korea).

Pulmonary function

Pulmonary function (i.e. FVC and FEV1) was measured on all subjects using a calibrated computerized pneumotachograph spirometer (Spirotouch; Burdick, Inc., Deerfield, Wisconsin USA.) according to American Thoracic Society (ATS) recommendations. Subjects performed three acceptable spirograms, of which the largest and second largest forced vital capacity (FVC) and FEV_1 values did not vary by more than 0.15 L, and the best FEV_1 and FVC maneuver kept for analysis.

Peak aerobic capacity

Each subject, wearing a nose clip, was required to run on a motorized treadmill (Landice, USA), which started at a speed of 1.7 mph, elevated at 10% and increased speed 0.8 mph and elevated 2% every 3 minutes (Bruce protocol) until volitional exhaustion. During the exercise test, HR was continuously monitored by ECG and breath-by-breath analysis of expired gases was accomplished by indirect open circuit calorimetry (Cortex Metamax 3B, Germany).

Blood collection and analysis

Blood samples were obtained from an antecubital vein. Plasma IgE and specific IgE were measured with the standard procedures of the certified clinical laboratory at King Chulalongkorn Memorial Hospital. Plasma vitamin C concentrations were determined by Colorimetric Method in plasma after derivatization with 2,4-Dinitrophenyldrazine using spectrophotometer. The serum malondialdehyde (MDA) levels, a marker of oxidative stress, was determined using thiobarbituric acid reaction.[21]

Rhinitis symptoms

Nasal symptoms were assessed using Total Nasal Symptom Score (TNSS) questionnaire.[22] The subjects were asked to score symptoms of persistent allergic rhinitis before and after each exercise protocol. The total nasal symptom scores were computed as the sum of four individual nasal symptom scores; nasal congestion, itching, sneezing, and rhinorrhea. The scores ranged from 0 to 3 scale (0 = none, 1 = mild, 2 = moderate, 3 = severe). All participants, including the control group were supplied with a questionnaire and instructed to record their daily nasal symptoms.

Peak nasal inspiratory flow

Peak nasal inspiratory flow (PNIF) was measured using a peak nasal inspiratory flow meter (Clement Clark International model IN-CHECK ORAL, UK.) attached to an anesthesia mask. During the procedure, the subjects placed a mask, which is turned onto a plastic cylinder through which the air passes during inspiration, over the nose and mouth and inspired forcefully through the nose, with lips tightly closed. Inside the cylinder, there is a diaphragm that moves to the airflow, and the maximum peak flow is registered in a scale range from 30-370 L/min. During the procedure, the subjects placed a mask over the nose and mouth and inspired forcefully through the nose, with lips tightly closed. PNIF was measured before and after exercise.

Nasal blood flow

Nasal blood flow (NBF) was measured by laser doppler flowmetry (DRT4 moor instrument, UK.). All subjects rested in a room for 1 hour before the test. They were advised to breathe normally and not to cough, speak or move during the test. A side delivery endoscopic probe with a flexible nylon sleeve with a diameter of 1.34 mm was placed on the anterior surface of the nose. The nasal blood flow values before and after exercise in each protocol were then measured.

Nasal challenge by house dust mite

Each subject underwent a nasal challenge to house dust mite allergen (ALK, Hørsholm, Denmark). Bilateral nasal provocation used a nasal spray (metered-dose bottle) delivering a fixed volume of 0.125 mL/puff, with 1 puff in each nostril containing 1000 AU/ml of *D. pteronyssinus*.[22] In pre-test and post-test, nasal secretion cytokine levels i.e. IL2 and IL4 were measured at baseline and after 5 minutes nasal challenge. Rhinitis symptoms, PNIF and NBF were measured at baseline, after 5, 15, 30, 45 and 60 minutes nasal challenge.

Nasal secretion collection and handing

Nasal secretion collection was performed bilaterally with filter paper strips (7 × 30 mm Whatman No.42, Whatman, Clifton, NJ). Three filter paper strips were sequentially placed on each anterior portion of the inferior turbinate for 10 min. The filter paper strips were collected and put in test tubes, and then centrifuged at 3,000 rpm for 5 min at 4 °C, after which the loose nasal secretions were immediately frozen at -70 °C until later analysis.

Cytokines analysis

Nasal secretion values of cytokines, IL-2 and IL-4 were acquired. The cytokine levels in nasal secretions were determined using the human Th1/Th2/Th9/Th17/Th22 13 plex FlowCytomix Multiplex kits (Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions. In brief, twenty-five microliters were incubated with two different sizes of polystyrol beads: 5.5 and 4.5 micron, coated with capture

antibodies. After incubation, biotinylated detector antibodies and streaptavidin-PE were added. Data were acquired 1500 events within small beads (R2 beads) using a flow cytometer (BD FACSCalibur Flow Cytometer, USA). All data were analyzed by FlowcytomixTM Pro software (eBioscience, USA.).

Statistical Analysis

Data were analyzed using SPSS version 17 for Windows statistical software. The normality of the distribution of the variables was tested using a Shapiro-Wilk test. A Two way (group × time: 3 × 2) analysis of variance, followed by LSD multiple comparison test, was used to determine the significant differences in general physiological characteristics, plasma vitamin C and MDA. Data are expressed as mean ± SEM. Rhinitis symptoms and total/specific IgE data were expressed as median values and compared by the Mann-Whitney test. Statistical difference was set at $p < 0.05$.

Results

As shown in **Figure 1**, the eligible participants were randomly allocated into three groups: sedentary control (CON; n = 11), aerobic exercise training group (EX; n = 11), and aerobic exercise training combined with vitamin C supplementation group (EX + Vit.C; n = 11). A total of 6 subjects dropped out of the study. The three control subjects dropped out from scheduling difficulties. Three subjects dropped out from the exercise groups due to scheduling difficulties and physical discomfort. Therefore, the CON, Ex and Ex + Vit. C group were comprised of 8 (Male = 3, Female = 5), 9 (Male = 3, Female = 6) and 10 (Male = 3, Female = 7) subjects, respectively.

General physiological characteristics

General characteristics are shown in **Table 1**. There were no significant differences in blood pressure, lung function and total and specific IgE among three groups of subjects. Both

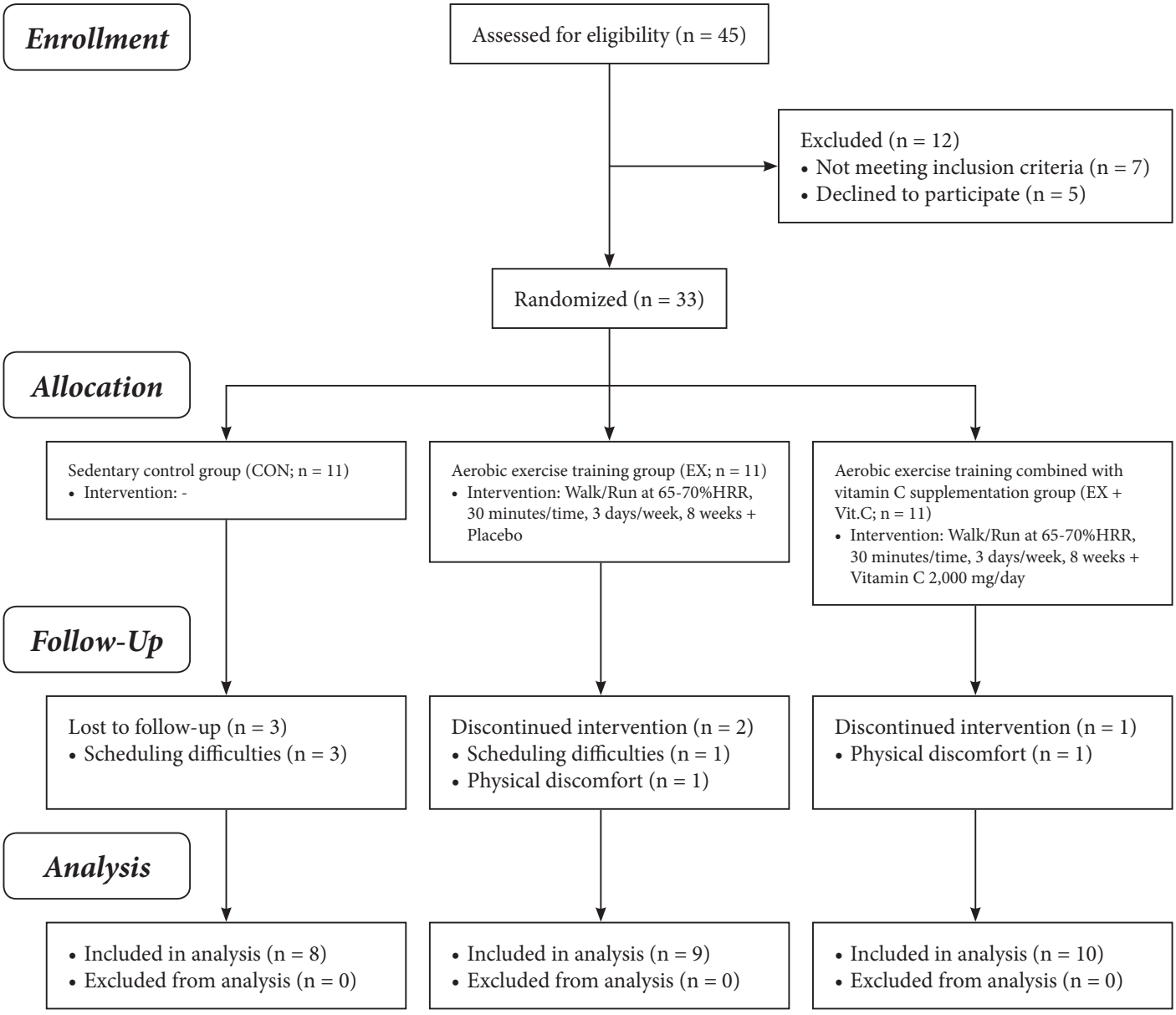

Figure 1. CONSORT 2010 flow diagram of participant allocation, follow-up and analysis.

Table 1. The comparison of percent difference of the general physiological characteristics variables among in control group (CON), exercise group (EX) and exercise combined vitamin C supplementation group (EX + Vit. C).

Variables	CON (n = 8)		EX (n = 9)		EX + Vit. C (n = 10)	
	Pre-test	Post-test	Pre-test	Post-test	Pre-test	Post-test
Resting heart rate (b/min.)	78.12 ± 2.52 (71.37, 84.87)	80.25 ± 1.46 (74.46, 86.03)	79.33 ± 3.17 (72.97, 85.69)	72.55 ± 2.43*† (67.10, 78.00)	83.40 ± 3.28 (77.36, 89.43)	75.60 ± 3.26*† (70.43, 80.77)
Systolic BP (mmHg)	118.89 ± 3.22 (109.18, 127.56)	114.11 ± 3.64 (115.72, 133.77)	113.16 ± 4.23 (107.22, 124.55)	113.00 ± 5.44 (105.60, 122.62)	112.10 ± 3.88 (103.88, 120.31)	108.10 ± 3.53 (100.02, 116.17)
Diastolic BP (mmHg)	74.12 ± 2.78 (65.86, 82.38)	74.12 ± 2.74 (67.54, 80.71)	73.11 ± 4.53 (65.32, 80.89)	69.22 ± 3.86 (63.02, 75.42)	70.10 ± 3.59 (62.71, 77.48)	65.50 ± 2.24 (59.61, 71.38)
FVC (Liters)	3.08 ± 0.25 (2.57, 3.59)	2.93 ± 0.27 (2.41, 3.45)	2.56 ± 0.20 (2.07, 3.04)	2.64 ± 0.20 (2.14, 3.13)	2.63 ± 0.23 (2.17, 3.08)	2.71 ± 0.23 (2.24, 3.18)
FEV_1 (Liters)	2.69 ± 0.25 (2.14, 3.23)	2.70 ± 0.24 (2.22, 3.17)	2.50 ± 0.17 (1.99, 3.01)	2.59 ± 0.16 (2.14, 3.04)	2.16 ± 0.28 (1.67, 2.64)	2.52 ± 0.23 (2.09, 2.95)
Body fat (%)	21.30 ± 3.59 (13.18, 29.41)	21.35 ± 3.52 (13.77, 28.92)	26.55 ± 4.26 (18.90, 34.20)	26.07 ± 3.90 (18.93, 33.22)	21.52 ± 3.21 (14.26, 28.77)	20.64 ± 2.97 (13.86, 27.41)
VO2peak (ml./kg./min.)	34.50 ± 2.44 (28.95, 38.79)	32.28 ± 2.72 (26.77, 37.22)	31.00 ± 1.76 (26.36, 35.63)	33.88 ± 1.46*† (28.96, 38.81)	33.11 ± 2.13 (29.70, 38.49)	35.44 ± 2.52*† (31.53, 40.86)
¥ Total IgE (IU/ml)	229.50	289.50	271.00	236.00	233.50	215.50
¥ *D.pteronyssinus* specific IgE (kUA/L)	0.18	0.21	15.89	25.71	9.04	13.66
Plasma Vit C (mg/dl)	1.33 ± 0.17 (0.99, 1.66)	1.10 ± 0.12 (0.83, 1.38)	1.27 ± 0.17 (0.95, 1.58)	1.13 ± 0.15 (0.87, 1.39)	1.19 ± 0.10 (0.89, 1.49)	1.54 ± 0.09*†‡ (1.29, 1.79)
MDA (μmol/L)	0.36 ± 0.07 (0.19, 0.52)	0.48 ± 0.13 (0.35, 0.61)	0.60 ± 0.07 (0.44, 0.75)	0.20 ± 0.01*† (0.79, 0.32)	0.57 ± 0.13 (0.42, 0.71)	0.24 ± 0.05*† (0.12, 0.35)

Data are presented as mean ± SEM. (¥ are median.) FVC = Forced Vital Capacity, FEV_1 = Forced Expiratory Volume in 1 sec, VO2peak = Peak oxygen consumption, MDA = Malondialdehyde * $p < 0.05$ vs. pre-test †$p < 0.05$ vs. CON ‡$p < 0.05$ vs. EX

the EX and EX + Vit. C groups had significantly increased VO2peak ($p = 0.004$, $p = 0.021$) and significantly decreased resting heart rate ($p = 0.003$, $p = 0.001$) and plasma malondialdehyde (MDA) levels ($p = 0.001$, $p = 0.001$) after 8 weeks of training and also significant difference (all $p < 0.05$) from CON group. Moreover, the plasma Vitamin C concentrations in the Ex + Vit.C group were significantly higher ($p = 0.012$) than pre-test values and significant difference from CON ($p = 0.022$) and EX ($p = 0.027$) groups. Total and specific IgE among three groups were not significant differences.

Rhinitis symptoms

After 8 weeks, the both EX and EX + Vit. C groups had significantly decreased in rhinitis symptoms such as nasal congestion ($p = 0.015$, $p = 0.002$), itching ($p = 0.009$, $p = 0.004$), sneezing ($p = 0.013$, $p = 0.005$), rhinorrhea ($p = 0.012$, $p = 0.014$) and total rhinitis symptoms ($p = 0.001$, $p = 0.002$). In addition, total rhinitis symptoms score was significantly lower in EX + Vit.C group (3.50) compared with pre-test (7.50, $p = 0.015$) and CON group (7.50, $p = 0.004$) (**Table 2**). Furthermore, Ex group had significantly decreased in total rhinitis symptoms difference from CON group ($p = 0.001$). After nasal challenge, no changes in total rhinitis symptoms was found in the CON group (**Figure 2A**) but the total rhinitis symptoms in both EX (**Figure 2B**) and EX + Vit. C (**Figure 2C**) groups had significantly decreased at 15, 30, 45 and 60 minutes compare with pre-test (all $p = 0.001$).

Peak nasal inspiratory flow and nasal blood flow

After 8 weeks, both the EX and EX + Vit. C groups had significantly increased ($p = 0.001$, $p = 0.016$) PNIF (**Figure 3A**) and significantly decreased ($p = 0.002$, $p = 0.018$) NBF (**Figure 3B**) compared with pre-test.

After nasal challenge for 60 minutes, the CON group did not showed any significant difference in PNIF (**Figure 4A**) and NBF (**Figure 4B**). The both exercise groups had a significantly

Table 2. The comparison of percent difference of the rhinitis symptoms variables among in control group (CON), exercise group (EX) and exercise combined vitamin C supplementation group (EX + Vit. C).

Variables	CON (n = 8)		EX (n = 9)		EX + Vit. C (n = 10)	
	Pre-test	Post-test	Pre-test	Post-test	Pre-test	Post-test
Nasal congestion	2.00	2.00	2.00	1.00*	2.00	1.00*†
Itching	3.00	2.00	3.00	1.00*	2.00	1.00*
Sneezing	2.00	2.00	2.00	1.00*	2.00	1.00*†
Rhinorrhea	2.00	2.00	2.00	1.00*	2.00	1.00*
Total rhinitis symptoms	9.50	7.50	8.00	5.00*	8.00	3.50*†

Data are Median. * $p < 0.05$ vs. pre-test †$p < 0.05$ vs. CON

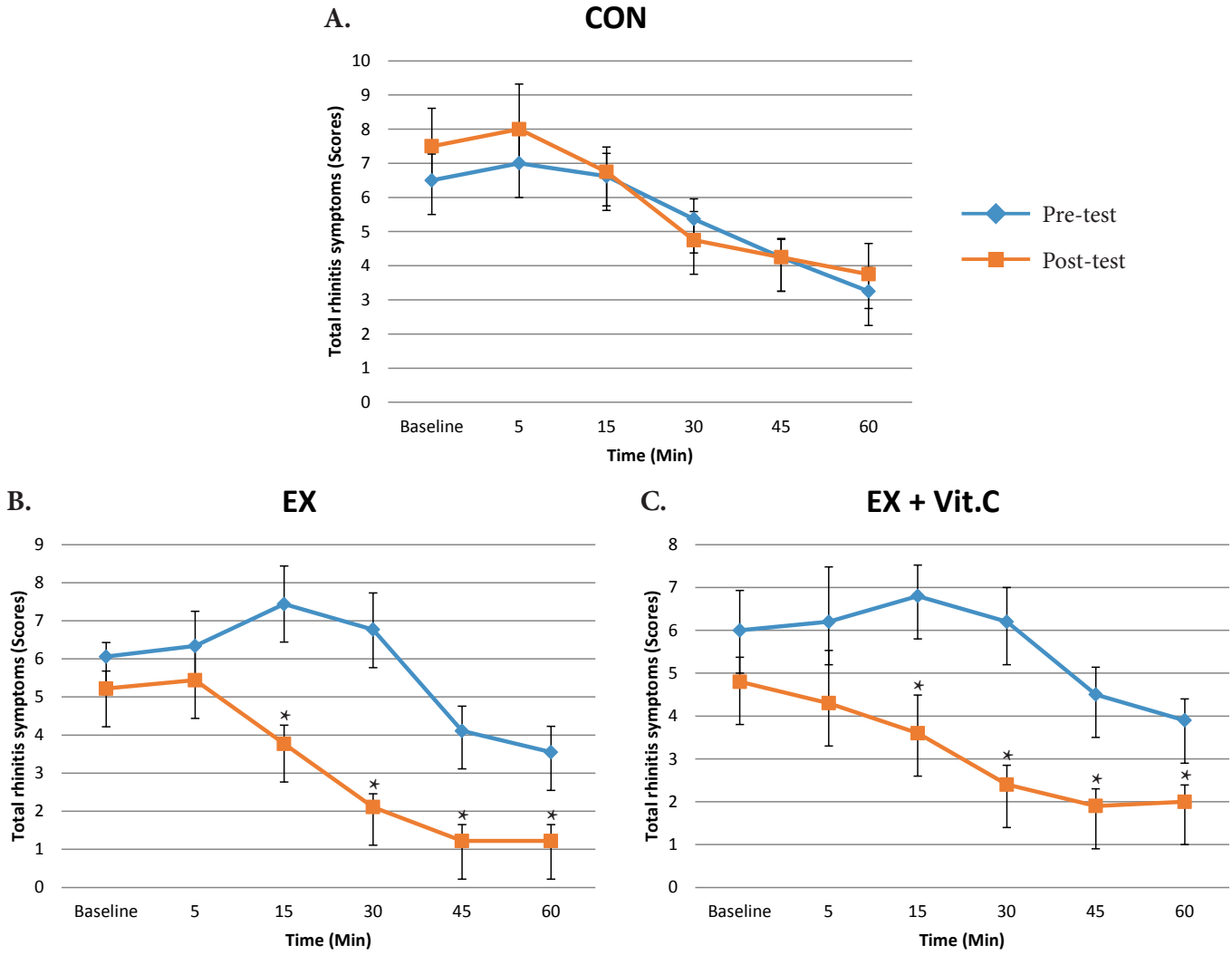

Figure 2. The comparison of total rhinitis symptoms after nasal challenge by house dust mite (*D.pteronyssinus*) between pre- and post-training in control group (A.), exercise group (B.) and exercise combined vitamin C supplementation group (C.).
Data are presented as mean ± SEM. * $p < 0.05$ vs. Pre-test.

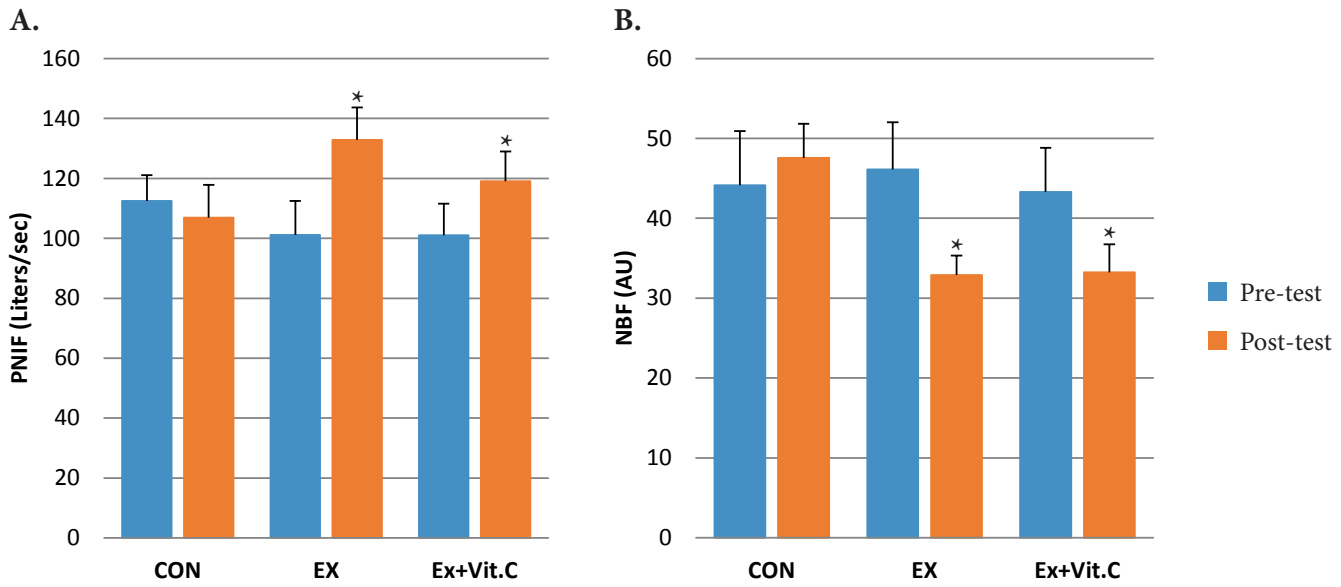

Figure 3. The comparison of peak nasal inspiratory flow (PNIF) (A.) and nasal blood flow (NBF) (B.) between pre- and post-training in control group (CON), exercise group (EX) and exercise combined vitamin C supplementation group (EX + Vit. C).
Data are presented as mean ± SEM. * $p < 0.05$ vs. Pre-test

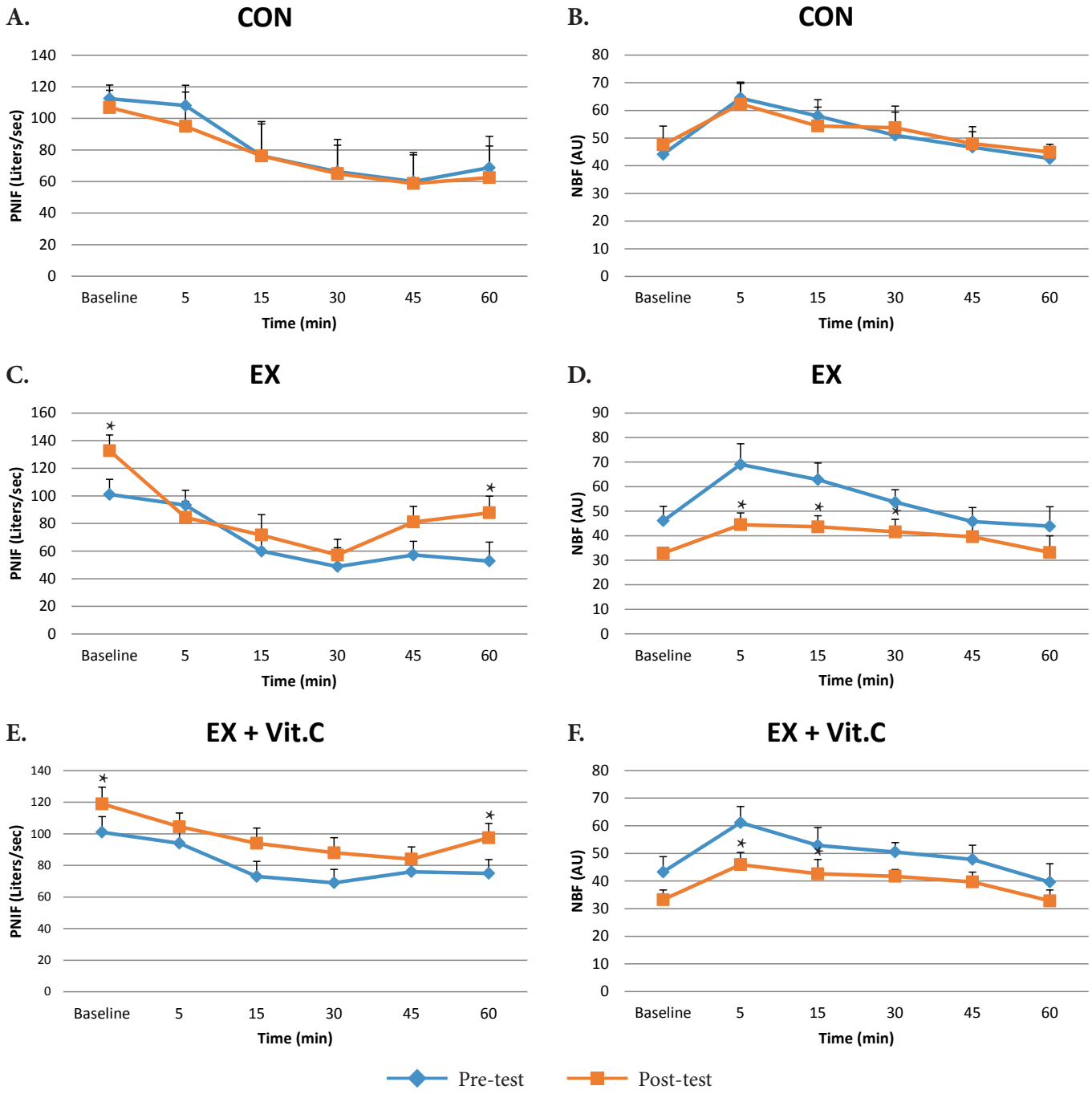

Figure 4. The comparison of peak nasal inspiratory flow (PNIF) and nasal blood flow (NBF) after nasal challenge by house dust mite (*D.pteronyssinus*) between pre- and post-training in control group (A. and D.), exercise group (B. and E.) and exercise combined vitamin C supplementation group (C. and F.).
Data are presented as mean ± SEM. *p < 0.05 vs. pre-test.

higher (all p = 0.001) PNIF when compared with pre-test at baseline and 60 minutes (**Figure 4C and 4E**) after nasal challenge. NBF decreased significantly after 5 (p = 0.001, p = 0.004) and 15 (p = 0.001, p = 0.048) minutes of nasal challenge compared with pre-test in the both EX and EX + Vit. C groups as shown in **figure 4D and 4F**.

