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# The unmet provision of allergy services in Hong Kong impairs capability for allergy prevention – implications for the Asia Pacific region

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## Abstract

**Introduction:** There is a high and rising prevalence of many allergic diseases in the Asia Pacific, including Hong Kong (HK), which is unmatched by a commensurate provision of clinical allergy services.

**Method:** This review highlights progress and deficiencies in allergy service and training in HK. The allergy work force was estimated from the numbers of doctors practicing allergy registered with the HK Medical Council Specialist Register in Immunology and Allergy; Paediatric Immunology and Infectious Diseases (includes allergy); Paediatrics; and Immunology (as a discipline of Pathology). The numbers of trainees were estimated from the trainee lists of the Hong Kong Colleges of Physicians, Paediatrics and Pathology. The numbers of allergy clinics were estimated from existing services in Hospital Authority public hospitals in HK.

**Results:** In the last 3 years, two new drug allergy clinics have been established in public hospitals, and for the first time in 20 years, Hong Kong has a trainee in adult allergy. The current ratio of allergists per head of population has improved slightly from 1:1.46 million in 2014 to 1:1.17 million, but it is still low compared to many countries. There are 5-fold more paediatric allergists than adult allergists per head of population in HK.

**Discussion:** Hong Kong is not equipped to take advantage of major public health advances in allergy prevention. If the unbalance of adult to paediatric allergists remains uncorrected, continuing care for allergic children as they grow into adulthood will be an increasing problem.

**Conclusion:** Hong Kong still has an unmet need for allergy specialists and is ill equipped to exploit recently discovered public health opportunities to prevent allergy. This review provides recommendations to improve allergy service provision and training, including the creation of Centres of Excellence in allergy to drive the growth of the specialty.

**Key words:** Hong Kong; Asia Pacific; allergy services; training; epidemiology; prevention; public health; allergy centre

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## Introduction

In 2015, the Hong Kong Allergy Alliance highlighted the rising trend of allergic diseases in Hong Kong and the heavy burden placed on local clinical services in the face of a relative paucity of allergy trained doctors and specialists.<sup>1</sup> It was estimated that there was only one allergy specialist for 1.46 million people, which was fewer than in many countries.<sup>2</sup>

Training in Paediatric Immunology and Infectious Diseases (PIID) in Hong Kong, which included allergy, was robust but there had not been any local trainee in adult Immunology and Allergy for nearly two decades. It was recommended that two new multi-professional Allergy Centres should be created under a hub-and-spoke model in the public sector.<sup>1</sup>

This review summarises some key discoveries on allergy prevention that could impact Hong Kong and the Asia Pacific. Progress in allergy service provision and training in Hong Kong is updated and case histories are presented to illustrate the continuing challenges. The findings suggest that despite some progress, Hong Kong is currently still unlikely to be able to fully deliver the marked changes required to implement international guidelines on preventing allergies.<sup>3-6</sup> Hong Kong's experience may parallel those of other Asian Pacific countries, many of which face similar challenges.

### **What is new in allergy prevention that could impact on Hong Kong and the rest of the Asian Pacific region?**

Asia Pacific is densely populated and is undergoing rapid urbanisation. With the loss of protective factors associated with a rural environment and emergence of risk factors associated with adopting a western lifestyle, the rising prevalence of allergic disorders in the region will likely continue to escalate in the next few decades,<sup>17</sup> thereby posing a severe burden on health services unless a preventative strategy can be implemented.

A number of early life modulators may play a central role in allergic sensitisation<sup>8-11</sup> and they could be manipulated to prevent allergies. The World Health Organisation recommends exclusive breastfeeding of infants in their first six months of life,<sup>12</sup> and many international guidelines had previously also recommended delaying introduction of allergenic foods such as egg, fish, shellfish, nuts, and wheat-based foods during infancy.<sup>13-15</sup> Thus, the recent publication of Learning Early About Peanut (LEAP) data was transformational because the study found early peanut introduction starting at 4-11 months of life in high-risk infants imparted a significant protective effect against the development of peanut allergy.<sup>16</sup> Based on such important, groundbreaking results, most international guidelines on infant feeding have already been revised.<sup>4-6</sup>

The LEAP study prompted world-wide interest in whether early introduction of other foods could also prevent allergy development. Perkin *et al* studied more than 1,300 infants who were randomly assigned to the introduction of 6 allergenic foods (peanut, cooked egg, cow's milk, sesame, whitefish and wheat) or to the standard UK recommendation of exclusive breast feeding to 6 months.<sup>17</sup> The children were assessed regularly until 3 years old for development of allergies to one or more of the 6 foods. While there was no significant difference between the two groups in the intention-to-treat analysis, the per-protocol analysis showed a significantly lower percentage of children in the early introduction group that developed any food allergy.<sup>17,18</sup> A benefit was also seen for preventing peanut allergy and egg allergy.