Cytokines levels in nasal secretion

At pre-test, there were no significant difference in IL-2 (**Figure 5A**) and IL-4 (**Figure 5C**) among three groups at baseline and after nasal challenge by house dust mite (*D.pteronyssinus*). After 8 weeks, the both EX and EX + Vit. C groups had significantly higher (p = 0.024, p = 0.019) baseline IL-2 (**Figure 5B**) and significantly lower (p = 0.012, p = 0.025) baseline IL-4 (**Figure 5D**) compared with the control group. Besides, both EX and EX + Vit. C groups had significantly decreased (p = 0.001, p = 0.008) in IL-4 when compared with pre-test (**Figure 5D**). After nasal challenge by house dust mite (*D.pteronyssinus*), the IL-2 in the both EX and EX + Vit. C groups was significantly higher (p = 0.018, p = 0.010) than the

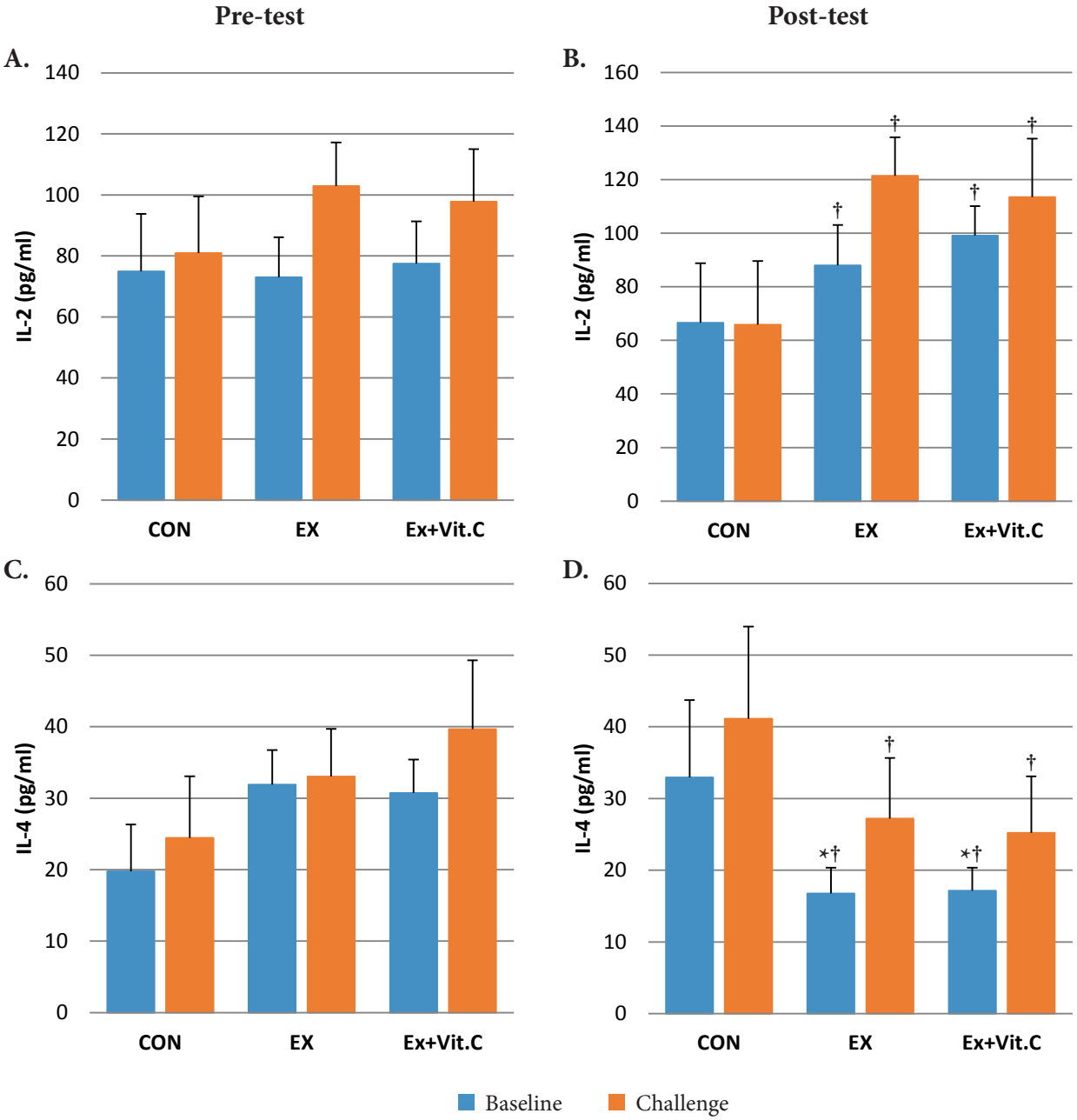

Figure 5. The comparison of cytokine levels in nasal secretion at baseline and after 5 minutes nasal challenge between pre-test and post-test and among three groups of subjects: control group (CON), exercise group (EX) and exercise combined vitamin C supplementation group (EX + Vit. C).
Data are presented as mean ± SEM. *p < 0.05 vs. pre-test †p < 0.05 vs. CON

CON group (**Figure 5B**) but the IL-4 in the both EX and EX + Vit. C groups were significantly lower (p = 0.009, p = 0.012) than the CON group (**Figure 5D**).

Discussion

The principal finding of the present study is that both exercise training alone and exercise training combined with vitamin C supplementation reduced rhinitis symptoms in allergic rhinitis patients. The improvement in clinical symptoms, as a consequence of aerobic training, was supported by reduced nasal blood flow and peak nasal inspiratory flow rate. Besides, the increased IL-2 and decreased IL-4 cytokine levels compared to control patients were also shown. However, the total and specific-IgE to house dust mites did not change between pre- and post-tests in either group.

The present study showed that aerobic exercise training reduced allergic rhinitis symptoms. The reduction in rhinitis symptoms following the aerobic exercise training may be due to decreased nasal resistance resulted from decreased sympathetic vasoconstriction in the nasal mucosa.[23] The fall in nasal resistance may be caused, at least partly, by reducing blood flow,[24] leading to reduced nasal congestion.[25] Our finding that PNIF increased following aerobic exercise training is in agreement with Marioni et al.[26] who reported that the mean PNIF after prolonged exhaustive exercise was

significantly higher than the mean PNIF value found before exercise. They suggested that PNIF sensitivity and reliability also in determining the changes in nasal patency, which occurred after physical exercise.

Allergic inflammation is associated with the production of IL-4, IL-13, and IL-5, which are responsible for IgE production by B cells, eosinophil activation and recruitment, and mucus production.[2] A recent study reported that the higher levels of IL-4 occur in the nasal fluid of allergic rhinitis patients compared to non-allergic controls.[27] In contrast, differentiated Th1 cells secrete interferon-γ and IL-2, which are important in intracellular destruction of phagocytosed microbes.[28] In the present study, no significant changes in terms of total IgE and specific IgE to house dust mites were detected after 8 weeks of aerobic exercise training. However, we did observe that, following the aerobic exercise training, IL-4 levels were significantly lower than pre-training at baseline and after nasal challenge with house dust mite. It has been suggested that aerobic exercise training could be attributed to decrease levels of IL-4.[29-30] Nevertheless, Shimizu et al.[31] found that moderate exercise training 5-days a week for 6 months did not change in IL-4 cytokine in blood.

In the present study, IL-2 levels in nasal secretion increased in patients following the moderate exercise training compared to those in the control group. Arai et al.[32] reported that long-term endurance training can enhance IL-2 production comparable to levels found in young male subjects indicating that chronic exercise could delay immunosenescence. According to our data, we suggest that the improvement in clinical symptoms in allergic rhinitis patients may be a consequence of a cytokine deviation after regular exercise training. Previous studies have reported that the anti-inflammatory effects of aerobic exercise training could be attributed to decrease levels of IL-4, IL-5, IgE, and also an increase in anti-inflammatory cytokines.[29-30] The potential mechanism as to how moderate exercise training modulates the cytokine response has not clearly been elucidated. One possible mechanism is that the improvement in VO2peak following the aerobic exercise training may attenuate oxidative stress which in turn, may alter the inflammatory cytokine expression pattern.[34] The moderate exercise training -induced improvement in inflammatory status may also result from the reduction of antigen-specific T helper cells migration due to decreasing chemokine receptor function in these subjects.[34] Since plasma MDA declined after 8 weeks of aerobic exercise training, we speculate that moderate exercise training conferred a beneficial effect by ameliorating oxidative stress, possibly as a result of a deviation of cytokine response in nasal secretion after exercise.

In the present study, it was clearly seen that nasal symptoms, peak nasal inspiratory flow, and nasal blood flow in both patient groups who underwent exercise training alone, and exercise training plus vitamin C supplement, were significantly improved compared to baseline symptoms and significantly better compared to data in the control patients; however, there was no significant difference between the aerobic exercise training and exercise training plus vitamin C group. As the normal range (0.6-2.0 mg/dL)[35] of vitamin C status in our allergic rhinitis participants, supplemental vitamin C is not likely to show any benefit. Our study suggests that the supplementary vitamin C in allergic rhinitis patients has no additional benefit in terms of clinical improvement if they are amply nourished and have adequate exercise. Future studies regarding the appropriate dosage of vitamin C supplementation for allergic rhinitis patients are warranted.

There are a number of limitations in the present study that should be emphasized. First the presently study did not include a group that performed no exercise but were vitamin C supplemented. Second, the number of subjects in each intervention group may be considered small. Third, the control group did not receive a placebo supplement.

Conclusions

The present study demonstrate that both aerobic exercise training alone and aerobic exercise training combined with vitamin C have beneficial effects in allergic rhinitis patients by reducing rhinitis symptoms. The extensive benefits on immune function were to improve cytokine deviation by increase IL-2 and decrease IL-4. Moreover, cardiorespiratory and clinical symptoms (PNIF and NBF) improvement as well as oxidative stress reduction were found in patients with allergic rhinitis following aerobic exercise training. This study clearly confirms that aerobic exercise significantly improved clinical of allergic rhinitis and cytokine profiles. However, due to the limited power of our small sample size, we were unable to determine as to whether adding vitamin C supplementation to exercise would confer a greater benefit than exercise alone in improving the clinical response in allergic rhinitis patients. A larger randomized controlled trial is thus warranted.

Acknowledgements

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ORIGINAL ARTICLE

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APJAI

First episode of preschool wheeze requiring hospitalization: a prospective study on the chance of recurrence and associated factors

Karaked Chantawarangul, Jitladda Deerojanawong, Suchada Sritippayawan

Abstract

Background: Although wheezing is very common in preschoolers, epidemiologic studies in Thailand are quite limited. The likelihood of having a second wheezing episode following the first attack remains unclearly established.

Objectives: This study aims to investigate the incidence of recurrent wheezing in preschool children presenting with first wheezing episode and identify the associated factors.

Methods: The study is an observational prospective study conducted at the inpatient pediatric department. Patients admitted with first episode of wheezing were followed up as an outpatient approximately one week after hospital discharge and subsequently followed up by telephone 3-monthly with a structured questionnaire seeking information concerning recurrent wheezing, defined as having a second wheezing episode requiring bronchodilator within a 1-year period.

Results: The total of 97 patients, aged 6 months to 5 years, were recruited from June 2014 to November 2015. Thirty-five patients were excluded because of inaccessibility for telephone follow-up. Amongst the remaining 62 patients, twenty-eight (45.2%) had recurrent wheezing within one year. The mean lapse duration was 4.7 ± 3.7 months after the first episode. Having an allergic sensitization to aeroallergen was a risk factor for recurrent wheezing (OR 2.48, 95%CI 1.81–3.4). Although not statistically significant, having an allergic sensitization to food seems to be another related factor (OR 2.36, 95%CI 1.75–3.18).

Conclusion: The recurrent rate of wheezing was 45%, which was considerably significant. Allergic sensitization to aeroallergen might increase the risk. These patients should be followed up, especially within the first year after their first wheezing episode.

Key words: First wheezing, Preschool, Hospitalized, Recurrent, Incidence, Associated factors

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Abbreviations:
EVW, episodic viral wheeze; ER, emergency room; HFNC, high flow nasal cannula; mAPI, modified asthma predictive index; MTW, multiple-trigger wheeze; OPD, outpatient department; PIS, pulmonary index score; RSV, respiratory syncytial virus

Introduction

Wheezing is very common in infants and young children due to age specific anatomical and physiological properties. One in three children under the age of 3 years have at least one episode of wheezing prior to their third birthday,[1] with a cumulative prevalence of up to 40% during the first 6 years of life.[2]

Various phenotypes of recurrent wheezing have been recognized. It could be classified as transient, late onset, and persistent wheezing according to population based cohort studies[3] or into episodic viral wheeze (EVW) and multiple-trigger wheeze (MTW), categorized by The European Respiratory Society Task Force on Preschool Wheeze.[4] Identification of these patterns of wheeze might allow the clinician to classify children during an office visit, which leads to effective treatment and follow up.[5-7] However, the classifications of episodic and

multiple-trigger wheeze might be unstable over time, with more than half of children switching to the other phenotype over the course of a year.[8]

In patients admitted for the first time with an acute wheezing episode, a commonly asked question from their parents is "Will my child wheeze again?". However, in Thailand, epidemiologic studies and long-term data are quite limited. A 5 -year prospective study at two tertiary hospitals in Khon Kaen revealed the recurrent wheezing of 61.8% in the first three years of follow-up, with the mean duration of 5.4 ± 7.2 months after the first episode of wheezing in children aged 1–24 months.[9]

The current study focused on investigating the incidence of having a second episode of wheezing in preschool children, aged 6 months to 5 years, presenting with a first wheezing episode and identifying its associated factors during a one year follow-up period.

Methods

This observational prospective study was conducted at the inpatient Pediatric department of King Chulalongkorn Memorial Hospital from June 2014 to November 2015. This study was reviewed and approved by the human rights and ethics committee of the Faculty of Medicine, Chulalongkorn University, Thailand. Written informed consent was obtained from the parents of each patient. In this study, the term "recurrent wheezing" was defined as having a second wheezing episode requiring treatment with nebulized bronchodilator at any hospital within a 1-year follow-up.

Participants and Procedure

Patients were included in the study if they were admitted with a first episode of wheezing. Other criteria were age (6 months to 5 years old), nationality (Asian), and consent from parents. The subjects were then excluded if they had congenital or chronic illness (including chronic lung disease, bronchopulmonary dysplasia, cerebral palsy, congenital heart disease, congenital anomalies, structural airway malformation, primary immunodeficiency, gastroesophageal reflux disease, illness that requires steroid use) or clinical of sepsis or septic shock. Data concerning the age of the patients at the initial wheezing episode, sex, gestation age (preterm/term), breast -feeding duration, parental history of asthma, exposure to secondhand smoke, modified asthma predictive index(mAPI), comorbidity (allergic disease, including allergic rhinitis, atopic dermatitis, food allergy, cow's milk protein allergy, sensitization to aeroallergens) were obtained through an interview questionnaire. The severity of the acute episode was assessed using pulmonary index score. Data regarding the length of stay, treatment needed (high flow nasal cannula (HFNC)), and viral pathogen (RSV or Non-RSV) were collected from the medical records. Viral antigen detection was tested on subjects through nasopharyngeal swabs at the discretion of the primary doctor.

As shown in **figure 1**, at the beginning of the study, there were 97 patients recruited. The patients were followed up as an outpatient approximately one week after being discharged and subsequently received an active follow up by telephone 3-monthly with a structured interview questionnaire seeking information on recurrent wheezing within a one year period. Out of the 97 originally recruited, sixty-two patients completed the follow up. The reason for drop-out was due primarily to the inability to contact their parents who may have changed their telephone number without automatic update.

Amongst the 62 subjects completing the telephone followed up, eight subjects reported having a positive skin prick test. The medical records of these subjects were subsequently reviewed. The skin prick tests were done by allergists in the outpatient allergy clinic at King Chulalongkorn Memorial Hospital. A wheal of 3 mm. or greater is considered a positive result. Types

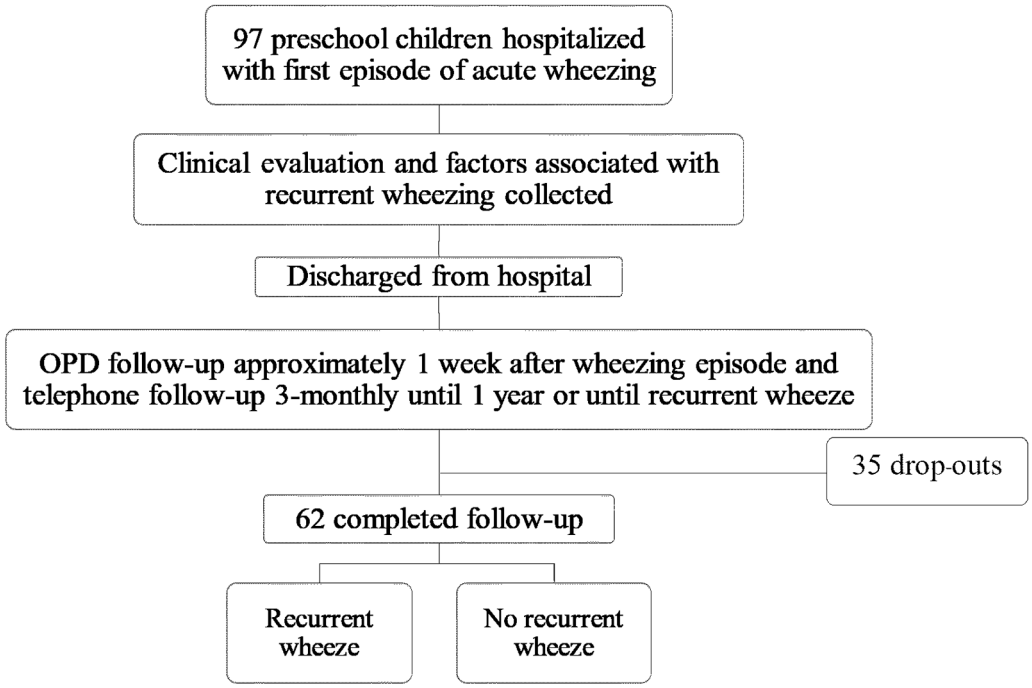

Figure 1. Patient disposition

of food allergens are Cow's milk, Casein, Egg yolk, Egg white, Soy bean, Peanut, Wheat, and Shrimp. Whereas aeroallergens consists of; D.pteronyssinus, D.farinae, German cockroach, American cockroach, Cat, Dog, Mold (Aspergillus, Alternaria, Cladosporium, Penicillium), Bermuda grass, and Johnson grass. This covers most of the allergens common in Thai population.[10]

Statistical Analysis

Data were analyzed using Statistical Package for Social Sciences (SPSS) version 22. The descriptive analysis included calculating percentages and mean ± SD for demographic variables. Logistic regression models were used to identify clinical variables associated with recurrent wheezing. Results from logistic models described by odds ratios and 95%CI. P-value < 0.05 was considered to indicate statistical significance.

Results

Demographic data

The total of 97 patients with the age of 6 months to 5 years old were diagnosed with first episode wheezing. Thirty-five patients were excluded because of inaccessibility of the parents for follow-up. Their characteristics are presented in **Table 1**. There were no significant differences in clinical and demographic characteristics between the subjects and dropouts indicating that the sample population was representative of the root population of 97 patients.

Of the completed 62 patients, 28 patients (45.2%) had recurrent wheezing while the remaining of 34 patients (54.8%) did not have it. The mean lapse duration of recurrence was 4.7 ± 3.7 months after the first episode.

Of the 62 patients included in the study, 31 (50%) were male subjects. The mean age of the patients at admission was 23 months (± 14.7 months). The weight and height of the patients are within the normal range for age. The entry points of study population varied, from visiting the emergency room (ER)/outpatient department (OPD) without appointment (88.7% being most of the case) to OPD with appointment and referred.

The record revealed that most of the subjects is of term, while 16.1% is of preterm. More than half (56.5%) were breast fed for over 6 months. About 44% had been exposed to household cigarette smoke. The majority of the subjects (88.7%) did not have co-morbid diseases, while only a few had atopic dermatitis (9.7%) and food allergy (1.6%).

Factors associated with recurrent wheezing

Table 2 shows the demographic and clinical characteristics of children with and without recurrent wheezing. The analysis revealed that demographic features and the severity of the wheezing episode; pulmonary index score, length of stay, and requirement of HFNC were not significant factors contributing to recurrent wheezing.

RSV infection was found positive in 21 subjects, eight (28.6%) in the recurrent wheeze group comparing to 13 (38.2%) in the other. There were 3 subjects tested positive for Rhinovirus, they all had recurrent wheezing. Four subjects were tested positive for other viruses each; Adenovirus, Bocavirus, Human metapneumovirus, and Influenza virus. These subjects did not have recurrent wheezing. However, some of the subjects were tested with RSV rapid test only, hence did not undergo other viral antigen detection. No significant association between respiratory viruses and recurrent wheezing was demonstrated in this study.

During the follow-up period, eight out of 62 subjects was found to have a positive skin prick test. In one subject, the skin prick test was done after having the wheezing episode, while the other 7 subjects underwent a skin prick test after having a second attack. Five were positive to aeroallergens, two were positive to food allergens, and 1 was positive to both. From the initial interview, three subjects were suspected to have food allergies. They all underwent a skin prick test for food allergens, and the tests were positive. Allergic sensitization to aeroallergen was a factor identified as being of significance (odds ratio 2.48, 95%CI 1.81–3.4) and allergic sensitization to food also contributes to the risk of recurrent wheezing (odds ratio 2.36, 95%CI 1.75-3.18). While, parental asthma, atopic dermatitis, eosinophilia > 4%, allergic rhinitis, and a positive mAPI were not significant factors influencing the recurrence of wheezing.

Table 1. Characteristics of study population and drop-outs

Factors	Subjects (n = 62)	Drop-outs (n = 35)	p-value
Demographic data			
Age at 1st wheezing (months)	23 ± 14.7	23.71 ± 14.05	0.811
Male sex	31 (50%)	17 (48.6%)	0.892
Preterm	10 (16.1%)	2 (6.3%)	0.174
Exposure to cigarette smoke	27 (44.3%)	2 (40%)	0.854
Allergy-related history			
Parental asthma	7 (11.3%)	3 (17.6%)	0.360
Atopic dermatitis	6 (9.7%)	1 (2.9%)	0.213
Allergic sensitization to aeroallergen	5 (8.1%)	0 (0%)	0.085
Eosinophilia > 4%	3 (4.8%)	0 (0%)	0.186
Allergic sensitization to food	3 (4.8%)	0 (0%)	0.186
Allergic rhinitis	2 (3.2%)	1 (2.9%)	0.920
Positive mAPI	13 (23.2%)	4 (11.4%)	0.161
Severity			
PIS before treatment	5.76 ± 2.01	5.95 ± 1.67	0.703
Required HFNC	7 (13.7%)	2 (5.7%)	0.233
Length of stay (days)	3.69 ± 2.85	3.97 ± 2.13	0.617
RSV positive	21 (33.9%)	12 (34.3%)	0.967

Values presented as frequency (%), Mean ± SD. and Odds ratio (95%; Confidence interval). P-value corresponds to Logistic regression analysis
HFNC, high flow nasal cannula; mAPI, modified asthma predictive index; PIS, pulmonary index score; RSV, respiratory syncytial virus

Table 2. Demographic and clinical characteristics of children with and without recurrent wheezing

Factors	Recurrent wheeze (n = 28)	No recurrent wheeze (n=34)	OR (95%CI)	p-value
Demographic data				
Age at 1st wheezing (months)	20.21 ± 13.06	25.29 ± 14.63	0.97 (0.94, 1.01)	0.161
Male sex	16 (57.1%)	15 (44.1%)	1.33 (0.76, 2.33)	0.444
Preterm	6 (21.4%)	4 (11.8%)	1.42 (0.78, 2.58)	0.326
Breast fed > 6 months	14 (50%)	21 (61.8%)	0.77 (0.45, 1.33)	0.443
Exposure to cigarette smoke	10 (35.7%)	17 (51.5%)	0.72 (0.4, 1.3)	0.31
Allergy-related history				
Parental asthma	1 (3.6%)	5 (14.7%)	0.35 (0.06, 2.11)	0.209
Atopic dermatitis	3 (10.7%)	3 (8.8%)	1.12 (0.48, 2.62)	1
Allergic sensitization to aeroallergen	5 (17.9%)	0 (0%)	2.48 (1.81, 3.4)	0.015*
Eosinophilia > 4%	1 (3.6%)	2 (5.9%)	0.73 (0.14, 3.7)	1
Allergic sensitization to food	3 (10.7%)	0 (0%)	2.36 (1.75, 3.18)	0.087
Allergic rhinitis	1 (3.6%)	1 (2.9%)	1.11 (0.27, 4.57)	1
Positive mAPI	8 (28.6%)	5 (17.9%)	1.51 (0.87, 2.6)	0.22
Severity				
PIS before treatment	5.7 ± 1.59	5.81 ± 2.34	0.97 (0.75, 1.26)	0.845
Required HFNC	4 (15.4%)	3 (12%)	1.31 (0.64, 2.66)	0.691
Length of stay (days)	3 ± 1.47	4.26 ± 3.54	0.77 (0.56, 1.05)	0.095
RSV positive	8 (28.6%)	13 (38.2%)	0.78 (0.42, 1.46)	0.59

Values presented as frequency (%), Mean ± SD. and Odds ratio (95%; Confidence interval). P-value corresponds to Logistic regression analysis
HFNC, high flow nasal cannula; mAPI, modified asthma predictive index; PIS, pulmonary index score; RSV, respiratory syncytial virus

Discussion

Several birth cohort studies have revealed the natural history of wheezing in preschool children.[11-13] In 2008, reclassification of preschool wheezing to EVW and MTW was recommended.[4] This study attempts to investigate the incidence of a second episode wheezing after only the first episode, which all of the subjects had a history of preceding viral upper respiratory tract infection. The incidence of recurrent wheezing of 45% found in this study corresponds with the results of prospective studies reported by Schultz *et al.* in 2010[8] and Kappelle *et al.* in 2012,[14] and in line with the retrospective study of Topal *et al.* in 2013,[15] as well as the results of a study in Thailand carried out by Teeratakulpisarn *et al.* in 2014.[9]

Schultz *et al.*[8] studied 38 cases of EVW for one year and found that 65.8% had recurrent wheezing. Which the phenotype classification remained as EVW in 31.6% of the cases, 34.2% changed to MTW, and the remaining 34.2% recovered. Kappelle *et al.*,[14] who studied 78 patients with severe EVW for 3.9 years, also reported that 66.7% of the patients had recurrent wheezing, while 33.3% recovered. The retrospective study of 236 cases by Topal *et al.* 2013 on short-term changes in phenotype of EVW and MTW concluded that EVW phenotype is not stable and may change over a short-term follow-up period.[15]

A positive mAPI for major criteria, and anti-inflammatory treatment at the time of diagnosis were identified as predictors of persistence of wheeze in preschool children with EVW by Topal *et al.*,[15] this present study did not find these elements to be of statistical significance.

Previous studies have shown the benefit of the mAPI in predicting asthma development in patients having 4 or more episodes of wheezing. Lowering the number of wheezing episodes to 2 (m2API) lowered the predictive value after a positive test.[16] This study used the mAPI to evaluate patients with only a first wheezing episode, and found no significant association between a positive mAPI and recurrent wheezing. Therefore, it could be implied that in patients with a first episode of wheezing, a positive mAPI does not indicate a higher risk of recurrence.

The severity of symptoms in this study, assessed by the Pulmonary Index Score, length of stay, and the requirement of HFNC was not found to have any differences between both groups. This differs from the study of Bessa *et al.* 2014,[17] which found that recurrent wheezers had more severe symptoms, nocturnal symptoms, and visits to emergency rooms and hospitalizations for wheezing and pneumonia, when compared to infants with occasional wheezing. However, all the subjects in

this study were hospitalized. This accounts for already having severe symptoms, which might be the reason for the insignificant differences of these factors between both groups. As for the factor of having an RSV infection, no association with recurrent wheezing was found, similar to previous studies.[18,19]

The present study identified one risk factor for recurrent wheezing in preschool children with first episode wheezing: allergic sensitization to aeroallergen. Allergic sensitization to food also contributes to the risk of recurrent wheezing, although it was not statistically significant. These results are similar to the Prevention of Asthma in Kids (PEAK) trial in 2004.[20] However, it is not a common practice in Thailand to perform skin prick tests in patients with only a first wheezing episode. It is usually done in cases of recurrent wheezing, which asthma is suspected. Although there was a trend for family history of asthma to be associated with recurrent wheezing, it was not conclusive in this study that it is a risk factor for recurrent wheezing. Other factors previously found to have significant association with recurrent wheezing are blood eosinophilia, atopic dermatitis, and a history of earlier episodes of wheezing in infancy.[21] These factors were not found to have an association with recurrent wheezing in this study.

The advantage of this study is that it is a prospective study. This justifies the ability to identify associated factors which are considered as risks for recurrent wheezing. However, the present study is considered to have the following limitations.

First, the study sample size was relatively small, limiting the ability to identify associated factors of recurrent wheezing during the one-year follow-up period. This is because this study only includes children admitted with first episode wheezing and excludes patients with already recurrent wheezing (either from the hospital records or the history suggested by their parents). The study population was representative of the initial population of 97 children positively identified as having a first episode wheezing (**Table 1**) in terms of age, gender, and history of allergic diseases. Further studies in larger prospective hospital-based cohorts of patients presenting with first episode wheezing are needed to confirm the results of the present study.

Secondly, the duration of follow-up was until the patient had a second episode wheezing, with a maximum duration of one-year period. Although recurrent wheezing was reported to occur mostly within the first year, with a mean duration 5.5 ± 7.2 months after the first episode,[9] which bears a resemblance to this study of 4.7 ± 3.7 months, other long-term follow-up cohort studies have shown that a number of patients had recurrent wheezing later in life and were diagnosed with asthma beyond the preschool age.[22-24] By extending the duration of the follow-up, the phenotypes of wheezing could be classified and the natural history of patients presenting with first episode wheezing could be more clarified.

A third limitation is that respiratory virus detection during episodes of wheezing was not performed in every subject. Previous studies have looked in to respiratory viruses in preschool wheeze and suggested that rhinovirus and respiratory syncytial virus may have impact on increasing the likelihood of persistent asthma.[9,25,26] However, viral testing is not necessary in children presenting with wheezing and is not recommended in clinical guidelines for preschool wheeze.[4] Therefore, data collected on respiratory viruses in this study was limited, and so no association between respiratory viruses and recurrent wheezing could be demonstrated.

The final limitation is that this study was conducted in a single center, which may limit the generalizability of the findings. Nonetheless, King Chulalongkorn Memorial Hospital is a tertiary hospital located at the city center of Bangkok. Hence, a number of patients visit the emergency room and the out-patient department each day, including patients referred from other hospitals. Therefore, it is believed that the subjects in this study are a representative of preschool children with first episode wheezing in a tertiary care center.

In conclusion, the incidence of recurrent wheezing in preschool children, age 6 months – 5 years, presenting with first wheezing is 45% which is of significance, with mean lapse duration of recurrence from the first episode of 4.7 ± 3.7 months. It remains a challenge for the clinicians to differentiate children with transient symptoms from children who will have recurrent wheezing. Having an allergic sensitization to aeroallergen could increase their risks. It is recommended to evaluate and follow up preschool children with first episode wheezing within the first year of their attack, especially patients with the risk factor above, so that clinicians could be able to early detect cases likely to develop recurrence

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ORIGINAL ARTICLE

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APJAI

Long-term effectiveness of omalizumab treatment in Thai severe asthmatic patients: A real-life experience

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Abstract

Background: To evaluate long-term effectiveness of omalizumab in 'real-life' setting of Thai asthmatic patients.

Methods: We conducted multi-center, observational study in severe asthma patients who received omalizumab in Thailand. Outcomes were asthma exacerbation (hospitalization and ER visit), asthma control test (ACT), and daily ICS dose. Data were evaluated at baseline, 16 Week, and 52 Week.

Results: A total of 78 patients received omalizumab treatment (average duration 16.9 months with range 16 weeks-2 years). The mean annualized rate of exacerbations was reduced from baseline (3.79) at Week 16 (3.54) and Week 52 (1.16), ($p < 0.05$), respectively. The mean hospitalization rate was reduced from 0.49 in previous year to 0.15 at Week 16 and 0.19 at Week 52. A reduction in ER visit rates was observed at Week 16 (0.15) and Week 52 (0.97) respectively from baseline (1.44) ($p < 0.05$). The ACT score increased from 15.4 at baseline to 20.6 at Week 16 ($p < 0.001$) and increased to 21.5 at Week 52 ($p < 0.001$). The number of patients with controlled asthma (ACT ≥ 20) increased from 16 of 51 at baseline to 32 of 45 at Week 16 and 25 of 32 at week 52, respectively. The median daily dose of ICS equivalent to fluticasone was reduced from baseline 680 mcg to 500 mcg at Week 52. In all, 22 patients discontinued omalizumab after 1 year. Six patients who discontinued omalizumab were restarted due to relapse of symptoms.

Conclusions: These data confirms the effectiveness of one-year duration of omalizumab treatment in Thai severe asthmatic patients. Furthermore, 27% of patients who discontinued treatment required restarting due to relapse of symptoms.

Key words: Omalizumab, Severe Asthma, Effectiveness, Long-term, Real life

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Background

Asthma is one of the major public health concerns in Thailand. In a survey study conducted in 2004, the burden of asthma was high, with 14.8% of respondents being hospitalized for their asthma from the past year and 9% reporting severe persistent asthma.[1] Asthma severity is classified based on the intensity of treatment required to achieve good control of symptoms. In general, patients with mild to moderate persistent asthma can be controlled with low-dose inhaled corticosteroids (ICS) plus a long-acting β_2 agonist (LABA) whereas patients with severe persistent asthma are often not well controlled despite treating with high dose ICS-LABA combination. Inadequately controlled patients are prone to recurrent exacerbations, hospitalization and increased mortality. Often, oral corticosteroids (OCS) are often additionally administered to suppress airway inflammation and improve symptoms; however, long-term use of OCS is associated with substantial side effects, including diabetes, osteoporosis and cataract formation, placing a major burden on patients and healthcare resources.[2-5]

Omalizumab, a humanized anti-immunoglobulin E (IgE) monoclonal antibody, was approved in Thailand in 2006 as an add-on therapy for severe allergic asthma, inadequately controlled with high dose ICS-LABA combination therapy. Omalizumab has been studied in several randomized controlled trials and observational real-life clinical practices involving different ethnic groups. Omalizumab has been shown to reduce asthma exacerbations and hospitalization rates, as well as requirements for oral corticosteroids, in patients with allergic asthma.[6-11] The clinical benefits of omalizumab in "real-world" settings has been demonstrated consistent with the results from clinical controlled trials, providing reassurance of the improvements in outcomes in patients with uncontrolled persistent allergic asthma.[12-14] The long term observational studies of omalizumab have proven its efficacy in terms of sustained asthma control without rebound effect on clinical or immunological parameters.[15-17] In addition, the safety of omalizumab has been confirmed in post-marketing surveillance.[18,19]

However, omalizumab is considered a high cost medication in Thailand with a lack of evidence to prove its clinical benefits among Thai patients suffered from severe persistent allergic asthma. The Omalizumab (Xolair) retrospective studY and reGistry in Thai asthmatic patiENts study (OXYGEN) was therefore initiated in 2009 to address the need to generate data specific to Thai patients and assess whether omalizumab has similar efficacy in this patient population. Patients with severe allergic persistent asthma treated with omalizumab both continuously and intermittently were included in this registry. The results of this study confirm the long-term effectiveness of omalizumab in a real-life setting among severe asthma patients in Thailand.

Methods

Study Design and Patient Population

We conducted a multi-center, cross-sectional observational study in allergy and pulmonology clinics from 10 medical centers across Thailand. Data were collected from paper and electronic hospital medical record between November 2009 and November 2013. A total of 78 patients were identified, including patients from Thammasat University (15 patients), Ramahibodi Hospital, Mahidol University (20 patients), Phramongkutklao Hospital (13 patients), Bhumiphol hospital (13 patients), the King Chulalongkorn Memorial Hospital, Chulalongkorn University (8 patients), Prince of Songkla University Hospital (4 patients), Siriraj Hospital, Mahidol University (3 patients) and Chiang Mai University (2 patients). All patients included in the study were confirmed to have received omalizumab treatment according to routine clinical practice based on the approved indication in Thailand as an add-on therapy for treatment of severe allergic asthma in patients inadequately controlled despite the use of high-dose inhaled corticosteriod (ICS) plus long-acting β2-agonists (LABA). Only patient's with age ≥ 6 years at time of omalizumab initiation, with a documented diagnosis of inadequately controlled severe persistent allergic asthma, with a documentation of asthma exacerbations prior to omalizumab initiation, and in whom omalizumab had been used for at least 16 weeks at time of enrollment were enrolled in the study. Patients with duration of omalizumab treatment less than 16 weeks were excluded, as the response to treatment is recommended to be evaluated at least 16 weeks after initiation of treatment. This study was approved by Institutional Review Board/Ethic Committees in each study center.

Data collection

The investigators from each center were asked to identify patients who received omalizumab and fulfilled the study criteria from their medical centers' patient database and review and collect data from the patients' medical records. Data collection included patient demographics, omalizumab dosing, concomitant asthma medications, exacerbations, hospital admissions, emergency visits, and Asthma Control Test (ACT) scores. Asthma exacerbations were defined as an increase in symptoms requiring treatment with oral corticosteroids (OCS) or doubling of the ICS dose. Data were collected at baseline (at the initiation of omalizumab), Week 16 (Month 4), and up to Week 52 (Year 1) after omalizumab treatment. Data beyond 1 year of treatment collected for exploratory purposes. The overall duration of omalizumab treatment up to the time of data collection was recorded. Data regarding treatment discontinuation and restart of omalizumab treatment were collected to determine treatment duration, reason of discontinuation, number of symptoms at the time of relapse and the time interval before restart of treatment. Asthma relapses after treatment discontinuation were defined as asthma exacerbations requiring OCS or doubling of ICS dose and/or persistent worsening or increase in asthma symptoms as judged clinically significant by investigators.

Omalizumab treatment and concomitant medications

Omalizumab doses were calculated according to the omalizumab dosing table based on individual patient's baseline IgE level and body weight. Omalizumab in Thailand is approved as an add-on therapy for the treatment of severe allergic asthma in patients age 6 or above who are inadequately controlled despite high dose ICS-LABA therapy.[20] Omalizumab was administered by subcutaneous injection every 2 or 4 weeks. There were no restrictions in use of concomitant asthma medications. The medications used, including controller medications, oral corticosteroids and allergic rhinitis drugs, were collected.

Effectiveness Outcomes

The primary purpose of this study was to evaluate the long-term effectiveness of omalizumab as an add-on therapy in local 'real-life' setting in Thai asthmatic patients. The main effectiveness outcomes were asthma exacerbation rate, hospitalization rate, ER visit rate, level of asthma control (ACT score), and daily ICS dose evaluated at baseline, Week 16, and Week 52. The rates of exacerbation, hospitalization, and ER visits were evaluated as the change in annualized rate from baseline. The level of asthma control was evaluated by change from baseline in ACT score and the change in proportion of patients with controlled and uncontrolled asthma as defined by ACT score. The daily ICS dose was converted into fluticasone-equivalent dose and then evaluated as a reduction from baseline at Week 16 and Week 52. Other concomitant asthma medications were evaluated as the reduction in percentage of patients with use of the medications as compared with baseline. The exploratory outcomes of this study were assessment of relapse rates and the frequency of restarting of treatment after discontinuation.

Statistical Analysis

The Stata version 14 (StataCorp, College Station, TX) was used for the entire analysis. A p-value < 0.05 was used to indicate a statistical significant difference, using Wilcoxon Signed Ranks test. Descriptive data were expressed as mean + standard deviation (SD) for continuous variables, and as number and percentage for categorical variables.

Results

Patient Characteristics

A total of 78 omalizumab-treated patients were reviewed. **Table 1** illustrates the baseline patient demographic and clinical characteristics of patients. The median age of patients was 58.5 (50.7-68.0) years, and the median duration of asthma was 10 years. The median baseline FEV_1% predicted was 67.45 (54.0-89.3), and the median PEFR was 310 (211-684) L/min. The median baseline total serum IgE was 257 (97-544) IU/mL. The median monthly omalizumab dose was 300 (150-1200) mg. The average duration of omalizumab use was 16.9 months with range of 16 weeks to 2 years. Sixty-one patients out of 78 patients (78.2%) had documented allergic test reports, either skin prick test or sIgE (RAST) , and 51 of which (83.6%) had positive allergic test results. The reports of the remaining 17 patients were missing. All patients were taking an inhaled corticosteroid plus inhaled long-acting β2-agonist; most patients were taking these as a fixed-dose combination. The median daily fluticasone-equivalent ICS dose was 680 mcg. Maintenance OCS were prescribed in 26.9% of patients. At baseline, 69.2% of patients were taking a leukotriene antagonists and 30.8% were taking an anti-histamine. Interestingly, 44.9% of patients were prescribed theophylline as their maintenance asthma medication. The most common concomitant allergic condition was allergic rhinitis (88.5%). The patient demographic and baseline characteristics are summarized in **Table 1**.

Reduction in Asthma Exacerbations, Hospitalization, and ER Visit Rate

The mean annualized rate of overall asthma exacerbations requiring an increased systemic corticosteroids and/or doubling dose of ICS was significantly reduced from 3.79 in the year prior to treatment to 3.54 at Week 16 ($p < 0.05$) and 1.16 at Week 52 ($p < 0.05$). The mean hospitalization rate was also reduced from 0.49 in previous year to 0.15 at Week 16 and 0.19 at Week 52. Similarly, a significant reduction in ER visit rate from baseline (1.44) was observed at Week 16 (0.15), and Week 52 (0.97) (*both* $p < 0.05$).

Table 1. Patient demographic and baseline characteristics.

Parameters	Study population (n = 78)
Gender (n, F/M)	51/27
Age, median and IQR (years)	58.5 (50.7-68.0)
12 -17 years, n (%)	8 (10.4%)
≥ 18 years, n (%)	69 (89.6%)
Body weight, median and IQR (kg)	62.7 (53.0-68.4)
Duration of asthma, median and IQR (years)	10 (2.75-23.25)
Baseline total IgE, median and IQR (IU/mL)	257 (97-544)
Proportion of patients with allergic tests	61 (78.2%)
Positive allergic tests, n (%) (aeroallergen: house dust mite, cockroach, cat)	51 (65.4%)
Baseline FEV_1% predicted, median and IQR (% predicted)	67.5 (54.0-89.3)
Baseline PEFR, median and IQR (L/min)	310 (211-684)
Concomitant allergic diseases, n (%)	78 (100%)
Allergic rhinitis	69 (88.5%)
Allergic conjunctivitis	9 (11.5%)
Atopic dermatitis, eczema	6 (7.7%)
Urticaria	4 (5.1%)
Omalizumab dose, median and IQR (mg/month)	300 (160-1200)
Daily ICS dose at baseline, median and IQR (mcg/day) (mcg equivalent to fluticasone per day)	500 (160-2000)

Abbreviation: IQR; interquartile range, IgE; immunoglobulin E, FEV_1; forced expiratory volume in the 1st second, PEFR; peak expiratory flow rate, and ICS; inhaled corticosteroid

Table 2. Mean annualized rate of asthma exacerbation, hospitalization, and emergency visit at baseline, Week 16, and Week 52.

Annualized rate[a]	Baseline (*n = 78*)	Week 16 (*n = 78*)	Week 52 (*n = 62*)
Asthma exacerbation rate	3.79	3.54[b]	1.16[c]
Hospitalization rate	0.49	0.15[b]	0.19[c]
Emergency visit rate	1.44	0.15[b]	0.97[c]

[a] Annualized asthma exacerbation, hospitalization and ER visit rates during treatment follow-up period compared with that of baseline (for p-value, statistically significant difference from baseline using Wilcoxon test).
[b] *$p < 0.05$, baseline vs week 16*
[c] *$p < 0.05$, baseline vs week 52*

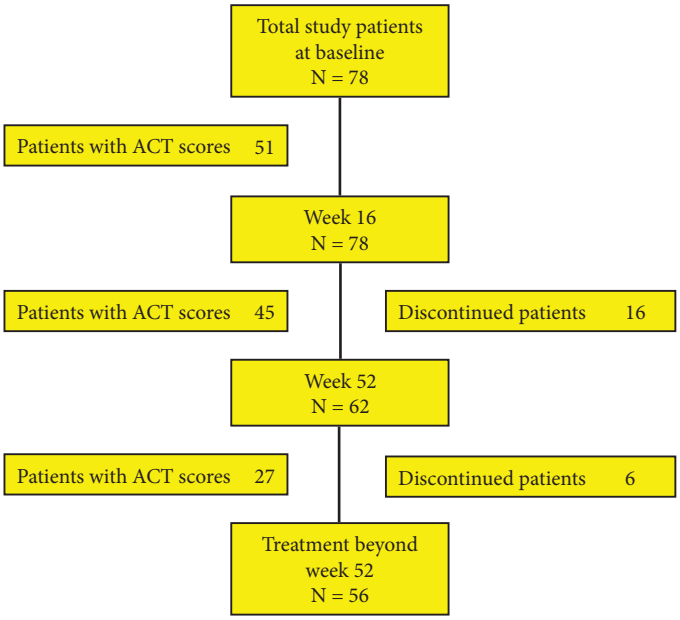
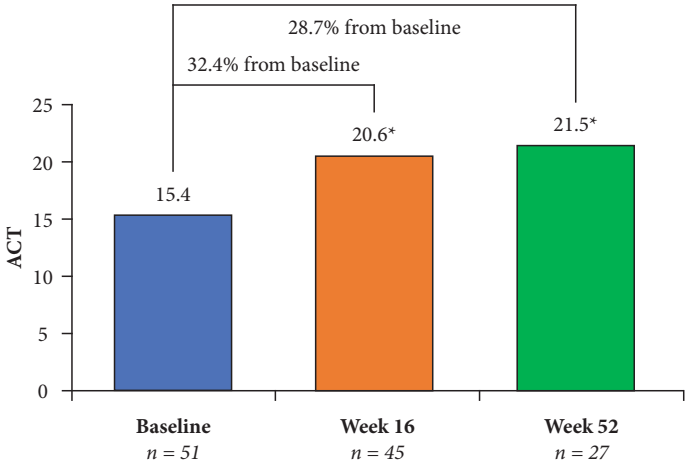


Figure 1. Summary of patient deposition



** p<0.001 vs baseline*

Figure 2. Level of asthma control improvement (a) ACT scores and percentage change from baseline to week 16 and week 52. (b) Proportion of patients with asthma control representing by numbers of patients who achieved ACT score >20 at different time points

Improvement in Level of Asthma Control

The level of asthma control was evaluated by ACT (Asthma Control Test) score. Only 51 patients had their ACT score documented. Summary of patient deposition was shown (**Figure 1**). Among these, ACT was increased from 15.4 at baseline to 20.6 at Week 16 (32.4% improvement, $p < 0.01$) and increased further to 21.5 at Week 52 (28.7% improvement, $p < 0.001$) **Figure 2**). For comparative purposes, patients were separated into 2 categories according to their asthma control as defined by ACT score – patients with controlled asthma (ACT ≥ 20) and patients with uncontrolled asthma (ACT < 20). At baseline, a high percentage of patients (68.6%) was uncontrolled. After 16 weeks of treatment; the proportion of patients with controlled asthma increased from 31.4% at baseline to 71.1%, corresponding to a decrease in the proportion of patients with uncontrolled asthma to 28.9%. This improvement persisted until 52 weeks of treatment as demonstrated with 78.1% of patients with controlled asthma and 21.8% of patients with uncontrolled asthma at this time point.