Hen's egg allergy is one of the commonest forms of food allergy and early-life sensitisation to egg is strongly associated with sensitisation to aeroallergens in preschool years. The two-step egg introduction for prevention of egg allergy in high-risk infants with eczema (PETIT) study tested whether early egg introduction could prevent egg allergy, but it also instituted aggressive treatment of eczema before and during feeding of allergenic food. 147 infants aged 4-5 months with eczema were randomised to have early introduction of egg or placebo.<sup>19</sup> The clinical trial was terminated early because the results of the scheduled interim analysis of 100 participants showed that

infants with early egg introduction had significantly fewer egg allergy (five [8%] versus 23 [38%] in the placebo group; risk ratio 0.222). The subjects did not experience increased risk of egg-induced allergic reactions. It was concluded that the introduction of heated egg in a stepwise manner along with aggressive eczema treatment was safe and efficacious to prevent egg allergy in high-risk infants.

Three studies on early egg introduction at 4-6 months of age failed to replicate its benefit for egg allergy in Caucasian babies.<sup>20-22</sup> In addition, 8.5% of infants who received egg early experienced significant allergic symptoms upon egg ingestion.<sup>20</sup> The inclusion of an aggressive eczema treatment strategy distinguished the PETIT study from the other clinical trials and thus this was a likely explanation for the difference in results.<sup>23</sup> An earlier randomised controlled trial of 118 Japanese neonates had also found eczema to be a significant risk factor for IgE sensitisation to egg white and that eczema development could be substantially reduced with the daily application of an emulsion-type moisturiser during the first 32 weeks of life.<sup>24</sup>

Researchers from Singapore investigated the timing of allergenic food introduction and food allergy outcomes from the Growing Up in Singapore Towards Healthy Outcomes (GUSTO) study.<sup>25</sup> They found that most of the infants had been introduced to egg, peanut, and shellfish after 10 months of age, but food allergy prevalence was very low between 12 and 48 months of age. There were no significant associations between the timing for introduction of allergenic foods and the development of food allergy.

The increasing numbers of reports on vitamin D deficiency among different North American<sup>26</sup> and European<sup>27</sup> populations over the past two decades have stimulated considerable debate on whether vitamin D deficiency contributes to allergic diseases. A nationwide, cross-sectional survey in 3720 Korean children aged 6-7 years reported the overall prevalence rates of vitamin D insufficiency (20-29 ng/mL) and deficiency (< 20 ng/mL) to be 64.0% and 18.4%, respectively.<sup>28</sup> For a 1 ng/mL decrease in serum 25-hydroxyvitamin D [25(OH)D] level, the adjusted odds ratios for allergic rhinitis, current atopic dermatitis and skin-prick test positivity were 1.020, 1.027 and 1.013, respectively. Another Korean study found significant relationship between 25(OH)D levels and the number and degree of food sensitisation and eczema severity.<sup>29</sup> Vitamin D deficiency also increased the risk of sensitization to food allergens.

Wang *et al* published high prevalence rates of vitamin D deficiency and insufficiency among 826 Hong Kong Chinese children.<sup>30</sup> They found 25(OH)D levels to be lower among children with eczema, and there were inverse associations between 25(OH)D levels and both short-term and long-term indices of eczema severity. Further, vitamin D-deficient patients had higher serum total IgE levels than those with insufficient and physiological 25(OH)D levels. The relationship between vitamin D and eczema might be modulated by a number of vitamin D pathway genes.<sup>31</sup>

The high prevalence of vitamin D deficiency was replicated in a population sample of 1,315 Taiwanese children aged 5-18 years who participated in the Prediction of Allergies in Taiwanese Children (PATCH) study.<sup>32</sup> However, serum 25(OH)D status was not associated with asthma, rhinitis, eczema, atopy and total serum IgE levels after adjustment for potential

confounders.

Notwithstanding some conflicting data in the literature, when taken together, there is compelling evidence from large, well-designed clinical trials that early introduction of certain foods, aggressive management of eczema and addressing low vitamin D levels might provide major opportunities for allergy prevention. But even in high-risk infants, any benefits could be food- and population-specific. Further research is required urgently to define the window of opportunity and criteria for intervention including the target populations. Against the background of the rising prevalence of allergic diseases, rapid progress may only be made by a larger network of specialists with expertise in allergy diagnosis and treatment than is currently available in many Asian Pacific countries to drive forward discovery science and clinical practice. This will inevitably require dedicated investments of resources but the payback for implementing a successful strategy could be substantial public health gains.

### **How many allergy specialists are there in Hong Kong?**

There are 4 Immunology and Allergy specialists listed in the Hong Kong Medical Council Specialist Register,<sup>33</sup> but only two of them practice allergy. Both of them were trained abroad and work in the private sector. Therefore, the ratio of registered adult allergists per head of population in Hong Kong remains around 1:2.8 million head of adult population,<sup>34</sup> which is exceedingly low.

There are 6 foundation fellows of PIID who spend on average about 40% of their working week on allergy, so Hong Kong has 2.4 full time equivalent (FTE) specialists in paediatric allergy. Hong Kong has about 1.3 million children under the age of 18 years old, so there is 1 paediatric allergist per 540,000 head of paediatric population.<sup>34</sup> This is less than the estimate 3 years ago of 1:460,000<sup>1</sup> as several paediatric allergists who worked full time previously can now only work part time due to increasing administrative and other responsibilities. This has implications for future manpower planning just to maintain the status quo let alone grow the discipline. The difference between numbers of paediatric and adult allergists is very striking and if this situation is not re-balanced, transitional and continuing care for children as they grow into adulthood will become an increasingly major problem.