Reduction in Daily ICS Dose and Other Concomitant Asthma Medications

The median daily dose of ICS was slightly reduced from baseline dose of 680 mcg equivalent to fluticasone per day to 500 mcg per day at Week 52 after treatment as shown in **Table 3**. As for other concomitant asthma medications, the proportion of patients using maintenance oral corticosteroids decreased from 26.9% (21/78 patients) at baseline to 14.1% (11/78 patients) at Week 16 and 6.4% (4/62) at Week 52 (**Table 3**). Similarly, the proportion of patients receiving concomitant treatment with LTRA, theophylline, and anti-histamine was also reduced during omalizumab treatment.

Treatment Discontinuation and Restart of Omalizumab Treatment

In all, 62 patients (79.4%) continued omalizumab treatment for at least 1 year. Of the 22 patients who discontinued omalizumab, the median treatment duration was 12 (from 4 to 48) months. The most common reasons for discontinuation of treatment were patient desire (50%), loss to follow up (13.6%) and physician desire (9.15%). Six patients (27%) who discontinued omalizumab treatment were restarted due to a relapse of symptoms. Among patients who restarted omalizumab, the mean dose of omalizuamb was 300 ± 164.3 mg, the mean duration of treatment prior to discontinuation was 9.7 ± 6.3 months and the mean duration of omalizumab discontinuation was 6.5 ± 3.4 months. The mean serum total IgE before restarting

Table 3. Changes in daily dose of inhaled corticosteroid (mcg equivalent to fluticasone per day) and number of patients on concomitant medications at each time point.

Asthma and concomitant mediations	**Baseline** (*n = 78*)	**Week 16** (*n = 78*)	**Week 52** (*n = 62*)
Mean ICS daily dose (mcg equivalent to fluticasone per day)	591.9	485.6[a]	356.5[b]
Median ICS daily dose (IQR) (mcg equivalent to fluticasone per day)	680	840	500[b]
Number of patients on concomitant medications, n (%)	**Baseline** (*n = 78*)	**Week 16** (*n = 78*)	**Week 52** (*n = 62*)
Oral corticosteroids	21 (26.9%)	11 (14.1%)	4 (6.4%)
LTRA	54 (69.2%)	44 (56.4%)	22 (34.9%)
Theophylline	35 (44.9%)	25 (32.1%)	15 (23.8%)
Anti-histamine	24 (30.8%)	17 (21.8%)	13 (20.6%)
Nasal ICS	16 (20.5%)	13 (16.7%)	9 (14.3%)

Abbreviation; ICS: inhaled corticosteroid, LTRA and leukotriene receptor antagonist
[a] *$p < 0.05$, baseline vs week 16*
[b] *$p < 0.05$, baseline vs week 52*

treatment was 355.7 ± 130.2 IU/ml. There were no serious adverse effects related to omalizumab treatment recorded.

Discussion

Despite the fact that there have been several randomized controlled trials demonstrating the clinical effectiveness of omalizumab in severe allergic asthma, real world studies for documenting effectiveness are also important. The advantages of real-life studies are maximizing external validity, increasing generalizability and being preferable evidence within healthcare systems.[21]

The results of our study included both adults and children with severe asthma. In addition, this study is the first to providereal world evidence of the effectiveness of omalizumab in Southeast Asian region. The average duration of omalizumab treatment in Thai asthmatics was 16.9 months (16 weeks-2 years) due to the regulation for reimbursement in the healthcare system in Thailand. Omalizumab effectiveness in our study was assessed in terms of clinically significant asthma exacerbation reduction rate and improvement of asthma control level (ACT) score similar to a previously reported pooled analysis comprising seven randomized studies.[22] However our asthmatic patients were older, had more severe airflow obstruction and had a higher baseline total serum IgE.[23] Our patient baseline characteristics were similar to those in a previously reported real world study conducted in France.[24]

Outcomes of asthma control can be measured by assessment of current symptom scores, frequency of asthma exacerbations and oral corticosteroid usage.[25] We found that a composite symptom score of asthma control, the asthma control test (ACT) score, was significantly improved at the 16th week of treatment in this study. However, there was no further improvement after the 52nd week of therapy. The improving of asthma composite score was similar to previous real world studies.[12,13,26,27]

More significant improvement of asthma outcome was found in our study using exacerbation frequency and reduction of oral corticosteroid usage. These findings support that omalizumab effectiveness was similar to the previous real world studies. Treatment with omalizumab has been associated with a reduction or discontinuation of added on oral corticosteroid required for controlling asthma.[11,12,24] In our study, omalizumab treatment was also associated with a trend toward a reduction in the dose of inhaled corticosteroid, although this trend did not reach statistical significance. In a much larger prospective observational cohort study of moderate-to-severe allergic asthma, omalizumab therapy was associated with a reduction in doses of ICS, SABA, and leukotriene modifiers over 2 years in a "real-world" setting.[14]

The recommended duration of omalizumab treatment has never been clearly established. Nevertheless, the result of a long term study of omalizumab treatment (7 years) demonstrated the additional benefit of extended treatment duration by reducing exacerbation rate.[17] Apart from sustained clinical efficacy, long term omalizumab treatment has also been associated with a sustained reduction of *in vitro* basophil allergen threshold sensitivity that persists beyond discontinuation of treatment.[15,16] Current Thai asthma guidelines limit long term treatment with omalizumab despite clinical evidence of improvement. For this reason, approximately one-third of our patients discontinued omalizumab injections. Twenty-seven percent of patients who discontinued treatment developed clinical relapse determined by the presence of asthma exacerbation requiring systemic corticosteroid that resulted in re-starting of omalizumab treatment. The asthmatic relapse developed after 6.5 month of discontinued treatment. This finding emphasizes the long term benefit of continuation of omalizumab treatment in a real life study.

The duration of follow up after discontinuation of omalizumab treatment is essential for determining the clinical relapse rate. In our study, the duration of follow up of patients who discontinued omalizumab was shorter than in the French study, and this reason may explain why the proportion of patients who developed clinical relapse was lower in our study compared to the French real world study.[24] The limitations of our real world study are incomplete data of asthma outcome measurement in the follow up period. In addition, approximately 20% of patients who received omalizumab were either current or former smokers, and we could not distinguish evere asthma from either COPD or asthma COPD overlap syndrome (ACOS) in this subset of patients.[28,30] However, the presence of fixed airflow obstruction, the long duration of diagnosed asthma, the high serum total IgE and skin prick test positivity to common aeroallergen in the majority of these patients are compatible with the ACOS definition. The study for comparing efficacy of adding anticholinergic or adding omalizumab to ICS/LABA in these patients with ACOS are needed.

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CASE REPORT

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APJAI

Biphasic buckwheat anaphylaxis: Case report and systematic review

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Abstract

Buckwheat anaphylaxis is commonly recognized in Europe and Asia, and there is only one case reported in Taiwan so far. Here, we report a case of biphasic buckwheat anaphylaxis in a 57 year-old male patient who lost consciousness twice in the same day after having buckwheat noodles. The serum test shows that *Dermatophagoides pteronyssinus* (Dp) immunoglobulin E (IgE) (42.4 kU/L) and buckwheat-specific IgE (81.5 kU/L) are unusually high. Although biphasic buckwheat anaphylaxis is rare, we should still be aware the second episode could be life-threatening and happen within a day after the exposure to the buckwheat antigen.

Key words: buckwheat, anaphylaxis, biphasic, allergy, predictor

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Introduction

Buckwheat (*Fagopyrum esculentum*) and tartary buckwheat (*Fagopyrum tartaricum*) are widely used in traditional Japanese, Korean, and European diets.[1] There is a growing consumption of health food in Taiwan, including buckwheat products. Besides, buckwheat is gluten-free and serves as a good alternative for celiac disease sufferers and other people intolerant of wheat flour.[2]

Direct ingestion is not the only pathway to cause buckwheat anaphylaxis, which could also be caused by buckwheat contamination during food preparation.[2] Therefore, the whole procedure of food preparation should be taken carefully for buckwheat-allergic patients. The incidence of buckwheat allergy is 0.22% in children.[3] However, as a hidden antigen, the risk of buckwheat ingestion is usually underestimated.

Buckwheat is a rare allergen in Taiwan. The only case report is of a woman with asthma who had anaphylactic shock, generalized urticaria, and an acute exacerbation of asthma five minutes after ingesting buckwheat.[4] To our knowledge, this is the first report of biphasic buckwheat anaphylactic shock in Taiwan.

Case report

A 57 year-old man had fainting, loss of consciousness and skin rash with wheal and flare developing 40 minutes after having buckwheat noodles. Immediately, he was brought to the emergency department by colleagues and was discharged two hours later after intramuscular (IM) injection of 0.5 mg epinephrine and 10 mg chlorphenamine. However, throat itching, generalized wheal and flare skin lesions, angioedema, fainting, and loss of consciousness were noted again within two hours of his return to his office. He was brought to the emergency department again right away. IM injection of 0.5 mg epinephrine and 10 mg chlorphenamine were again given at the emergency room, and he was discharged after a 2-hour observation. He followed up in our outpatient department for further diagnosis and management one week later.

He has a past history of tongue numbness, dizziness, wheal and flare skin rash with pruritis, palpitations, and loss of consciousness that developed at the age of 54 within 30 minutes after ingesting buckwheat noodles. Besides, generalized wheal and flare skin rash developed at the age of 55 within 1 hour after ingesting whole grain bread (including buckwheat). There were no allergic symptoms (i.e., urticaria, itching throat, fainting, or angioedema) noted in his medical history after

ingesting noodles or bread not containing buckwheat. The patient's history was negative for drug allergy.

His serum was tested for specific IgE to Dp, cat and dog danders, cockroach, and food allergens including egg white, milk, fish, wheat, and buckwheat (**Table 1**) with Pharmacia CAP system (Uppsala, Sweden), and only test results for Dp (42.4 kU/L, class 4) and buckwheat-specific IgE (81.5 kU/L, class 5) were positive. Other blood test results were: hemoglobin 14 gm/dL, hematocrit 40.9%, white blood cell count 11,100/μL, platelet count 351,000/μL, blood sugar 97 mg/dL, glycosylated hemoglobin (HbA1c) 5.8%, and IgE 950 IU/mL.

Table 1. Specific IgE of the patient

Antigen	Level (ku/L)	class
D. pteronyssinus	42.4	4
Cat dander	< 0.35	0
Dog dander	< 0.35	0
Cockroach	< 0.35	0
Egg white	< 0.35	0
Milk	< 0.35	0
Fish	< 0.35	0
Wheat	< 0.35	0
Buckwheat	81.5	5

Class 0: < 0.35 kU/L
Class 1: 0.35~0.7 kU/L
Class 2: 0.7~3.5 kU/L
Class 3: 3.5~17.5 kU/L
Class 4: 17.5~50 kU/L
Class 5: 50~100 kU/L
Class 6: > 100 kU/L

Discussion

IgE-mediated hypersensitivity to buckwheat is common in Korea, Japan, and some other Asian countries.[4] It is also reported that the proportion of anaphylaxis was highest in the patients allergic to buckwheat in Korea. Therefore, the risk of buckwheat anaphylaxis should not be underestimated. We summarize (**Table 2**) the most common foods causing anaphylaxis, the prevalence of biphasic anaphylaxis, and the fatal cases in Asia from recent reports.[5-14]

Biphasic anaphylaxis refers to the second episode of anaphylaxis and has been reported to develop in up to 20% of reactions.[15] There are no significant differences in baseline characteristics between patients with and without biphasic anaphylaxis, such as atopic diseases, triggers, precipitating factors (i.e., food, exercise, and medication), clinical manifestations, and treatments.[6] However, it is known that the risk of biphasic anaphylaxis increases with multiple doses of epinephrine during the initial treatment. For instance, in one of the reports, pediatric patients received a dose of 0.01 mg/kg (maximum dose = 0.5 mg).[7] The risk also increases in pediatric patients taking a longer time to resolve from the first episode compared to the uniphasic reactors[16] or having delayed administration of epinephrine.[17] Additionally, we noted a delayed onset for the development of symptoms after the initial exposure to the antigen,[18] and oral ingestion of antigen has been noted as a potential predisposing factor in two reports.[18,20]

Although double-blind placebo-controlled food challenge (DBPCFC) is the gold standard to confirm buckwheat allergy,

Table 2. summary of food anaphylaxis reports in Asian

Authors	Country	Numbers of study participants	population	type of food anaphylaxis	Biphasic anaphylaxis	Rate of death	Time from contact to onset
Wiparat Manuyakorn et al.[5]	Thailand	160	Children (aged 3 months-18 years)	Seafood* (53.3%) Wheat* (18.3%) Hen' egg* (11.7%) Cow's milk* (1.7%)	8.7%	0	Not available
Ratchaya Lertnawapan et al.[6]	Thailand	171	the median age: 20.67 years	Seafood* (24.7%) Fried-insect* (23.6%)	6.3%	0	30 minutes in uniphasic group 120 minutes in biphasic group
Naoyuki Inoue et al.[7]	Japan	61	Children (aged 2 months-14 years)	Egg* (41.1%) Milk* (21.4%) Wheat* (14.3%)	3.3%	0	Not availables
Woei Kang Liew et al.[8]	Singapore	108	Children (aged 3 years-11 years)	Peanut (19%) Egg (12%) Shellfish (10%) Bird's nest (10%)	3.6%	0	Not available
Kim M et al.[9]	Korean	29842	Children (aged 6-16 years)	Peanut (0.08%) Cow's milk (0.07%) Buckwheat (0.06%) Hen's egg (0.06%)	0.97%	0	Not available
Yang MS et al.[10]	Korean	138	no age limit (only 1 patient < 15 years old)	Wheat (4.3%) Buckwheat (4.3%) Seafood (2.9%)	2.2%	0.0001%	Not available
Vezir E et al.[11]	Turkey	96	Children (mean age is 7.4 years)	Peanuts* and nuts (30%) Cow's milk* (23.3%) Egg's white* (20%)	5.2%	0	Not available

Table 2. (Continued)

Authors	Country	Numbers of study participants	population	type of food anaphylaxis	Biphasic anaphylaxis	Rate of death	Time from contact to onset
Jiraponsananuruk O et al.[12]	Thailand	101	no age limit (aged 2.8 months to 81.3 years)	Seafood* (45%) Wheat* (8%) Milk* (4%)	7% in pediatric group 2% in adult group	0.00019%	30 minutes (50%), more than 60 minutes (32%), 30 to 60 minutes (10%)
Civelek E et al.[13]	Turkey	843	no age limit (aged 1-79 years)	Milk (8%) Nut (6%) Egg (2.7%) Fish (2.1%) Legume (1.2%)	4.3%	0	Not available
Jeong KG et al.[14]	Korean	1353	Children (aged <18 years)	Cow' milk* (27.5%) Hen's egg* (21.9%) Wheat* (11.3%) Walnut* (10.5%) Peanut* (5.9%)* Buckwheat* (4.2%) Pine nut* (3.0%) Shrimp* (1.8%) Kiwi* (1.4%) Almond* (1.2%) Soybean* (0.8%)	Not available	Not available	Not available

* indicates that the statistics only include food allergens.

it is time-consuming and can be life-threatening. This risk can be avoided with measuring the food-specific IgE concentration in serum. Thus, the detection of crude buckwheat extract -specific IgE by ImmunoCAP (f11) (Phadia AB, Uppsala, Sweden) is widely used to diagnose buckwheat allergy.[21]

Sohn et al. concluded that a cutoff level of 1.26 kU/L of specific IgE for buckwheat was adequate for diagnosis in children with a strong clinical history.[26] In this report, we utilized Pharmacia CAP to obtain the buckwheat-specific IgE concentration of 81.5 kU/L in serum, which is significantly higher than the cutoff value (1.26 kU/L) of the method mentioned above. The buckwheat-specific IgE concentration is extremely high, which is compatible with his severe allergic symptoms. We thought it was dangerous for the patient to try an oral food challenge. The specific IgE of wheat is significantly low (< 0.35 IU/L, class 0) in this patient. Besides, food containing wheat is common in Taiwan, and there are no related allergic symptoms found in the patient's history. Hence, we conclude that the patient is allergic to buckwheat without further examinations (e.g., the measurement of the major allergen in wheat, omega 5-gliadin).

Adrenaline (epinephrine) is essential to all patients experiencing anaphylaxis, which should be given by IM injection into the mid-outer thigh. The safety profile of IM adrenaline is excellent, although patients may experience transient pallor, palpitations, and headache. IM adrenaline (1 mg/mL) should be given at a dose of 0.01 mL/kg of body weight to a maximum total dose of 0.5 mL. When using adrenaline auto-injectors, patients weighing 7.5–25 kg should receive a 0.15 mg dose with patients being moved to a 0.3 mg dose at 25–30 kg. The adrenaline dose can be repeated after at least a 5-minute interval.[15]

Oral (PO) or intravenous (IV) glucocorticosteroids are commonly used in anaphylaxis and are thought to possibly prevent protracted anaphylaxis symptoms, particularly in patients with concomitant asthma and biphasic reactions.[9] The dose of hydrocortisone for adults and children depends on age: > 12 years and adults: 200 mg IM or IV slowly; > 6 to 12 years: 100 mg IM or IV slowly; > 6 months to 6 years: 50 mg IM or IV slowly; < 6 months: 25 mg IM or IV slowly.[23]

With regard to monitoring and discharge arrangement, patients who present with respiratory compromise should be closely monitored for at least 6–8 hours, and patients who present with hypotension require close monitoring for at least 12–24 hours.[6] The education is important to the patients, including the of early recognition of anaphylactic symptoms and the avoidance of direct and indirect contacts with allergens, especially food allergens. Before discharge, the risk of future reactions should be assessed and an adrenaline auto-injector prescribed to those at risk of recurrence. Patients should be provided with a discharge advice sheet, including allergen avoidance measures (where possible) and instructions for when and how to use the adrenaline auto-injector. Referral to an allergy specialist is recommended to investigate possible triggers, assess and, where possible, to intervene to minimize the risk of further reactions, and ensure that patients and caregivers are optimally equipped and trained to manage any further reactions; and, if food is involved, referral to a registered dietitian.[6]

As buckwheat is becoming a popular health food in Taiwan, there is increasing prevalence of buckwheat anaphylaxis. When taking a history about exposure to allergens, buckwheat should be included. Besides, the correct management of this emergency can reduce the risk of biphasic anaphylaxis.

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ORIGINAL ARTICLE

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Randomized comparison of caregivers' ability to use epinephrine autoinjectors and prefilled syringes for anaphylaxis

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Abstract

Background: Caregivers often incorrectly use epinephrine autoinjectors. It is unclear whether this is due to insufficient training or a difficult-to-use tool. Furthermore, the high costs of epinephrine autoinjectors may limit their availability; so low-cost prefilled syringes may be the alternative.

Objectives and Methods: We performed a prospective randomized trial to compare successful epinephrine administration at four stages: after reading written instructions, and immediately after, 6 weeks, and 3 months following video training. The time required for successful epinephrine administration and failed steps in the administration of epinephrine autoinjectors and prefilled syringe were also investigated.

Results: Complete data analysis of 113 participants (prefilled syringe group, n = 57; EpiPen, n = 56) was performed. Significantly more participants correctly demonstrated the use of prefilled syringes compared to EpiPen after reading instructions, and immediately following 6 weeks, and 3 months after video training. ((adjusted OR 26.17 (95%CI 8.25-83.04), adjusted OR 4.07 (95%CI 1.29-12.86), adjusted OR 14.01 (95%CI 3.62-54.22)) and adjusted OR 31.44 (95%CI 5.73-172.39), respectively) Four key step errors would likely result in failure of administration and were more common with EpiPen (14.0% vs. 2.3%, $p < 0.001$). There were no statistically significant differences in time of successful administration between the two groups ($p > 0.05$).

Conclusion: Epinephrine prefilled syringe was significantly easier to use with a higher rate of correct use compared to EpiPen over time. All four key step errors in the administration were more likely with EpiPen. The time required for successful epinephrine administration was not significantly different.

Key words: anaphylaxis, epinephrine, autoinjectors, prefilled syringe, food allergy, caregivers' ability

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Introduction

The prevalence of anaphylaxis appears to be increasing.[1-3] This may be due to more effective diagnosis by physicians, or due to a genuine increase in prevalence. It is is mainly caused by foods, drugs, and insects.[4] The most common leading cause of anaphylaxis in children is food allergy.[3,5] Epinephrine is the drug of choice for life-threatening allergic reactions and is needed immediately in anaphylactic patients in community and healthcare settings.[6] Epinephrine administration by injection into the muscle layer of the anterolateral thigh is an appropriate position.[4]

The rise in prevalence of anaphylaxis is leading to the increased prescription of epinephrine. All patients with a history of anaphylaxis or patients who are allergic to foods that are likely to cause a severe allergic reaction, especially in patients

allergic to nuts, fish and seafood, need to carry epinephrine at all times.[7] Therefore, patients and caregivers must be aware of the indications for use of epinephrine, which is the initial treatment for anaphylaxis before reaching the hospital, including the need to be able to use it correctly and quickly. If this condition cannot be diagnosed or the initial treatment is delayed or incorrect, it may lead to death.[4,8] Most anaphylaxis deaths occur in community settings rather than in healthcare settings.[8] Thus, epinephrine injectors need to be easy to use and user-friendly devices. Previous studies have reported that only 25-50 percent of anaphylactic patients carry epinephrine.[9,10] Moreover, the patients carry epinephrine, but they don't use epinephrine (73 percent in adults[11] and 83 percent in children).[12] The reasons for patients or caregivers not using epinephrine might be lack of knowledge about indications for drug use, or how to use medication correctly, or a lack of adequate training. In addition, equipment design may make it difficult to use and thus reduce user confidence. Previous studies showed that patients or parents could use self-injectable epinephrine correctly in only 1 in 3 cases.[9]

Epinephrine delivery systems used to treat anaphylaxis differ in their designs. Commercially available epinephrine autoinjectors are expensive, causing restrictions on use. Moreover, previous studies have found they are difficult to use and autoinjector accidents occur frequently, including finger injuries.[13,14] Many studies have compared the functionality or usability of epinephrine autoinjector devices.[15-18] In many countries worldwide, epinephrine autoinjectors remain unavailable or unaffordable, patients at risk for anaphylaxis are often provided with a manual prefilled syringe containing a premeasured epinephrine dose, however, there are some problems with stability and sterility about 2-3 months after preparation.[19,20]

EpiPen is the only autoinjector brand available in Thailand. The previous study assessing the use of self-administered EpiPen devices found that only 38% of patients/parents, 21% of attending pediatricians and 36% of pediatric residents could accurately demonstrate the EpiPen device.[9] Based on its difficult usage, the EpiPen was redesigned. The subsequent study found that more than 80% of parents and hospital staff correctly demonstrated all steps in use of the redesigned EpiPen.[16] An epinephrine prefilled syringe is a low- cost therapeutic alternative to epinephrine autoinjectors. From the previous literature review, no study has compared the performance of the two devices.

In this study, a prospective randomized controlled trial, we compared the ability of caregivers of food-allergic children to successfully inject epinephrine using autoinjectors, the redesigned EpiPen and epinephrine prefilled syringe, after reading instructions, receiving standard video training, and whether they more easily recalled this information with usage at six weeks (primary outcome), three months after training. The secondary outcomes were evaluating the time required for successful epinephrine administration and the failed steps of epinephrine administration in each tool.

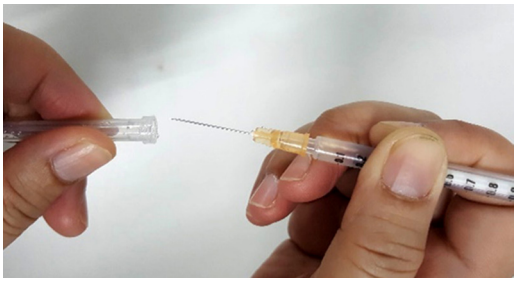
Methods

This prospective randomized controlled trial was approved by the Institutional Ethics Committee of the Faculty of Medicine, Vajira Hospital, Navamindradhiraj University. Inclusion criteria were participants who were responsible for taking care the food allergic children that they were diagnosed with combined skin prick test, serum IgE and clinical symptoms and gave informed consent prior to commencing this study. Exclusion criteria were participants who were healthcare personnel, unable to understand Thai, a history of, or having a child with, anaphylaxis, or having a child with high risk of anaphylaxis such as from tree nuts, peanut or seafood, or having a child with food allergy and a history of asthma which may be the reason for prescribed epinephrine injectors,[7] have received previous education in use of an epinephrine injector, and have significant psychiatric problems. The participants were excluded if their children were prescribed epinephrine injectors during the study. The study was registered with www.clinicaltrials.in.th (TCTR20171127001).

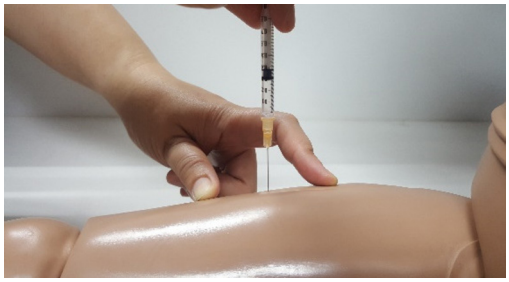
We allocated caregivers of food-allergic children who met the inclusion criteria to a computer-generated randomization list to either the EpiPen group or prefilled syringe group and asked them to demonstrate the use of a 'trainer' device with a manikin after reading instructions and immediately after receiving standard video training. Epinephrine prefilled syringe was prepared from 0.3 ml of a 1 mg/ml epinephrine solution in disposable plastic 1 ml. syringe with 25-guages and 1 inch (2.54 cm) needle. After evaluating their ability to use epinephrine devices following video training, all participants in the prefilled syringe group and EpiPen group were individually shown the correct technique for their assigned epinephrine trainer device by a single specialist pediatric allergist and asked to immediately demonstrate its use with complete accuracy and confidence. Participants' ability to recall the correct use of each device at 6 weeks and 3 months after initial training in a standard situation was evaluated. Before the reassessment (at 6 weeks and 3 months), the participants were asked to confirm that they had not been trained in the use of epinephrine devices during the intervening period. Participants were asked to suddenly demonstrate the trainer without retraining. All demonstrations were evaluated by the same two investigators. All demonstrations were videotaped for futher evaluation in case of disagreement between the two investigators.

Outcome measures

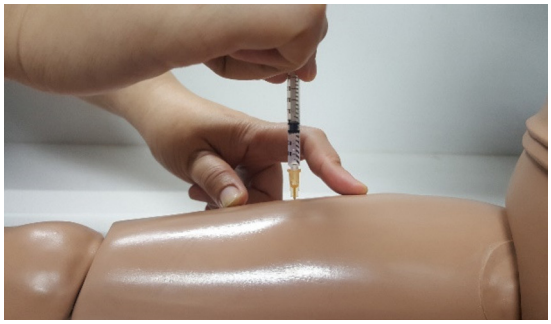
Primary outcome was the comparison of successful epinephrine administration using epinephrine autoinjectors, redesigned EpiPen, and prefilled syringe at six weeks after training. The four key steps for EpiPen administration were as follows: (i) remove blue safety cap; (ii) place the orange end of the device against the thigh; (iii) push down to activate; and (iv) hold device in place for 3 seconds for successful epinephrine delivery.[17,21] For prefilled syringes the key steps were: (i) remove needle cap; (ii) place needle against the thigh; (iii) push down the needle on the thigh and needle up to the needle hub; and (iv) push syringe tabs completely for successful epinephrine administration. (**Figure 1**) The drug administration must be demonstrated step by step that cannot skip the step.



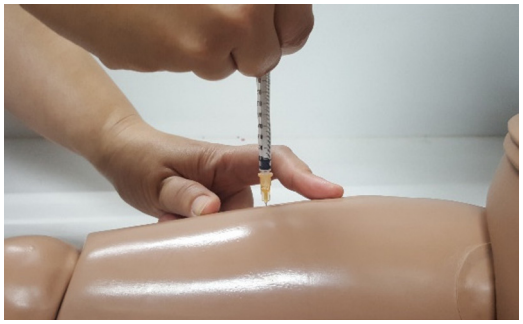
(i) remove needle cap



(ii) place needle against the thigh skin



(iii) push down the needle on the thigh and needle up to the needle hub



(iv) push syringe tabs completely for successful epinephrine administration

Figure 1. The four key steps for epinephrine prefilled syringes administration

Secondary outcomes included comparing the rate of successful epinephrine administration between the two devices at three months after training, the time required for successful epinephrine administration, and the failed steps of epinephrine administration for each tool.

Statistical analysis

Data were analysed using SPSS (Statistical Package for the Social Sciences for Windows) version 22.0. Sociodemographic characteristics were compared to ensure comparability between the two groups. Categorical data were described as frequencies and analyzed with the Chi-square test. Continuous data and the time required for successful epinephrine administration at each time between groups were presented as mean±standard deviation or median and analyzed with the Student t-test or Mann–Whitney U-test for continuous data if they were non-normal distribution. The proportion of participants for each successful administration was compared between two groups at each of the time points using Pearson's chi-squared statistic or Fisher's exact test, and multiple logistic regression for adjusted analyses of binary outcomes to calculate adjusted odds ratios (OR) and 95% confidence intervals (95%CI). Whether the differences in rates of successful epinephrine administration after reading instructions immediately, 6 weeks and 3 months after training was statistically significant or not was analyzed by McNemar test (within group). The cut-off of $p < 0.05$ was used to determine statistical significance.

Results

The flow chart of participants is shown in **Figure 2**. Complete data analysis of 113 participants was performed (prefilled syringe group, n = 57; EpiPen group, n = 56). Characteristics of participants and food-allergic children are shown in **Table 1**. The randomized groups were similar, except there was a difference in the respiratory manifestations of food allergy in the EpiPen group ($p = 0.037$).

Ability to successfully administer epinephrine after reading instructions, and immediately following, 6 weeks, and 3 months after video training

After reading instructions, only 28.6% of caregivers in the EpiPen group were able to perform a successful administration of epinephrine, while 89.5% in the prefilled syringe group were able to perform successfully ($p < 0.001$). Six weeks after video training, the ability to recall steps in the use of the assigned trainer device was significantly higher in 53 of 57 (93%) of the prefilled syringe group, compared to 34 of 56 (60.7%) in the EpiPen group, after adjusting for age and education of caregivers, other underlying allergic diseases and the number of food allergies in patients (adjusted OR 14.01 (95%CI 3.62-54.22). Only 58.9% of participants demonstrated correct EpiPen use at 3 months, compared with 96.5% for prefilled syringe users. Successful epinephrine administration following reading the instructions, immediately, and 3 months after video training was significantly higher in the prefilled syringe group compared to the EpiPen group (adjusted OR 26.17 (95%CI 8.25-83.04)), (adjusted OR 4.07 (95%CI 1.29-12.86 and adjusted OR 31.44 (95%CI 5.73-172.39), respectively (**Table 2**).

There was a significant difference in increased success rate of participants in EpiPen group after reading instructions and immediately after video training ($p < 0.001$) (**Figure 3**). The differences in rates of successful epinephrine administration after complete training and 6 weeks, 3 months later decreased in the EpiPen group, however, this was not statistically significant ($p > 0.05$, respectively). There was an increased rate of successful epinephrine administration after complete training and a

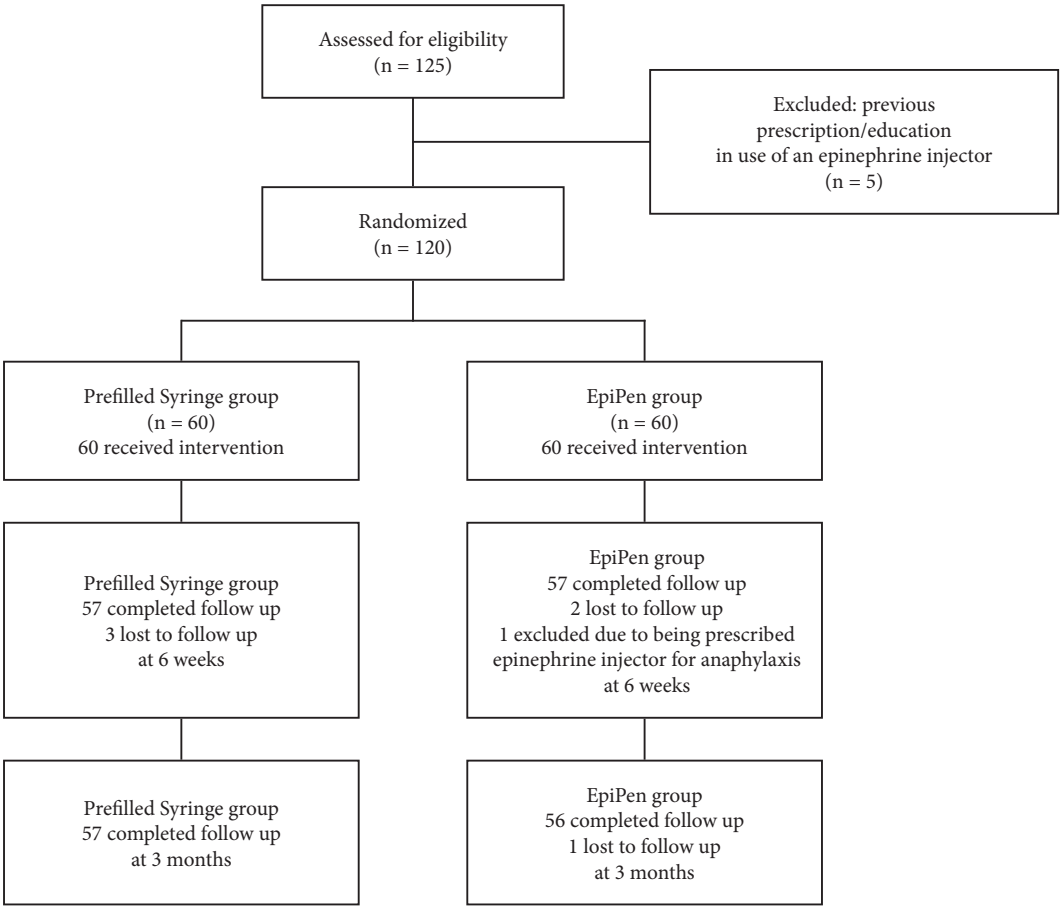


Figure 2. Study flow chart

Table 1. Demographic characteristics

	Prefilled syringe group n = 57 (%)	EpiPen group n = 56 (%)
Demographic characteristics of participants		
Age (years)		
Median (IQR)	39.33 (35.00-49.00)	41.42 (32.50-49.00)
Sex		
Female	43 (75.4)	48 (85.7)
Relationship to patients		
Father	8 (14.0)	4 (7.1)
Mother	27 (47.4)	30 (53.6)
Grandfather/Grandmother	9 (15.8)	13 (23.2)
Others	13 (22.8)	9 (16.1)
Education level of caregivers		
Elementary School	7 (12.3)	12 (21.4)
High School	18 (31.6)	20 (35.7)
College	8 (14)	8 (14.3)
Univesity	23 (40.4)	15 (26.8)
Postgraduate	1 (1.7)	1 (1.8)
Family income per month (baht/month)		
< 10,000	4 (7)	7 (12.5)
10,001-30,000	39 (68.4)	37 (66.1)
30,001-50,000	12 (21.1)	8 (14.2)
50,001-80,000	2 (3.5)	2 (3.6)
> 80,001	0 (0)	2 (3.6)

	Prefilled syringe group n = 57 (%)	EpiPen group n=56 (%)
Demographic characteristics of food-allergic children		
Age (years)		
Median (IQR)	3.0 (1.10-8.00)	3.3 (1.20-6.30)
Clinical manifestation of food allergy*		
Skin changes	49 (86)	48 (85.7)
Respiratory symptoms	8 (14.0)	17 (30.4)
Gastrointestinal symptoms	10 (17.5)	8 (14.3)
Cardiovascular symptoms	0 (0)	1 (1.8)
Neurological symptoms	0 (0)	0 (0)
Other allergic diseases of patients		
Atopic dermatitis	11 (19.3)	8 (14.3)
Allergic rhinitis	5 (8.8)	12 (21.4)
Asthma	1 (1.7)	0 (0)
The number of food allergies		
1	41 (71.9)	40 (71.4)
2	10 (17.6)	9 (16.1)
≥ 3	6 (10.5)	7(12.5)

*Some children had more than 1 clinical manifestation of food allergy
IQR = interquartile range

Table 2. Comparision of successful epinephrine administration of prefilled syringe or EpiPen each time

	Prefilled syringe group n = 57 (%)	EpiPen group n = 56 (%)	OR (95%CI)	*p* value	Adjusted OR* (95%CI)	*p* value
After reading instructions	51 (89.5)	16 (28.6)	21.25 (7.62-59.26)	< 0.001*	26.17 (8.25-83.04)	< 0.001*
Immeaditely after video training	52 (91.2)	40 (71.4)	4.16 (1.41-12.32)	0.010*	4.07 (1.29-12.86)	0.017*
6 weeks after training	53 (93)	34 (60.7)	8.57 (2.72-27.06)	< 0.001*	14.01 (3.62-54.22)	< 0.001*
3 months after training	55 (96.5)	33 (58.9)	19.17 (4.24-86.58)	< 0.001*	31.44 (5.73-172.39)	< 0.001*

OR = odds ratio; 95%CI = 95% confidence interval
*Adjusted OR (95%CI); adjusted by age and education of caregivers, underlying other allergic diseases and the number of food allergies in patients

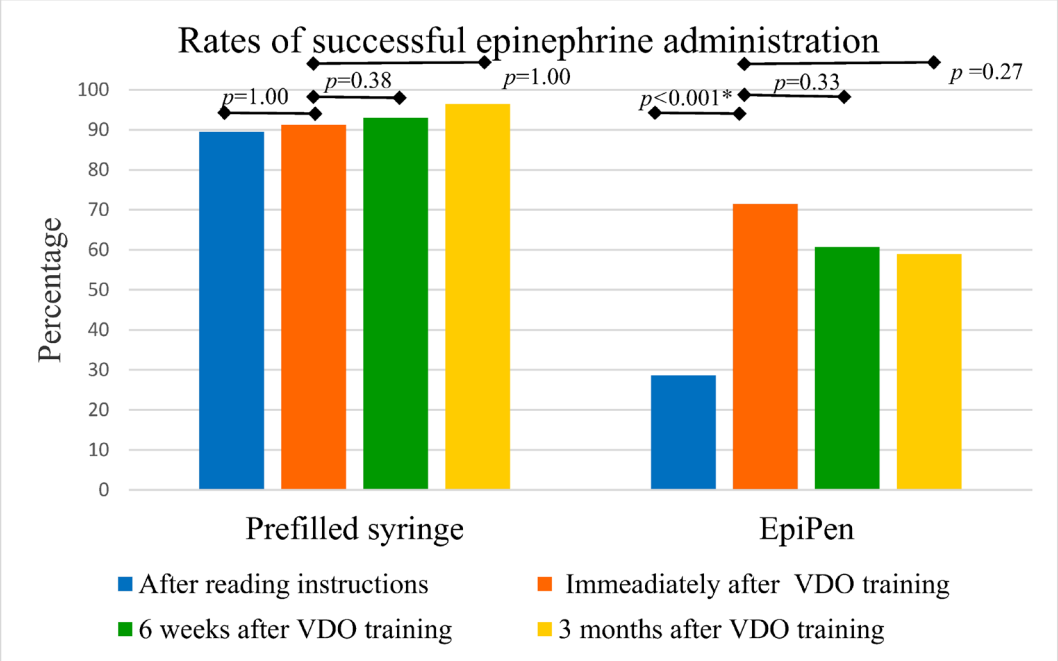


Figure 3. Rates of successful epinephrine administration in prefilled syringe group or EpiPen group after reading the instructions, and immediately, 6 weeks and 3 months after video training.

3-month period in the prefilled syringe group, however, there was no significant difference ($p > 0.05$) (**Figure 3**).

Number of successful administrations of prefilled syringe or EpiPen device

Participants in the prefilled syringe group were significantly more likely to demonstrate correct administration technique on all four assessment occasions compared to those in the EpiPen group (47, 82.5% in prefilled syringe group vs 9, 16.1% EpiPen group; $p < 0.001$).

Comparison of failed steps in epinephrine administration

There are 'four key steps' for each device that if performed incorrectly result in failure to deliver epinephrine. We compared the two devices for failed steps each time and for total assessments (**Figure 4**). The cause of failure to deliver epinephrine may be due one or more steps. The frequent errors with EpiPens were failure to remove the blue safety cap (17%), apply enough pressure to trigger the device (15.6%), hold the device for a few minutes (12.9%) and place orange end on thigh (10.3%). For prefilled syringes it was not placing needle on thigh (3.1%), pushing syringe tab completely (2.2%), removing safety cap (1.3%) and pushing the needle into the thigh (1.3%).

Comparision of the steps needed to succesfully administer epinephrine and subsequent failures to demonstrate correct technique showed that the EpiPen group failed significantly more in all four key steps (step 1-4) compared to the prefilled syringe group in total assessments ($p < 0.05$) (**Figure 4**).

Adverse event

In the EpiPen group, 3 participants, 1 participant and 2 participants choosing the wrong end of the device for placement would have injected the digit after reading instruction, immediately after, and 3 months after video training, respectively. This adverse event did not occur in the prefilled syringe group hroughout the study. However, there was no significant

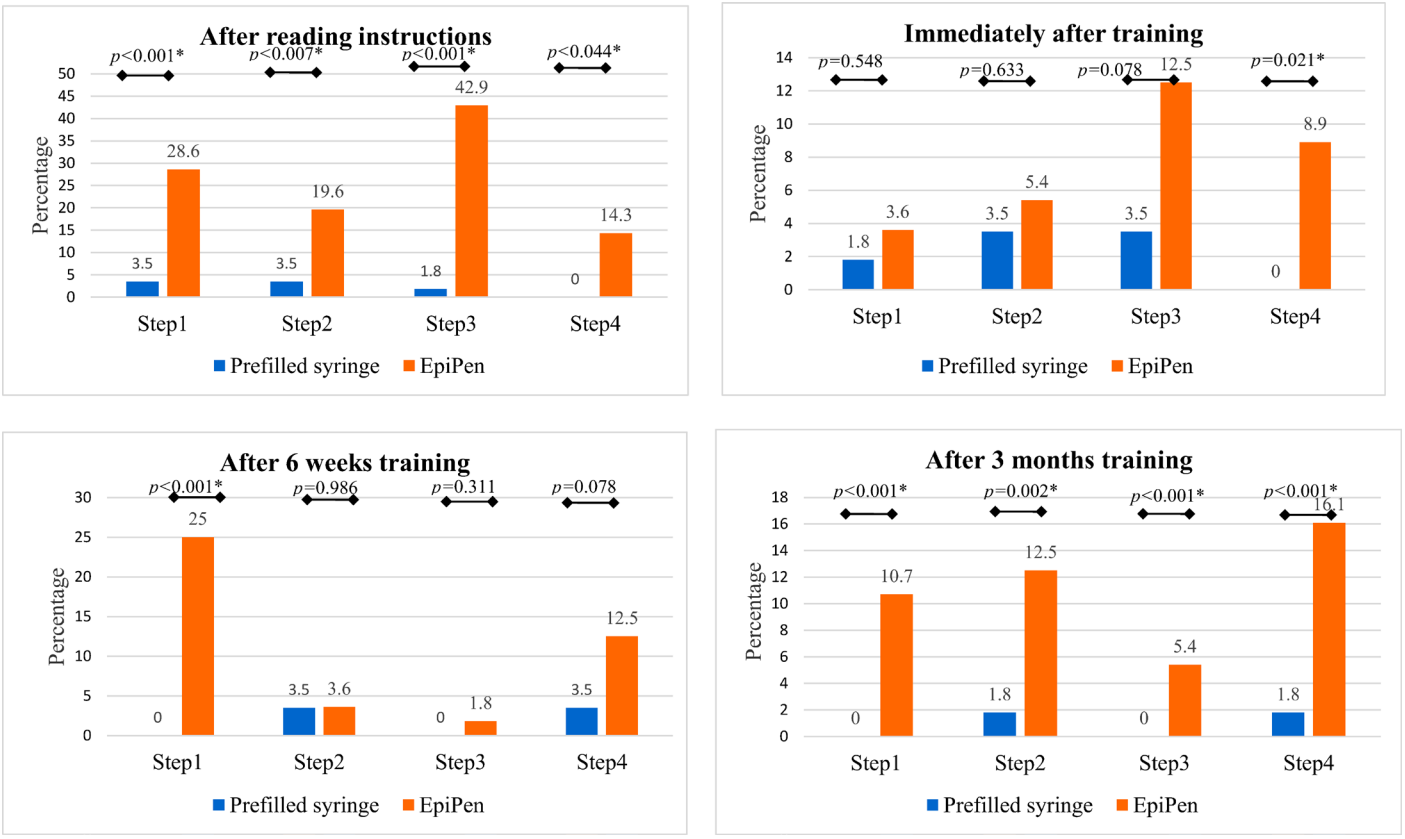


Figure 4. The failed steps of epinephrine device demonstration
Step 1: removal of safety cap.
Step 2: placement of correct end of the device against the thigh.
Step 3: push down to activate EpiPen, push down the needle on the thigh and needle up to the needle hub in prefilled syringe.
Step 4: holding device in place for 3 seconds in EpiPen, and pushing syringe tabs completely in prefilled syringe.

Table 3. The time required for successful epinephrine administration

	Prefilled syringe group	EpiPen group	*p*-value
After reading instructions	n* = 51	n* = 16	0.686
Median time (sec) to administer (IQR)	15.27 (11.24-25.39)	17.33 (10.82-24.65)	
After video training	n* = 52	n* = 40	0.506
Median time (sec) to administer (IQR)	11.92 (8.34-16.19)	10.92 (7.81-16.33)	
6 weeks after training	n* = 53	n* = 34	0.429
Median time (sec) to administer (IQR)	11.86 (9.70-15.45)	10.58 (8.57-14.27)	
3 months after training	n* = 55	n* = 33	0.207
Median time (sec) to administer (IQR)	12.43 (9.87-15.31)	10.58 (8.27-13.64)	

n*= the number of participants who successfully administered epinephrine in each time
IQR = interquartile range
sec = second

difference between groups with the frequency of accidental digital injection ($p > 0.05$).

The time required for successful epinephrine administration

The median time for successful administration was 11.86 sec (IQR 9.70-15.45) using prefilled syringe devices, and 10.58 sec (IQR 8.57-14.27) using EpiPens at 6 weeks after video training. There was no statistically significant difference. Additionally, the time required for successful epinephrine administration was not significantly statistically different between the two groups in the other 3 assessments. ($p > 0.05$, **Table 3**)

Discussion

To our knowledge, this is the first study comparing epinephrine autoinjectors, the redesigned EpiPen, and prefilled syringe in ease of use and the ability to recall and demonstrate correct use at 6 weeks and 3 months after training.

Surprisingly, 89.5% participants were able to correctly demonstrate the use of prefilled syringes without prior training when allowed to read the instructions while only 28.6% of participants in the EpiPen group were able to demonstrate correct use. These results can be generalized to the general population, when anaphylaxis occurs in everyday life, that prefilled syringes are easier to use without training. The reading instructions appear to be an important factor supporting

correct use, thus healthcare personnel should prepare the prefilled syringe, stored in a pencil case with written instructions, about when and how to use this device. Additionally, successful demonstration in the prefilled syringe groups was significantly 4 times, 14 times and 31 times more likely compared with the EpiPen group immediately after, 6 weeks and 3 months after video training. The findings may be generalized to caregivers that the prefilled syringe is significantly easier to use with high rates of success in epinephrine administration. Furthermore, there was a decline in the ability to correctly use Epipen (but not prefilled syringe) after a 6-week and 3-month period ($p > 0.05$, **Figure 3**).

Many studies have previously documented difficulties with the use of epinephrine autoinjectors by patients, caregivers and even medical practitioners.[9,15,22] These findings were similar to previous studies of the original EpiPen that reported high rates of failed EpiPen use. A study of the redesigned EpiPen reported that most participants, including parents and hospital staff, correctly demonstrated all steps in the use of the redesigned EpiPen before, after training and 3 months of use (89, 100 and 87%, respectively).[16] However, a survey of community pharmacists found that only 62.1% could accurately demonstrate to a 'patient' how to use the redesigned EpiPen.[23] Similarly, our result found that 71.4% of participants were able to successfully administer the redesigned EpiPen after immediate video training. The participants in the EpiPen group were able to demonstrate the increasingly correct use immediately after video training ($p < 0.001$). These findings found that 60% of the participants who initially did not use their EpiPen device correctly were then able to do so immediately after receiving standard video training. This suggests that the EpiPen is difficult to use when reading instructions and also that video training can greatly assist with correct use. This confirms that caregiver training has an important role in correctly using autoinjectors.[24,25] After being optimally trained, completely accurate and confident in using their trainer, only 60.7% of participants correctly demonstrated the four key steps in EpiPen use at 6 weeks compared with 93% for the prefilled syringe (adjusted OR = 14.01; 95%CI 3.62-54.22). A previous study showed that among mothers of food-allergic children, 42.5% were able to administer epinephrine using a redesigned EpiPen in a simulated anaphylaxis scenario at 6 weeks following training.[17] Our study occurs in the standard scenario where levels of stress are likely to be lower so successful administration is higher.

The prefilled syringe is easier to use than the EpiPen in 4 assessments. These findings may be explained by the fact that prefilled syringes were prepared from an ampule of epinephrine and a disposable plastic 1-mL syringe with a needle that is similar to the injection of other medications or vaccinations, so the participants are familiar with the device. Interestingly, two participants in the prefilled syringe group massaged the site after injection in spite of it not being included in the instructions. It was suggested that they are familiar with this device in daily life. Additionally, previous studies have found that the length of the epinephrine autoinjectors' needle is sometimes not long enough to reach muscular tissue in the thighs of obese individuals.[26] An epinephrine prefilled syringe was prepared from disposable plastic 1 mL syringes and suitable needle lengths can be chosen to adequately give epinephrine intramuscularly. Thigh circumference, body mass index (BMI) and body weight are useful predictors for assessing needle length.[27]

Four key step errors that would result in failure to administer epinephrine were also more common with EpiPens compared to prefilled syringes in all 4 assessments. Our study adapted the 4 steps needed for successful EpiPen administration. This included holding the device in place for epinephrine delivery for 3 seconds because a previous study on epinephrine absorption suggested that holding the device in place for 1 second might be just as effective,[28] thus EpiPen instructions suggest that the injection hold time is 3 seconds.[21] The most common four key step errors in the use of the EpiPen, both after reading instructions and immediately after video training, was the failure to push down to activate. Similarly, the most frequent errors made with the old EpiPen were not exerting enough pressure to activate and quickly punching and holding the autoinjector for less than 5 seconds.[9,22] Failure to remove the blue safety cap and failure to hold for 3 seconds were the most common key step errors at 6 weeks and 3 months, respectively. These results were similar to those reported by Umasunthar et.al, which showed that two common reasons for failure were 28.8% failed to remove all safety caps and 17.8% applied the autoinjector for less than 5 seconds using the redesigned EpiPen at 6 weeks.[17]

We compared step by step the causes of the failed step of epinephrine device demonstration. Failure to remove the blue safety cap (step 1) and failure to hold for 3 seconds (step 4) were significantly more common when using EpiPen compared to prefilled syringe in 3 assessments ($p < 0.05$, **Figure 4**). Participants took the EpiPen out of the skin immediately after the device had been triggered. These results were similar to a previous study that reported that only 47% using the current design of EpiPen could hold it in place.[29] The results suggested these are important points for epinephrine autoinjector device developers to focus on and emphasize to caregivers all steps, especially the two common step errors, in action plans.

Accidental digital injection using the EpiPen was 2.68% but this adverse event did not occur in the prefilled syringe. Unintentional injection has led to digital ischemia in the caregivers[30] and failure to receive epinephrine to the child in need of it. This finding supported Simons et al,[13] who reported in a systematic review of the hazards of unintentional injection of epinephrine autoinjectors that accidental injection is increasing.

The time required for successful epinephrine administration was not statistically significantly different between groups throughout the study. We demonstrated that video training on epinephrine injectors for caregivers shortened the time required for successful administration by 6 seconds in EpiPen and by 3 seconds in the prefilled syringe on average. The time to successfully administer in each device was similar in 2 assessments at 6 weeks and 3 months after video training (median time 12 seconds in prefilled syringe and 11 seconds in EpiPen)

The strengths of our study includes that it is a prospective randomized design investigating caregivers with food allergic children. All participants are representatives who may, in the

near future, have to actually use this device. Because food allergy is the most common cause of anaphylaxis in children.[3,5] A previous study found that participants, including hospital staff, were commonly able to administer epinephrine using an epinephrine trainer but the results cannot be generalized to people who will actually use this device.[16] Epinephrine autoinjector devices are restricted or unavailable in some countries. One recent study reported that the most common problem in the use of epinephrine was lack of availability.[31] Thus, our results suggested epinephrine prefilled syringe is a user-friendly and available device. Moreover, with epinephrine autoinjectors it is impossible to give an accurate dosage for infants and many children by using currently available autoinjectors with fixed epinephrine doses (0.15 or 0.3 mg). In contrast,prefilled syringes are prepared with an epinephrine dose based on mg/kg for body weight of patient but should be prepared by trained healthcare professionals for accurate epinephrine doses and prompt use.[32] However, the limitation of epinephrine prefilled syringes is the need to replace them every 3 months due to stability.[19,20]

Our study has some potential limitations. Firstly, this study was not performed in settings with actual intances of anaphylaxis. It is possible that more frequent errors would have occurred in the stressful environment of acute anaphylaxis. Secondly, this study was performed with demonstrations using the trainer and a manikin. The prefilled syringe with a needle was also a trainer so the participants could not demonstrate with their children because of ethical considerations. Needle phobia may also be a key barrier to the use of epinephrine prefilled syringe in real life situations. Thirdly, there was the absence of gold standard test to diagnose food allergy, double blind placebo controlled food challenge. (DBPCFC). Lastly, this study cannot blind the investigators, it may be caused the measurement bias, thus we observed with the same two investigators for reduced bias. If a disagreement between the two investigators occurred, video recordings were used to decide whether epinephrine would have been successfully delivered.

In conclusion, our study suggested that epinephrine prefilled syringe can be an appropriate alternative device for anaphylactic patients because of ease, ability to recall usage, and safety. Additionally, the low cost of prefilled syringes increases patients access to them.

Acknowledgements

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Conflict of interest

The authors declare no conflict of interest.

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ORIGINAL ARTICLE

Investigation of mast cell toll-like receptor 3 in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis and Systemic Mastocytosis using the novel application of autoMACS magnetic separation and flow cytometry

Cassandra Balinas,[1,2] Thao Nguyen,[1,2] Samantha Johnston,[1,2] Peter Smith,[2] Donald Staines,[1,2] Sonya Marshall-Gradisnik[1,2]

Abstract

Background: Viral infections and hypersensitivities are commonly reported by Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) patients. Mast Cells (MC) uniquely mediate type 1 hypersensitivities and resolve viral infections via toll-like receptor 3 (TLR3).

Objective: To characterise and compare mast cell progenitors (MCPs) in CFS/ME participants with a known MC disorder, Systemic mastocytosis (SM), and secondly, to investigate the role of MC TLR3 in CFS/ME participants following Polyinosinic:polycytidylic acid (Poly I:C) stimulation.

Methods: A total of 11 International Consensus Criteria defined CFS/ME participants (40.42 ± 10.31), 9 World Health Organisation defined systemic mastocytosis (SM) participants (47.00 ± 10.37) and 12 healthy controls (HC) (36.36 ± 9.88) were included. Following autoMACS magnetic separation, $CD117^{+}/Lin^{-}$MCPs were stimulated with Poly I:C for 24 hr. MCP purity (CD117 and Lin2), maturity (CD34 and FcεRI), interaction receptors and ligands (CD154 and HLA-DR), and SM-specific (CD2 and CD25) markers were measured using flow cytometry.

Results: There was a significant decrease in $HLA\text{-}DR^{+}/CD154^{-}$ expression between CFS/ME and SM groups pre and post Poly I:C stimulation. There were no significant differences in maturity MCPs, CD154, and CD2/CD25 expression between groups pre and post Poly I:C stimulation.

Conclusion: This pilot investigation provides a novel methodology to characterise MCPs in a rapid, inexpensive and less invasive fashion. We report a significant decrease in $HLA\text{-}DR^{+}/CD154^{-}$ expression between CFS/ME and SM participants, and an observed increase in $HLA\text{-}DR^{-}/CD154^{+}$ expression post Poly I:C stimulation in CFS/ME participants. Peripheral MCPs may be present in CFS/ME pathophysiology, however further investigation is required to determine their immunological role.

Key words: Chronic Fatigue Syndrome; Mast Cells; Myalgic Encephalomyelitis; Systemic Mastocytosis; Toll-like Receptor 3

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Introduction

Mast cells (MC) are multifunctional leukocytes of the immune system, proficient in responding to both allergen specific and nonspecific danger stimuli.[1] In the peripheral bloodstream, MCs circulate as $CD34^{+}/FcεRI^{-}$ uncommitted hematopoietic progenitors.[2,3] Mature MCs do not typically circulate in the peripheral blood. MCs migrate as immature progenitor cells to

vascularised tissue, such as the skin, mucosa, brain and airways, to differentiate and mature into functional connective (or serosal) tissue and mucosal MCs.[2] Stem cell factor (SCF), also known as CD117 (c-kit), is the primary ligand that mediates MC proliferation, development and survival. MCs are responsive to immune modulators within the MC microenvironment, such as growth factors, cytokines and chemokines that influence their functional, structural and biochemical phenotype.[4] MC phenotype is also determined by protease content and surface expression levels of Cluster of Differentiation (CD) markers such as c-kit, FcεRI, and CD34. CD34 is a primary immaturity marker of MCs. In contrast, differentiated, mature or activated MCs express FcεRI, HLA-DR and CD154, whereby the former is a primary maturation marker of MCs. HLA-DR is expressed on B and T lymphocytes, monocytes/macrophages and dendritic cells, whereas CD154 is expressed on activated T cells.[1]

MC activation and degranulation can occur following IgE cross-linkage to the FcεRI receptor during hypersensitivity responsesor via pattern recognition receptors following direct pathogen interactions, such as Nod-like receptors, C-type lectins, CD48, and toll-like receptors (TLR). Additional MC activation receptors include: other immunoglobulin receptor (FCγR), cytokines, chemokines, neuropeptides, interaction molecules with surrounding immune cells (MHC-II, CD40L), complement receptors and G-protein-coupled receptors.[1]

Traditionally, *in vitro* culture of mast cell progenitors (MCP) have been characterised from the bone marrow and tissue in pathological diseases using predominant methods of microscopic analysis,[5] histochemical staining[6] and fluorescence imaging.[7] However, this method continues to be a challenge in providing adequate numbers for assessing MC maturation and function as isolation of live tissue-resident MCs do not readily proliferate and have a limited survival period following isolation. Alternatively, several scientific groups have developed protocols for *in vitro* differentiation and culture of human MCs from different progenitors to establish a method that could easily provide mature, abundant and functional MCs. Similarly to tissue-resident MCs, MCPs can be strongly influenced by isolation techniques[8] and require a significantly long development and maturation period *in vitro*. $CD34^+$ MCPs have been the predominant precursor cell for MC differentiation *in vitro* from the bone marrow, peripheral blood and cord blood. However, this precursor cell demonstrates phenotype variation dependent on *in vitro* stimulants such as IL-3, IL-6, IL-9, and SCF.[9]

Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) is a debilitating disorder hallmarked by unexplained fatigue that is associated with immune, neurological (including autonomic), musculoskeletal, cardiovascular and gastrointestinal systems.[10] Although the underlying aetiology of CFS/ME is not clearly defined, immunological dysfunction has been consistently implicated in this condition. A significant reduction in natural killer (NK) cell cytotoxicity is a consistent feature in CFS/ME patients compared with healthy controls, as well as atypical pro- and anti-inflammatory cytokines, nitric oxide production, and hypersensitivity responses.[11] A high prevalence of viruses has also been commonly reported by CFS/ME patients prior to the onset of CFS/ME symptoms. Although no universal virus or pathogen has been identified in CFS/ME patients, a number of viruses have been consistently reported by CFS/ME patients such as Herpes human viruses (e.g. Epstein Barr virus), adenoviruses, measles, rubella, influenza, cytomegaloviruses, *Coxiella burnetti*, and Ross River virus. A similar feature exhibited by these viruses is the activation of TLR3.[12]

TLRs are type I membrane receptors that induce antimicrobial immune responses by recognising pathogen-associated molecular patterns (PAMP). Currently, eleven human (TLR1-11) TLRs have been identified and are subclassed on subcellular localisation and selective PAMP recognition. Group one TLRs (1, 2, and 4-6) are present at the plasma membrane, whereas group two (TLR3, and 7-9) localize within intracellular compartments, such as endosomes. All TLRs utilise the universal MyD88-dependent pathway adaptor to initiate TLR signalling, with the exception of TLR3.[13] Human TLR3 is activated by double stranded ribonucleic acid (dsRNA). The synthetic analogue, Polyinosinic:polycytidylic acid (Poly I:C), has been predominantly used by researchers to mimic the effects of viral dsRNA.[14,15] Although MC reactivity against bacteria, primarily through TLR2 and TLR4, has been characterised more than viruses; an anti-viral mechanism has recently emerged via TLR3.

MC TLR3 also responds to dsRNA and Poly I:C, which activates the nuclear factor kappa beta pathway following the MyD88 independent TRIF pathway.[13] Subsequently, various pro-inflammatory cytokines (notably type I and type III interferons) and chemokines are released which can collectively enhance the recruitment of multiple inflammatory cells including eosinophils (eotaxin), NK cells (IL-8), and neutrophils (IL-2 and TNF-α).[1] Interestingly, type I hypersensitivity responses have been reported during viral infections due to a possible synergistic signal and cross-linkage between MC TLR3 and the FcεRI receptor during prolonged viral infections.[16] Given MCs have not been investigated in CFS/ME and are a key innate immune cell involved in both inflammation and hypersensitivities, TLR3 on MCs has been proposed to possibly contribute to the consistent viral reports and inflammation exhibited by CFS/ME patients. To better understand the role of MCs in CFS/ME, it is advantageous to compare this disorder with a known MC disorder, such as Mastocytosis.

Mastocytosis is a heterogeneous group of disorders characterized by a myeloproliferative neoplasm of MCs, with both cutaneous and systemic manifestations.[17] Systemic mastocytosis (SM) is characterised by significant systemic involvement of MCs in one or more extracutaneous organs, a somatic KIT gene mutation (D816V) of the SCF receptor (c-kit), organomegaly, abnormal serum tryptase levels, and atypical CD2 and CD25 expression.[17] SM patients often experience sudden attacks, lasting approximately 15-30 minutes. These attacks are characterised by various clinical manifestations ranging from constitutional signs, mediator-related symptoms, muscular skeletal disease-related, and dermatological symptoms, some of which overlap with CFS/ME including: fatigue, flushing, headache, syncope, abdominal pain, bone pain, arthralgia, and myalgia. Dermatological symptoms such as pruritus, blistering, and urticaria pigmentosa are not commonly exhibited by CFS/ME patients, however are hallmark symptoms of SM.[18]

Currently, the bone marrow is recognised as the most useful biopsy site as it is the primary extracutaneous tissue infiltrated

by MCs in mastocytosis patients. Examination of the bone marrow both reveals diagnostic infiltrates and allows evaluation of the hematopoietic marrow, which provides important prognostic information. Immunohistochemical staining of bone marrow biopsies with antitryptase is currently the method of choice to characterise and visualize MCs in paraffin-embedded decalcified specimens.[17,18] However, these methodologies are poorly reproducible, expensive, extremely invasive and biologically unrepresentative *in vivo*.[9] As MC leakage from the tissues into the peripheral blood has been found in SM patients,[19] a number of studies have attempted to characterise MCPs from alternative routes, such as the peripheral blood, using flow cytometric methods. Aberrant co-expression of CD2 and/or CD25 by flow cytometry and immunohistochemistry has been found on neoplastic $CD117^+$ MCs from bone marrow aspirates, which has further refined the diagnostic options for mastocytosis patients.[20] Currently, aberrant expression of CD2 and/or CD25 is acknowledged as one minor inclusion criteria by the WHO case definition of Mastocytosis.[18,21]

As active MCs are not typically found in the peripheral blood, the primary aim of this project was to identify peripheral MCPs in CFS/ME participants and compare these MCPs with an established MC disorder, such as SM (positive control group), in addition to a HC group. The use of two control groups enabled CFS/ME to be compared with a disorder hallmarked with high MC activity, as well as a normal MC activity group. The rationale for this project was to develop a less invasive and less expensive procedure to characterise and analyse MC activity in CFS/ME and other MC activation disorders. A secondary aim was to analyse the immunological role and contribution of TLR3 on MCs via Poly I:C stimulation to determine the extent of viruses in the pathomechanism of CFS/ME.

Materials and methods

Study Participants

Participants were sourced from the National Centre for Neuroimmunology and Emerging Diseases (NCNED) research database for CFS/ME. Participants aged between 18 and 65 years were recruited from community support networks in the South East Queensland and Northern New South Wales region of Australia. All participants completed a screening questionnaire reporting their sociodemographic details, medical history, and symptoms. All participants provided written consent prior to participation and completed a self-reported questionnaire on their current symptoms and history of illness. CFS/ME participants were aged and sex-matched with SM and HC groups. CFS/ME participants were defined in accordance with the Fukuda and International Consensus Criteria (ICC) symptom requirements.[10,22] The HC group reported no chronic illness or symptoms of CFS/ME and SM. SM participants were defined by the World Health Organisation (WHO) case definition of SM[17,18] and diagnosed by a clinician. Participants were excluded if they were pregnant or breastfeeding, or reported a previous history of smoking, alcohol abuse or chronic illness (for example, autoimmune diseases, cardiac diseases and primary psychological disorders).

Participants donated 85 ml of whole blood which was collected in ethylendiaminetetraacetic acid tubes between 8:00 am and 10:30 am. Routine pathology screening was further performed to exclude participants who demonstrated parameters outside the normal ranges. All participants provided written informed consent and the study was approved by the Griffith University Human Research Ethics Committee (HREC/15/QGC/63).

Peripheral blood mononuclear cells and $CD117^+/Lin^-$ Magnetic Bead Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by centrifugation over a density gradient medium (Ficoll-Paque Premium; GE Healthcare, Uppsala, Sweden) to separate granulocytes (such as neutrophils, basophils and eosinophils). PBMCs were stained with trypan blue stain (Invitrogen, Carlsbad, CA) to determine total cell count and cell viability and adjusted to a final concentration of 1×10^8 cells in 20 ml. CD117 Microbead and Lineage Cell Depletion kits were used to isolate PBMCs to $CD117^+/Lin^-$ cells by magnetic bead separation on the autoMACS® Pro Magnetic Separator as described by the manufacturers' instructions. The CD117 Microbead kit was used to separate $CD117^+$ cells, a primary MC marker, and the Lineage Cell Depletion Kit labelled Lin^- cells by negative selection with a cocktail of biotin-conjugated antibodies against lineage-specific antigens (CD2, CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD123, and CD235a), followed by magnetic labelling of Anti-Biotin microbeads. Following magnetic bead separation, $CD117^+/Lin^-$ cells were divided into two 5 ml polystyrene round-bottom FACS tubes where one tube was stimulated with 2 ul/1000 ul of Poly I:C. $CD117^+/Lin^-$ cells were incubated for 24 hr at 38°C. Post the 24 hr incubation, stimulated and unstimulated $CD117^+/Lin^-$ cells were pelleted down and resuspended in 200 ul of autoMACS running buffer (bovine serum albumin).

$CD117^+/Lin^-$ cells were labelled with CD117 for 15 minutes, followed by a number of fluorochrome-conjugated antibodies correspondent to MCP: purity (CD117 and Lin2), maturity (FcεRI and CD34), interaction receptors and ligands (CD154 and HLA-DR) and SM-specific markers (CD2 and CD25) for 25 minutes in the dark. A Lin2 monoclonal antibody cocktail (anti-CD3, anti-CD14, anti-CD19, anti-CD20 and anti-CD56) was used as a negative MCP marker to label and exclude additional immune cells (such as, T cells, B cells, NK cells, monocytes, eosinophils and neutrophils). Cells labelled with the myeloid receptor, anti-CD117 were gated as $CD117^+$ committed MCPs. $CD117^+/Lin^-$ cells were further labelled with anti-CD34 and anti-FcεRI to distinguish four different MC maturity MCPs, including $CD34^+/FcεRI^-$ (MC-monocyte committed MCP), $CD34^+/FcεRI^+$ (late-committed MCP), and $CD34^-/FcεRI^+$ (mature MCP). Labelled cells were resuspended with stain buffer (BD Bioscience, San Jose, CA) prior to flow cytometric analysis as previously described.[23]

Flow cytometry

MCPs were determined using LSR Fortessa™ X-20 flow cytometry as previously described.[23] All samples were collected at 10,000 events and all antibodies were purchased from BD Bioscience, unless otherwise stated. Four separate panels were designed to investigate MCP: purity, maturity, interaction receptors and ligands, and atypical SM characteristics. MC purity was measured by a Lin2 monoclonal antibody cocktail

and anti-CD117, which were used to gate for CD117$^+$/Lin MCPs. There were no significant differences in MCP purity between groups. The mean MCP purity of all groups was 84.72 ± 7.93. CD117$^+$/Lin$^-$ MCPs with purity more than 80% were gated for the following maturity MCPs: mature MCP (FcεRI$^+$/CD34$^-$), late-committed MCP (FcεRI$^+$/CD34$^+$), MC/monocyte committed MCP(FcεRI$^-$/CD34$^+$) and non-MC (FcεRI$^-$/CD34$^-$). Mature MCPs were further characterised by T and B lymphocyte interaction markers, HLA-DR (MHC-II) and CD154 (CD40L). CD2 and CD25 marker expression was used on the total MC population to determine possible atypical SM characteristics.

Statistical analysis

Flow cytometry data were exported directly from BD FACS LSR Fortessa X-20 and the four panels were separated into pre and post Poly I:C stimulation data sets. Data were compared between the three test groups (CFS/ME, SM and HC) with statistical analyses performed based on the distribution of each variable. Data were analysed using IBM SPSS Version 22. Demographics of participants was normally distributed and the one-way ANOVA test was used to test for significance at $p < 0.05$. Shapiro-Wilk normality tests were conducted to determine the distribution of data, in addition to skewness and kurtosis tests to determine data normality. The Kruskal-Wallis H test was performed to determine the statistical significance of MCP: purity, maturity, interaction receptors and ligands, and atypical SM characteristics. Statistical significance was reported at $p < 0.05$ and a Bonferroni correction was applied to adjust for multiple test parameters.

Results

Participant Demographics

From a total of 32 participants, 11 participants were defined by the Fukuda and ICC criteria for CFS/ME, 9 participants met the WHO case definition for SM, and 12 participants met the criteria for HCs. There were no significant differences in age and gender between groups (**Table 1**). Similarly, there were no significant differences between groups for routine pathology tests (**Table 2**).

Table 1. Demographic results between CFS/ME, SM and HC groups

Parameters	HC	CFS/ME	SM	p value
Age (years)	36.36 ± 9.88	40.42 ± 10.31	47.00 ± 10.37	0.083
Gender				
Male	36.4%	41.7%	77.78%	
Female	63.6%	58.3%	22.22%	

Table 2. Routine pathology blood results between CFS/ME, SM and HC groups

Parameters	HC	CFS/ME	SM	p value
White Cell count ($\times 10^9$/L)	$5.77 \times 10^9 \pm 4.43 \times 10^8$	$4.70 \times 10^9 \pm 7.34 \times 10^8$	$6.05 \times 10^9 \pm 5.99 \times 10^8$	0.515
Neutrophils ($\times 10^9$/L)	$3.69 \times 10^{10} \pm 3.49 \times 10^9$	$2.21 \times 10^{10} \pm 6.14 \times 10^9$	$3.44 \times 10^{10} \pm 3.79 \times 10^9$	0.214
Lymphocytes ($\times 10^9$/L)	$1.82 \times 10^{10} \pm 1.63 \times 10^9$	$1.77 \times 10^{10} \pm 1.19 \times 10^9$	$2.22 \times 10^{10} \pm 1.98 \times 10^9$	0.221
Monocytes ($\times 10^9$/L)	$3.34 \times 10^8 \pm 2.48 \times 10^8$	$2.98 \times 10^8 \pm 3.71 \times 10^7$	$3.05 \times 10^8 \pm 2.00 \times 10^7$	0.548
Eosinophils ($\times 10^9$/L)	$1.47 \times 10^8 \pm 2.62 \times 10^7$	$2.11 \times 10^8 \pm 2.62 \times 10^7$	$1.67 \times 10^8 \pm 3.93 \times 10^7$	0.189
Basophils ($\times 10^9$/L)	$4.00 \times 10^7 \pm 7.07 \times 10^6$	$2.75 \times 10^7 \pm 4.12 \times 10^6$	$2.27 \times 10^7 \pm 2.37 \times 10^6$	0.151
Platelets ($\times 10^9$/L)	$2.51 \times 10^{11} \pm 1.16 \times 10^{10}$	$2.68 \times 10^{11} \pm 2.49 \times 10^{10}$	$2.51 \times 10^{11} \pm 1.10 \times 10^{10}$	0.635
Haemoglobin ($\times 10^9$/L)	144.25 ± 4.02	132.25 ± 4.62	143 ± 3.66	0.124
Haematocrit ($\times 10^9$/L)	0.43 ± 0.01	0.40 ± 0.01	0.43 ± 0.007	0.149
Red Cell count ($\times 10^{12}$/L)	$4.89 \times 10^{12} \pm 1.25 \times 10^{11}$	$4.44 \times 10^{12} \pm 1.46 \times 10^{11}$	$4.87 \times 10^{12} \pm 1.05 \times 10^{11}$	0.057
MCV ($\times 10^9$/L)	87.83 ± 1.06	89.88 ± 0.95	87.64 ± 0.93	0.223

Identification of human peripheral maturity mast cell progenitors

There were no significant differences between groups for maturity MCPs pre and post Poly I:C stimulation (**Figure 1**). However, there was an observed increase in MC/monocyte committed (CD34$^+$/FcεRI$^-$) and late-committed MCPs (CD34$^+$/FcεRI$^+$) pre and post Poly I:C stimulation for CFS/ME participants compared with SM and HCs. Conversely, mature (CD34$^-$/FcεRI$^+$) MCPs were the predominant MCP in SM participants pre and post Poly I:C stimulation. The HC group demonstrated no distinct MCP pre and post Poly I:C stimulation.

Comparison of CD154 and HLA-DR expression between CFS/ME, SM and HC groups

Surface markers CD154 and HLA-DR were compared between groups pre and post Poly I:C stimulation. There was a significant decrease in HLA-DR$^+$/CD154$^-$ expression between HC and SM participants, as well as CFS/ME and SM participants pre Poly I:C stimulation. This significant decrease in HLA-DR$^+$/CD154$^-$ expression remained post Poly I:C stimulation between CFS/ME and SM participants (**Figure 2**). Although there were no significant differences between groups for mature MCPs (CD34$^-$/FcεRI$^+$), higher levels of HLA-DR/CD154$^+$ were expressed on these mature MCPs (CD34$^-$/FcεRI$^+$) in CFS/ME participants compared to HCs and SM participants post Poly I:C stimulation.

CD2 and CD25 expression between CFS/ME, SM and HC groups

Across the four SM MCPs, there was no significant difference between groups for CD2 and CD25 expression pre and post Poly I:C stimulation (**Figure 3**).

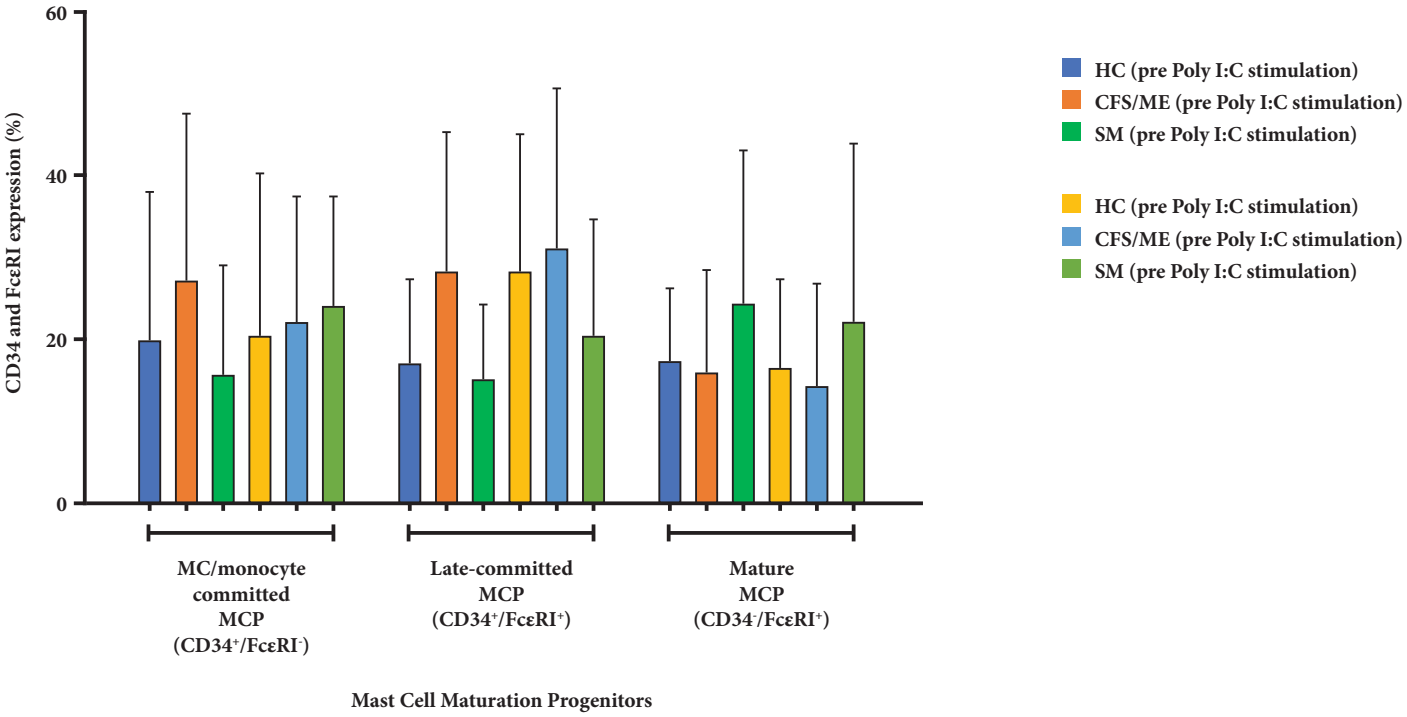

Figure 1. Bar graph plots for CD34 and FcεRI expression are shown as percentage of parent CD117$^+$/Lin$^-$ cells in CFS/ME, SM and HC groups pre and post 24hr Poly I:C stimulation.

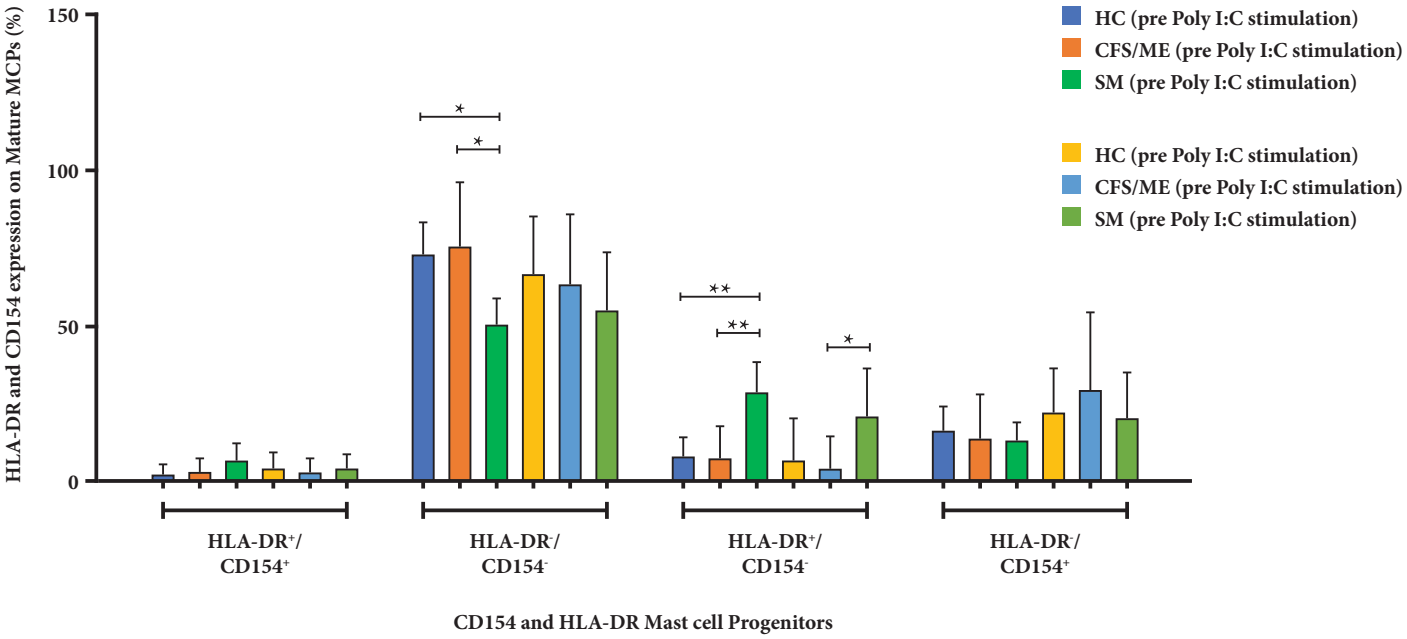

Figure 2. Bar graph plots for CD154 and HLA-DR expression are shown as percentage of parent CD117$^+$/Lin$^-$ mature MCPs (CD34$^-$/FcεRI$^+$) in CFS/ME, SM and HC participants pre and post 24hr Poly I:C stimulation.

* refers to significant difference where $p < 0.05$, using Bonferroni post-hoc test.
** refers to significant difference where $p < 0.001$, using Bonferroni post-hoc test.

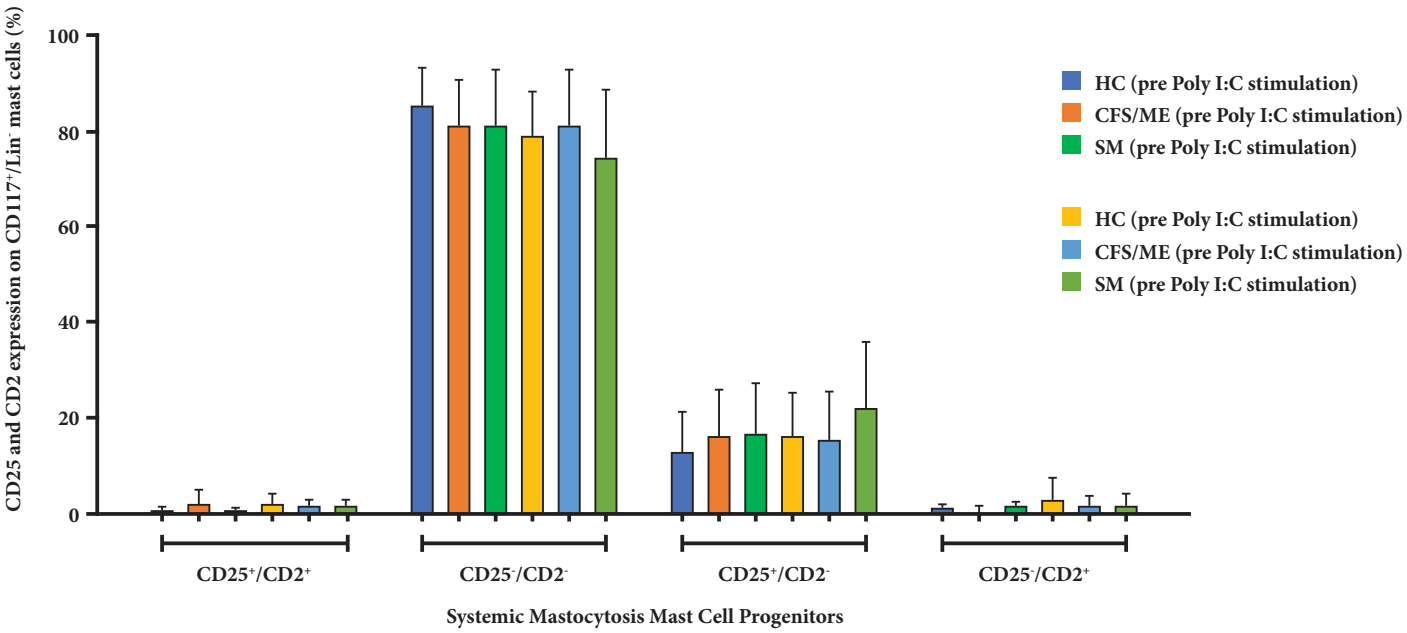

Figure 3. Bar graph plots for CD2 and CD25 expression are shown as percentage of parent CD117$^+$/Lin$^-$ cells in CFS/ME, SM and HC groups pre and post 24hr Poly I:C stimulation

Discussion

This present study supports our pilot study[23] that identified MCPs in PBMCs from CFS/ME and HC participants. Additionally, this investigation is the first to characterise peripheral MCPs with a known MC disorder, SM.[24] Importantly, we report novel findings of MCPs following pre and post Poly I:C stimulation of TLR3 in CFS/ME participants. A significant decrease in HLA-DR$^+$/CD154$^-$ expression was reported in CFS/ME pre ($p < 0.001$) and post ($p < 0.05$) Poly I:C stimulation compared with SM participants. This pilot investigation identified for the first time peripheral MCPs through flow cytometric methods and possible immunological dysfunction in disease-compromised patients (CFS/ME and SM). This novel method may have significant implications for analysing MCPs compared with traditional methods, such as bone marrow biopsies, that are poorly reproducible, expensive and extremely invasive.[20]

We confirm our previous findings that identified MC/monocyte committed (CD34$^+$/FcεRI$^-$) and late committed (CD34$^+$/FcεRI$^+$) MCPs in CFS/ME participants compared with HCs as demonstrated in **Figure 1**.[23] This observed increase in MCPs pre and post Poly I:C stimulation in CFS/ME participants may suggest increased mobilisation of MCPs following a latent viral infection. MCPs predominantly circulate in the bloodstream and lymphatics and traverse from the bone marrow to peripheral tissues during heightened physiological and inflammatory settings, such as asthma and hypersensitivities.[11] MCs recognise viruses by detecting dsRNA or Poly I:C via TLR3. Following PAMP-TLR3 stimulation, a collection of inflammatory cytokines (TNF-α, IL-6, IL-8, IFN-β, IFN-γ, and IL-1α) and chemokines (CCL4, CCL5, CXCL8 and CXCL10) are released following NF-κβ and IFN-regulatory pathway stimulation.[1] An excessive release of these pro- and anti-inflammatory mediators can disrupt the inflammatory homeostasis and induce systemic inflammation following prolonged MC TLR3 stimulation (viral latency). An imbalance in the inflammatory pathways may influence the tissue microenvironment, and consequently affect the proliferation, differentiation and recruitment of MCPs to these inflammatory sites. This may explain the altered pro-inflammatory cytokine profiles exhibited in CFS/ME patients.[11,25,26]

MCs are primarily involved in innate immunity. However, MCs also mediate adaptive immune responses with other immune cells, such as B and T lymphocytes, through their extensive collection of cell surface receptors and ligands. Prior to Poly I:C stimulation, HLA-DR$^+$/CD154$^-$ expression was significantly reduced in HC ($p < 0.001$) and CFS/ME ($p < 0.001$) participants compared with SM participants. This significant decrease remained in CFS/ME participants ($p < 0.05$) compared with SM participants post Poly I:C stimulation as demonstrated in **Figure 2**. Although it has been reported that MCs express HLA-DR following antigen-dependent interactions with effector CD4$^+$T cells,[27] the current findings do not demonstrate this interaction. A possible rationale is that high HLA-DR expression is a novel occurrence, transpiring between MCs and T cells only after tissue infiltration and localised tissue inflammation,[28] such as in SM. As shown in **Figure 1**, the CFS/ME group acquired the lowest percentage of mature MCPs (CD34$^-$/FcεRI$^+$) compared with SM and HC participants. This observed decrease in mature MCPs (CD34$^-$/FcεRI$^+$) and significant decrease in HLA-DR$^+$/CD154$^-$ expression may possibly suggest that MCs in CFS/ME do not acquire a comparable MC abundance to cause tissue infiltration as in SM.

Interestingly, although CFS/ME participants acquired the lowest percentage of mature MCPs (CD34$^-$/FcεRI$^+$) (refer to **Figure 1**), HLA-DR$^-$/CD154$^+$ expression on these mature MCPs (CD34$^-$/FcεRI$^+$) was the highest in CFS/ME than HCs and SM participants post Poly I:C stimulation as demonstrated in **Figure 2**. CD154 is a critical marker for immune and inflammatory response. It is primarily expressed on B lymphocytes and mediates B cell co-stimulation.[29] Our data suggest a possible association between MC TLR3 activation and B cell co-stimulation via FcεRI. Binding of CD154 and B cells may cause an

increase in B cell proliferation, subsequently promoting the expression of the MC activating immunoglobulin, IgE.[30] Elevations in circulating IgE provides increased stimuli to activate these mature MCPs ($CD34^-/Fc\varepsilon RI^+$). This potentially may explain the type I hypersensitivity responses reported by CFS/ME patients during viral infections.[31] These MC interactions with B cells may possibly provide a plausible explanation for the elevated B cell populations documented in CFS/ME patients.[32,33]

SM is characterised by over-proliferative MC populations, causing tissue infiltration and subsequent MC release into the peripheral bloodstream.[17] Aberrant expression of CD2 and/or CD25 expression by bone marrow, peripheral blood or other extracutaneous tissue MCs is currently a minor WHO diagnostic criterion for SM.[34] Given no significant differences were observed between groups pre and post Poly I:C stimulation across the four SM MCPs ($CD2^+/CD25^+$, $CD2^-/CD25^-$, $CD2^+/CD25^-$ and $CD2^-/CD25^+$) (refer to **Figure 3**), these findings suggest that MCs in CFS/ME patients may not acquire a comparable abundance as in SM. Given previous studies have characterised MCs from bone marrow tissue aspirates and other extracutaneous organs, the source of these MCPs may rationalise this finding as MCs can phenotypically change with different activation, anatomical sites and cultured settings and only constitute approximately 0.053% of PBMCs.[35] Thus, further investigations in cell culturing and immunofluorescence staining of these MCPs may provide additional support to further evaluate the progenitor state of these MCPs.

Conclusion

This pilot investigation identified for the first time, peripheral MCPs in CFS/ME, SM and HC participants following MC TLR3 stimulation. The dual application of autoMACS magnetic separation and flow cytometry with these sample groups demonstrates the potential application to analyse MCPs through an alternative method that is inexpensive, less invasive and hence ethically preferred compared with traditional methods to potentially diagnose other MC activation disorders.

The results of this study present a novel field for immunological MC investigation in CFS/ME. The observed increase in MC/monocyte committed ($CD34^+/Fc\varepsilon RI^-$) and late-committed ($CD34^+/Fc\varepsilon RI^+$) MCPs in CFS/ME pre and post Poly I:C stimulation represents a finding not previously noted in clinical situations other than SM. This increase in MCP mobilization suggests a possible dysregulation of the inflammatory pathways and alteration of the microenvironment following excessive MC TLR3 activation on tissue-resident MCs.

The significant decrease in $HLA\text{-}DR^+/CD154^-$ expression suggests that CFS/ME participants may not acquire a comparable MC abundance to cause significant tissue infiltration as in SM. Conversely, the observed increase in $HLA\text{-}DR^-/CD154^+$ expression on mature MCPs ($CD34^-/Fc\varepsilon RI^+$) in CFS/ME participants post Poly I:C stimulation suggests possible associations between MCs and B lymphocytes, which may elucidate the hypersensitivities reported by CFS/ME patients during viral infections. Further investigation is required to determine the immunological contribution of MCs in the pathophysiology of CFS/ME.

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Author contributions

The authors in this article were involved in the design, drafting, and development of this manuscript. All authors have reviewed and approved the final version of and declare no conflict of interest in the research presented.

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ORIGINAL ARTICLE

Hamster IFN-γ⁺CD4⁺ and IL-4⁺CD4⁺ T cell responses against leptospires are significantly higher than those of mice

Yaowarin Nakornpakdee,[1,3] Rasana W Sermswan,[2,3] Santi Maneewatchararangsri,[4] Surasakdi Wongratanacheewin[1,3]

Abstract

Background: Leptospirosis is a bacterial disease caused by the *Leptospira interrogans*. The hamster is considered a susceptible host while the mouse is resistant. The knowledge of hamster T cell immunity is limited compared to the mouse. The reason why the hamster and the mouse give different responses to leptospires remains unclear.

Objective: To determine the differential responses of CD4⁺ T cells between hamsters and mice using *Leptospira interrogans* as an infectious model.

Methods: The CD4⁺ T-cell reactivity and their intracellular cytokine responses after infection with live *L.interrogans* serovar Autumnalis or leptospiral antigens, or injection with recombinant LipL32 protein (rLipL32) were elucidated. For secondary immune responses, mononuclear cells were re-stimulated with leptospiral crude antigens (LAg) or rLipL32. Intracellular cytokines and CD4⁺ T cells were determined using flow cytometry.

Results: There were no significant differences between the percentages of hamster and mouse CD4⁺ and CD25⁺CD4⁺ T cell responses to live bacteria. Mouse CD4⁺ (24.50 ± 1.98%) and CD25⁺CD4⁺ T cells (3.83 ± 0.88) responded significantly higher than those of hamster (15.07 ± 2.82% and 2.00 ± 0.37%) when infected and re-stimulated with LAg. The numbers of IFN-γ and IL-4 producing cells in hamsters at 1.76 ± 0.10% and 0.82 ± 0.25% for IFN-γ⁺CD4⁺ and IL-4⁺CD4⁺ T cells were significantly higher than those in resistant mice at 0.10 ± 0.02% and 0.23 ± 0.03% for IFN-γ⁺CD4⁺ and IL-4⁺CD4⁺ T cells.

Conclusion: Hamsters responded significantly higher in secondary stimulation especially in the levels of the IFN-γ⁺ and IL-4⁺CD4⁺ T cells. The mechanisms of this dissimilarity remain to be elucidated.

Key words: Leptospirosis, LipL32, *L.interrogans* serovar Autumnalis, T cell response, CD4

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Introduction

Leptospirosis is a worldwide zoonotic disease caused by the pathogenic *Leptospira* genus and there is variable host susceptibility toward pathogenic *Leptospira* strains. The commonly used animal models for leptospirosis studies are hamsters and guinea pigs while mice and rats are generally resistant to leptospirosis and are often found to be reservoirs of the bacteria.[1] Although the clinical aspects and progression of the disease are well understood, knowledge of host factors which determine the outcome of infection is limited.[2] As the hamsters and mice give different responses to leptospires especially in pathogenesis and protection, the variances in these models were therefore studied. The animal models with different susceptibilities to leptospires may help discovering the crucial factors for survival of the infection. The Syrian hamster is highly susceptible to many organisms and has been used as an excellent experimental model for several infectious diseases caused by microorganisms, such as *Treponema pallidum*,[3] *Leishmania* spp.,[4] *Opisthorchis viverrini*,[5] and *Leptospira interrogans*.[6] It is still unclear, however, why the hamster is extremely susceptible to such infections and gives different outcomes to leptospirosis compared to the

well characterized mouse model. In the mouse model, the interaction between host and pathogens can induce chemokine expression and different levels of host susceptibility can give differential chemokine profiles. BALB/c mice are considered as the most resistant mouse model against leptospirosis and gave the highest level of chemokine expression compared to C3H/HeJ and C3H/HePas which are sensitive and have intermediate susceptibility.[7] Due to the highest resistance level in BALB/c mouse, it was accordingly selected as the model to compare with hamsters which are susceptible to this pathogen. Furthermore, previous studies reported that antigen-presenting cells (APCs) present the processed leptospiral antigens to $CD4^+$ T cells through MHC Class II molecules, leading to their activation and production of cytokines such as IL-4 and IFN-γ to support the role of B cells in protection against leptospires.[8,9] Therefore, the responses of $CD4^+$ T-cell subsets and their intracellular cytokines, IFN-γ and IL-4, were studied between susceptible hamsters and resistant BALB/c mice infected with virulent *L.interrogans* serovar Autumnalis or the recombinant LipL32 protein (rLipL32) and were compared by flow cytometry in this study. In addition, the differences in responses of mice and hamsters to *L.interrogans* might also reflect the dissimilarities between hamster and mouse immunities.

Methods

Animals and ethics

Outbred 4 week old female Syrian golden hamsters obtained from the Animal Laboratory Breeding Unit, Faculty of Medicine, Khon Kaen University and four-week-old inbred female BALB/c mice purchased from Nomura Siam International Co. Ltd. were used in this study. All animals were maintained in the animal care unit at Faculty of Medicine, Khon Kaen University. All experiments were approved by the Animal Ethics Committee of Khon Kaen University (No. AEKKU 6/2558 and AEKKU-NELAC 3/2558, No. 0514.1.75/1) and performed in accordance with institutional guidelines.

Antigen Preparation

1. Leptospiral crude antigens (LAg)

LAg was prepared as described.[10] Briefly, *L.interrogans* serovar Autumnalis UI13372 was cultured in *Leptospira* medium Ellinghausen–McCullough–Johnson–Harris (EMJH) (Becton, Dickinson and Company, Maryland, USA) at 30 °C for 7-10 days to yield a cell density of 10^8 cells/ml. Bacteria were harvested by centrifugation at 10,000 ×*g* for 10 minutes and killed with 0.5 mg/l sodium azide for 30 minutes. The bacteria were washed twice in 0.01 M phosphate-buffered saline (PBS), pH 7.4, resuspended in PBS, and frozen at -20 °C for 7 days. They were centrifuged at 10,000 ×*g* for 30 minutes at 4 °C after being thawed. The pellets were washed two times with PBS, resuspended in PBS, and sonicated on ice at 20 kHz (High intensity ultrasonic processor model VC/VCX 750, Sonics) for 3 periods of 3 minutes each.

LAg was filtered with 0.2 μm pore size filter membranes (Whatman, Buckinghamshire, England) and the protein concentrations were determined using Bradford reagents (Bio-rad, CA, USA). The sterility of proteins was confirmed by absence of bacterial growth on Luria Bertani (LB) agar plates at 37°C and EMJH media at 30°C. The contaminated endotoxins were determined by the *Limulus amebocyte* lysate (LAL) assay using Pierce LAL Chromogenic Endotoxin Quantitation Kits (Thermo Fisher Scientific, MA, USA). The proteins were kept at -20 °C until used.

2. Recombinant LipL32 protein (rLipL32)

rLipL32 was produced from BL21(DE3) *E.coli* carrying the recombinant *lipl32*-pET23a(+) plasmid as described previously with modifications.[11,12] Briefly, the transformed *E.coli* was grown in LB with 100 μg/ml ampicillin (LB-A) at 37 °C with shaking at 200 rpm. The rLipL32 protein expression was induced by 0.2 mM IPTG at 37 °C for 3 hours.

The His6-tagged rLipL32 was purified from crude solubilized protein prepared from bacterial inclusion bodies by a Ni -NTA affinity column (GE Healthcare, Uppsala, Sweden) under a denaturing condition. The rLipL32 was concentrated and its buffer was exchanged to RPMI1640 plain medium (Gibco, Thermo Fisher Scientific, MA, USA) using a 3 kDa cut-off Amicon Ultra-tubes (Merck Millipore, County Cork, Ireland) at 4 °C, followed by filtration with a 0.2 μm filter membrane (Whatman, Buckinghamshire, England). Protein concentration was measured by a BCA protein assay kit (Thermo Fisher Scientific, MA, USA) and the aliquots of proteins were stored at -20 °C.

The rLipL32 protein was analyzed by reverse phase nano -liquid chromatography (Dionex, Surrey, UK) coupled with MicroToF Q II mass spectrometry (Bruker, Bremen, Germany) and the mass spectrometric result was identified using the MASCOT search engine 2.2 (Matrix Science, Ltd.). Protein purity of rLipL32 protein was verified under 13% SDS-PAGE and colloidal Coomassie Briliant Blue G-250 stain. Antigenic specificity of rLipL32 was confirmed by Western blotting using anti-6x His antibody "(R&D Systems, MN, USA)". The protein sterility and contaminated endotoxins in the rLipL32 were determined as the same in LAg preparation.

Leptospira infection and rLipL32 injection

1. Live *L.interrogans* serovar Autumnalis infection

Hamsters and BALB/c mice were divided into three groups, 3 per group, including a non-injected group as a normal control, an EMJH-injected, and a 10^2 live *L.interrogans* serovar Autumnalis-infected group. Hamsters and BALB/c mice were injected intraperitoneally with EMJH or 10^2 live *L.interrogans* serovar Autumnalis on day 0. After 10 days of infection, all animals were sacrificed and spleens were collected.

2. rLipL32 injection

Hamsters and BALB/c mice were divided into four groups, 3 per group, including a non-injected group as a normal control, an RPMI1640-injected, a TiterMax gold adjuvant (Sigma-Aldrich, USA) injected group and a 20 μg of rLipL32 emulsified in adjuvant-injected group. Hamsters and BALB/c mice were injected intraperitoneally with RPMI1640, TiterMax gold adjuvant, or rLipL32 on day 0. The same antigens were subcutaneously injected at multiple sites on the backs of the hamsters and BALB/c mice on days 7, 14, and 21. Spleens were collected 3 days after the last injection.

Flow cytometric analysis

Fluorescent antibodies

Anti-mouse antibodies used in this study were CD4-PE/Cy7 (GK1.5), CD25-Pacific blue (PC61), IFN-γ-FITC (XMG 1.2), and IL-4-PerCP/Cy5.5 (11B11). Isotypic controls were rat IgG2b-PE/Cy7 (RTK4530), rat IgG1-Pacific blue (RTK2071), rat IgG1-FITC (RTK2071), and rat IgG1-PerCP/Cy5.5 (RTK2071). All antibodies were purchased from Biolegend.

Cell stimulation and surface immunofluorescence staining

Splenic mononuclear cells were isolated from all experimental animals with Ficoll-Paque solution (GE Healthcare, Uppsala, Sweden) according to the manufacturer's protocols. Splenic mononuclear cells were sequentially stained with fluorescent antibodies and analyzed by flow cytometry. Besides flow cytometric analysis, hamster and mouse splenic mononuclear cells derived from live serovar Autumnalis infections were stimulated with RPMI1640 alone as an unstimulated control or with 20 μg/ml of LAg at 24 and 48 hours. Those derived from rLipL32 injections were stimulated with RPMI1640 alone as an unstimulated control or 20 μg of rLipL32 at 48 hours. In brief, splenic mononuclear cells were suspended in complete RPMI1640 medium (RPMI1640 medium supplemented with 10% FBS, 100 U/mL Penicillin and 100 μg/mL Streptomycin). One million cells were cultured with or without mentioned antigens in 48-well plates and 5 μg/ml of Brefeldin A (Biolegend, CA, USA) as a protein transport inhibitor was added into the cultures 12 hours before harvesting. The stimulated cells were centrifuged at 350 ×*g* for 5 minutes at 4 °C. The culture cells were then washed in fluorescence-activated cell sorting (FACS) staining buffer (PBS, 5% FBS, and 0.1% sodium azide) and resuspended in 50 μl of FACS staining buffer containing an optimal concentration of the desired fluorescent antibodies and 2% of normal rat serum as Fc receptor blocking. Cells were washed with FACS staining buffer after being incubated for 30 minutes at room temperature. Intracellular staining was subsequently performed.

Intracellular cytokine staining

To analyze intracellular cytokine production, the cell surface marker stained cells were fixed with Cytofix/Cytoperm solution (BD Biosciences, CA, USA) for 20 minutes at 4 °C and washed twice with Perm/Wash solution (BD Biosciences, CA, USA). Fixed and permeabilized cells were thoroughly resuspended in 50 μl of Perm/Wash solution containing an optimal concentration of anti-IFN-γ-FITC (XMG1.2), anti-IL-4-PerCP/Cy5.5 (11B11), or isotype control antibody (Biolegend, CA, USA) and incubated at 4 °C for 1 hour in the dark. The cells were then washed twice with Perm/Wash solution and resuspended in FACS staining buffer prior to flow cytometric analysis.

All samples were analyzed on a FACS Canto II flow cytometer (BD Biosciences, CA, USA) and data were analyzed by FlowJo software 10.2 (FlowJo LLC, OR, USA). Lymphocytes were gated based on an FSC-SSC gate. The stained anti-CD4 mAb areas were subsequently gated and defined as percentages of CD4+ T cells of lymphocytes. Further gating adjustments were performed based on the expressions of CD25, IFN-γ, and IL-4. Percentages of each cell subpopulation were calculated. Isotype controls of each antibody were included in each staining protocol.

Statistical analysis

Data are shown as means ± standard deviations (SDs). The one-way ANOVA was used to analyze multiple groups and Student's *t*-test was used to compare data between mouse and hamster in each parameter. The comparison data with *P* values < 0.05 were considered as statistically significant differences.

Results

Mouse CD4+ and CD25+CD4+ T cells respond significantly higher than those of the hamster when infected with L.interrogans

Hamster and mouse T cell subsets that responded to *L.interrogans* infection were investigated using commercially available anti-mouse antibodies (**Table 1**) which had previously been tested for cross-reactivity in the Syrian golden hamster. The CD4+ and CD25+CD4+ T cells derived from spleens of hamsters and BALB/c mice with or without live *L.interrogans* serovar Autumnalis infection were identified by flow cytometry. There were no significant differences between percentages of hamster and mouse CD4+ and CD25+CD4+ T cells responding to live bacteria in both *L.interrogans* infected and control groups although mouse CD4+ T cells were slightly increased in both infected and control groups. The %CD4+ T cells in the mouse model under *in vitro* LAg re-stimulated conditions at 48 hours were significantly higher than those of the hamster. This phenomenon was also found in CD25+CD4+ T cells except that the responses of hamster CD25+CD4+ T cells at 24 hours were significantly greater than in the mouse. In addition, *in vitro* LAg re-stimulation exhibited higher responses of CD4+ and CD25+CD4+ T cells compared to conditions without re-stimulation (**Figure 1A-B**). The overall CD4+ and CD25+CD4+ T cell responses in the mouse were significantly higher than in the hamster.

Table 1. Antibodies used in this study

Antigens	Clones	Host species	Reactivity	Isotype	References
CD4	GK1.5	Rat	Mouse	IgG2b	Dondji et al., 2008; Hammerbeck et al., 2011
CD25	PC61	Rat	Mouse	IgG1	Kaewraemruaen et al., 2016
IFN-γ	XMG1.2	Rat	Mouse	IgG1	Kaewraemruaen et al., 2016
IL-4	11B11	Rat	Mouse	IgG1	Kaewraemruaen et al., 2016

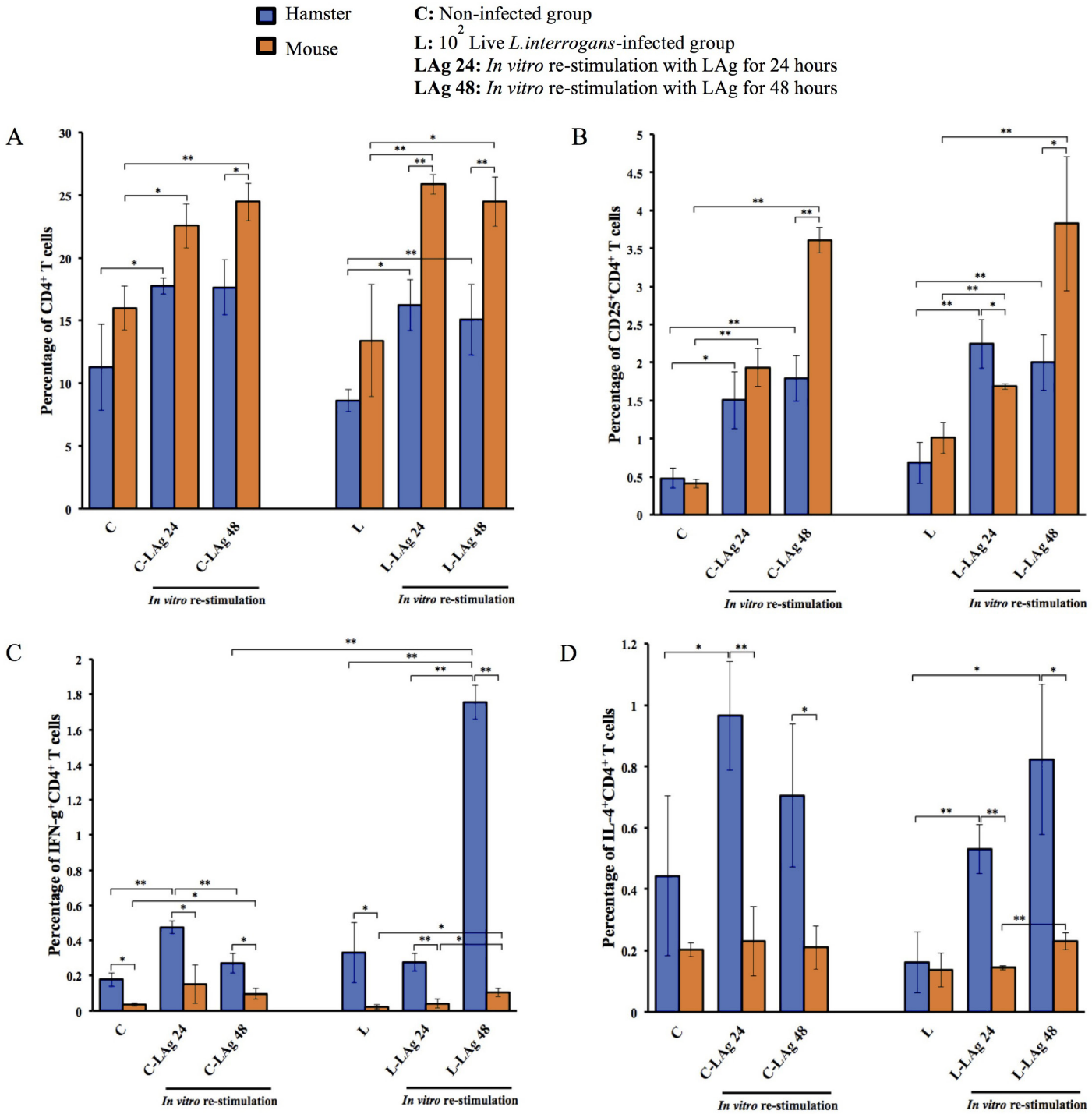

Figure 1. Quantification of CD4⁺ T cells (A) CD25⁺CD4⁺ T cells (B) IFN-γ⁺CD4⁺ T cells (C) and IL-4⁺CD4⁺ T cells (D) derived from *Leptospira* infection. Hamsters and BALB/c mice were intraperitoneally injected with EMJH or 10^2 live *L.interrogans* serovar Autumnalis on day 0. The non-injected group served as a control. Spleens were collected after 10 days of infection and splenic mononuclear cells were isolated. Splenic mononuclear cells were analyzed by flow cytometry and also cultured with or without 20 μg/ml of LAg for 24 and 48 hours. Cells were stained with anti-mouse (CD4, CD25, IFN-γ, and IL-4) mAbs. Data are reported as means ± standard deviations for three animals per group. Statistically significant differences were evaluated using one-way ANOVA and Student's *t*-test. The asterisks (*) and (**) indicate statistical significance at $p < 0.05$ and $p < 0.01$ when compared with controls.

The IFN-γ⁺CD4⁺ and IL-4⁺CD4⁺ T cells of hamsters responded significantly higher than those of mice

In contrast to the percentage of CD4⁺ T cells, the percentages of IFN-γ⁺CD4⁺ and IL-4⁺CD4⁺ T cells in hamsters were significantly higher than those in mice among all groups. *L.interrogans* serovar Autumnalis-infected hamsters with LAg re-stimulation for 48 hours gave the strongest response of IFN-γ⁺CD4⁺ T cells (1.76 ± 0.10%). *In vitro* LAg re-stimulation conditions showed significantly higher responses of IL-4⁺CD4⁺ T cells than conditions without LAg re-stimulation. This circumstance, however,

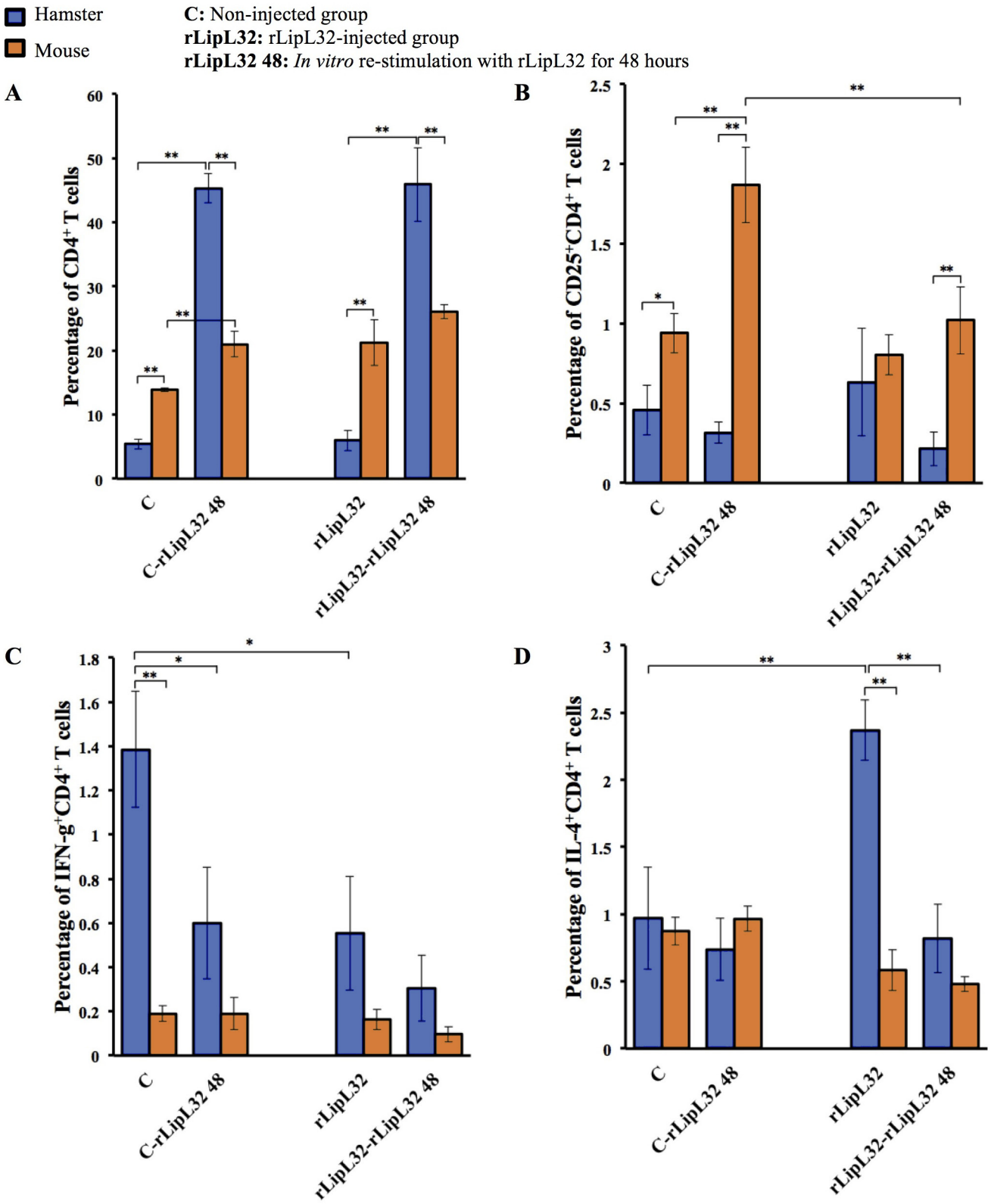

Figure 2. Quantification of CD4⁺ T cells (A) CD25⁺CD4⁺ T cells (B) IFN-γ⁺CD4⁺ T cells (C) and IL-4⁺CD4⁺ T cells (D) derived from rLipL32 injection. Hamsters and BALB/c mice were intraperitoneally injected with RPMI1640, Adjuvant (TiterMax gold adjuvant), or 20 μg of rLipL32 on day 0. The non-injected group served as a control. The same antigens were subcutaneously injected at multiple sites on the backs of hamsters and BALB/c mice on days 7, 14, and 21. Spleens were collected 3 days after the last injection and splenic mononuclear cells were isolated. Splenic mononuclear cells were analyzed by flow cytometry and also cultured with or without 20 μg/ml of rLipL32 for 48 hours. Cells were stained with anti-mouse (CD4, CD25, IFN-γ, and IL-4) mAbs. Data are reported as means ± standard deviations for three animals per group. Statistically significant differences were evaluated using one-way ANOVA and Student's *t*-test. The asterisks (*) and (**) indicate statistical significance at $p < 0.05$ and $p < 0.01$ when compared with controls.

occurred only in hamsters pre-infected with live *L.interrogans* (**Figure 1C-D**). This might indicate the hamster $CD4^+$ T cells produced either IFN-γ or IL-4 differently from mice.

Hamster $CD4^+$ T cells responded against rLipL32 differently from the mice

As LipL32 is the common surface protein of pathogenic leptospire serovars, it was then used as the stimulation antigens in this study for the investigation of differential responses between hamsters and mice. Similar to live infections, the numbers of mouse $CD4^+$ T cells (21.23 ± 3.55%) were significantly higher than the hamsters (5.98 ± 1.59%). Interestingly, after *in vitro* re-stimulation with rLipL32, hamster $CD4^+$ T cells (45.90 ± 5.80%) were significantly greater than mouse cells (26.05 ± 1.06%) while the hamster $CD25^+CD4^+$ T cells (0.22 ± 0.11%) were significantly lower than mouse cells (1.02 ± 0.21%) (**Figure 2A-B**). While the IFN-γ^+CD4^+ and IL-4^+CD4^+ T cells of both animals were comparable; LipL32 stimulated slightly higher, but not significantly, hamster IFN-γ^+CD4^+ T cells (0.55 ± 0.26% and 0.31 ± 0.15% for LipL32 injections and injections with *in vitro* re-stimulation) than in the mouse (0.16 ± 0.05% and 0.10 ± 0.03% for LipL32 injections and injections with *in vitro* re-stimulation) (**Figure 2C**). In contrast, the hamster gave a significantly higher number of IL-4^+CD4^+ T cells than those of the mouse in the primary response (**Figure 2D**). This result indicates the striking difference of both animal models to the common pathogenic leptospiral antigens. These data suggested that T cell responses elicited against *Leptospira* protein, LipL32, are different between hamster and mouse models.

Discussion

The humoral-mediated immune response is known to be a major immune system component against leptospirosis as leptospires are extracellular pathogens[13] while the knowledge of the T cell response to this disease remains poorly understood. Several animal models have been used to elucidate host immune responses and leptospirosis pathology. Hamsters, guinea pigs, and gerbils are susceptible to leptospirosis while mice and rats are resistant.[1] In order to discover the crucial factors for the host defense mechanisms in survival to leptospirosis and provide more strategies to control this disease, *Leptospira*-specific $CD4^+$ T-cell subsets and the cytokine release associated with different host susceptibilities to leptospires were analyzed between susceptible hamsters and resistant BALB/c mice. Although the Syrian hamster is highly susceptible to many organisms and has been used as an excellent experimental model for several infectious diseases, it remains unclear why the hamster is extremely susceptible to such infections and gives the different outcomes in leptospirosis compared to the well characterized mouse model. It might be because of limited availability of immunological reagents, specific monoclonal antibodies (mAbs), and molecular tools to study the immune system of this hamster model. The production and development of new specific mAbs is time-consuming and expensive. Several commercially available anti-mouse mAbs including anti-mouse CD4 clone GK1.5,[14] anti-rat CD8β clone 341,[15] anti-mouse CD25 clone PC61, anti-mouse IFN-γ clone XMG1.2, and IL-4 clone 11B11[5] are available which have previously been shown to cross-react with hamsters and were thus used to determine the responses of $CD4^+$ T-cell subsets and their intracellular cytokines, IFN-γ and IL-4, between leptospirosis susceptible hamsters and resistant BALB/c mice in this study. Although the outbred hamsters were used to compare with the inbred mice in this study, most of the available hamsters were extensively line bred from the same mother and litters so that they could be closely related to inbred stock. Inbred hamsters are usually unhealthy with shorter life spans than those constantly outcrossed. Thus, a limitation regarding this point could not be excluded. The severity of outcomes in leptospirosis has been based considerably on the environment, pathogen virulence, and host susceptibility.[16] Host immune responses are hypothesized to be the more significant ones to exhibit the dramatic symptoms of the disease than virulence of the pathogen.[1] In this study, the *ex vivo* phenotypes of $CD4^+$ T-cell subsets were compared among different groups. The results demonstrated that there were no differences between the hamster or mouse models. This indicates similar $CD4^+$ T-cell stimulation of leptospiral antigens in both animals. After *in vitro* re-stimulation with rLipL32 for 48 hours, the responses of the mouse $CD4^+$ and $CD4^+CD25^+$ T cells were significantly higher than those of the hamster. This might be due to different secondary immune responses leading to the more rapid production of chemokines which are important for recruitment and activation of T cells in the resistant model compared to susceptible models. Several studies compared the immune responses of the host with different susceptibilities to leptospires. The immune responses of the susceptible Syrian golden hamster were compared with the resistant Oncins France 1 (OF1) mouse in terms of histological analysis, cytokine mRNA expression, and the quantification of leptospire loads in target organs and blood. Severe outcomes such as hemorrhage, inflammation, and augmentation of leptospire burdens were found in hamster organs, while a rapid clearance was observed in the mice resulting in limited changes in histological observations. The pro-inflammatory cytokines TNF-α, IL-1β, cyclo-oxygenase-2, and IL-6 and anti-inflammatory cytokine IL-10 were delayed and vast overexpression in the hamster occurred while rapid induction was found in mice. The same result was also observed for the chemokines, IP-10/CXCL10 and MIP-1α/CCL3. The rapid cytokine production and recruitment of immune cells, especially T cells, in resistant mice might be the important factor to rapidly controlling leptospires and limiting pathological lesions.[17] Although the numbers of mouse $CD4^+$ T cells was higher than those of hamster $CD4^+$ T cells, these cells produced low levels of IFN-γ and IL-4. The high production of hamster IFN-γ^+CD4^+ T cells may lead to the marked inflammation of infected hamsters causing animal death. This finding was also reported in previous data by the present authors[18] when heat-killed vaccine protected hamsters from leptospirosis with lower levels of IFN-γ^+CD4^+ hamster cells. Another explanation might be due to the various subpopulations of $CD4^+$ T cells with distinct cytokine profiles between hamsters and mice giving the different responses. As the antibody used for determination of the number of hamster $CD4^+$ and $CD4^+CD25^+$ T cells were anti-mouse antibodies, therefore, the low reactivity to hamster cells could not be excluded. The *L.interrogans* serovar Autumnalis-injected hamsters with LAg re-stimulation for 48 hours gave the greatest response of IFN-γ^+CD4^+ T cells among all samples (**Figure 1C**). This indicates that the primary infection

with *L.interrogans* serovar Autumnalis primes the populations of antigen-specific hamster CD4⁺ T cells resulting in the high level of IFN-γ production when re-stimulated with LAg correlated with host susceptibilities to this infection. The study of specific CD4⁺ T cell reactivity in various clinical outcomes of leptospirosis patients reported that the response of IFN-γ⁺ CD40L⁺CD4⁺ T cells derived from whole-blood specimens stimulated with the leptospiral antigen *in vitro* and was correlated with the severity of leptospirosis in these patients.[9]

LipL32 is derived only from pathogenic strains of *Leptospira* and is a well-known outer membrane protein.[19] According to this previous study, the *in vivo* gene expression of *Leptospira* LipL32 was quantified in blood of animal models with different susceptibilities to leptospires; the susceptible Syrian golden hamster and the resistant BALB/c mouse. Their results indicated that the *lipl32* expression in hamsters was significantly higher than in mice.[20] This result may correlate with the present data in which the responses of hamster LipL32-specific CD4⁺ T cells were higher than those of the mouse model. Although the response of hamster CD4⁺ T cells was dramatically increased in *in vitro* re-stimulation with rLipL32, lower levels of IFN-γ and IL-4 producing CD4⁺ T cells were detected. This might be because of the different stimulations of epitopes in mice and hamsters.

Conclusion

Taken together, the results of the present study appear to be the first report demonstrating the different CD4⁺ T cells and CD25⁺CD4⁺ T cells responses between hamster and mouse models when infected with live *Leptospira*. Although there were a similar number of CD4⁺ T cells and CD25⁺CD4⁺ T cells in the primary response, the IFN-γ and IL-4 producing cells were different especially when re-stimulated with LAg or LipL32 antigens. The significantly higher levels of the IFN-γ⁺ and IL-4⁺CD4⁺ T cells in hamsters might make them to be more susceptible of such infections. The mechanisms of this phenomenon remain to be elucidated when reagents for hamsters are more available.

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Conflict of Interest

none

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Author Contributions

- Yaowarin Nakornpakdee did almost all experiments.
- Rasana W Sermswan designed the leptospiral experiments.
- Santi Maneewatchararangsri designed, cloned, and expressed rLipL32.
- Surasakdi Wongratanacheewin designed all experiments except leptospiral work, wrote grants, and wrote and edited the manuscript.

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