The number of allergists in Hong Kong will increase by the end of 2017 when two paediatric trainees complete their training in PIID and another immunologist completes her training. However, these graduates can only work part-time in allergy because of the manpower constraints in the public hospital services.

Therefore, we estimate that by the end of 2017, in addition to two full time adult allergists, two immunologists and 8 paediatricians will spend on average about 40% of their working week in allergy. Assuming Hong Kong has a population of around 7 million, it will have one full time equivalent (FTE) allergist per 1.17 million population,<sup>34</sup> which is slightly better than the 1:1.46 million figure estimated in 2014,<sup>1</sup> but this would still be inadequate to deliver a robust service.

These figures could have underestimated the available expertise because many patients with allergic diseases are treated by non-allergy specialists and General Practitioners. These

doctors are not included in the calculations because the extent of their allergy training cannot be ratified by any existing mechanism. In addition, a few clinicians will have received dual training overseas in Allergy and another specialty, but they are only permitted by the Hong Kong Medical Council to be registered under one specialty, so may have decided to register under a non-allergy specialty. Nevertheless, it is the authors' experience that Hong Kong has very few doctors who have a wide breadth of allergy experience (including but not exclusive to the use of specific allergen immunotherapy, food challenges and specialised allergy testing) who are not already registered as specialists in Immunology and Allergy; Immunology; or PIID. Even if the few doctors who are dually trained are included in the metrics, it would be unlikely to detract from the conclusion that the Hong Kong community is inadequately served by trained allergists.

The authors know of only one published global survey of the ratio of allergists per head of population<sup>2</sup> (Tables 1 and 2). Hong Kong ranks 6<sup>th</sup> out of 7 countries in the Asia Pacific, Australia and New Zealand; only Malaysia has fewer allergists. In the rest of the world, Hong Kong ranks 30<sup>th</sup> out of 36 countries with Ecuador, France, Honduras, Mongolia, Peru and South Africa having less representation. The data need to be updated as the figures (apart from Hong Kong) were published 11 years ago and there could have been significant improvements in some countries, which might make Hong Kong's comparative position even worse.

Hong Kong is among the top 14 countries in the world for gross domestic product per capita<sup>35</sup> with an average annual growth rate of 2.6% and prides itself in having an excellent and mature health service.<sup>36</sup> Therefore, it is both surprising and concerning that Hong Kong, despite its abundance in wealth, is not only insufficiently served by allergy specialists locally, but also very low when compared to the neighbouring region and the rest of the world.

**Table 1. Allergists per head of population in Asia pacific, Australia and New Zealand**

Country	Allergists per head of population
Australia	1:140,000
Hong Kong	1:1,170,000
	Adult 1:2,800,000
	Pediatrics 1:540,000
Japan	1:61,200
Malaysia	1:25,000,000
New Zealand	1:440,000
Philippines	1:66,115
Thailand	1:1,000,000

Data adapted from ref 2. Permission granted by S Karger AG, Basel. The Hong Kong data is 2017 data whereas the others were extracted from ref 2.



**Table 2. Allergists per head of population rest of world**

Country	Allergists per head of population	Country	Allergists per head of population
Argentina	1:46,353	Lebanon	1:121,000
Belgium	1:900,000	Mexico	1:175,000
Brazil	1:100,000	Mongolia	1:2,250,001
Bulgaria	1:106,250	Paraguay	1:200,000
Canada	1:200,000	Peru	1:1,360,000
Chile	1:500,000	Portugal	1:63,334
Colombia	1:571,428	Romania	1:197,577
Czech Republic	1:17,543	Sebia/ Montenegro	1:500,000
Denmark	1:135,000	South Afica	1:1,666,666
Ecuador	1:2,400,000	Spain	1:44,000
El Salvador	1:470,000	Sweden	1:42,857
Finland	1:94,545	Switzerland	1:36,649
France	1:1,240,000	Turkey	1:1,076,923
Germany	1:16,000	United Kingdom	1:1,083,333
Greece	1:183,333	Ukraine	1:94,441
Honduras	1:1,380,000	Uruguay	1:110,000
Hungary	1:50,000	United States	1:65,546
Israel	1:52,000	Venezuela	1:109,090
Italy	1:43,200		

Data adapted from ref 2. Permission granted by S Karger AG, Basel.

**The lack of allergists can create significant problems as illustrated by the following case histories:**

**Case 1:** A 53-year old man was recently reviewed at a specialist clinic of a public hospital by an allergy trainee after decades of waiting for an allergist’s evaluation. For the past 20 years, he had been admitted to different public hospitals around Hong Kong for idiopathic anaphylaxis. He had first experienced urticaria and facial angioedema about 20 years ago while rushing his daughter to school after eating bread for breakfast. Since then, he has experienced more than 10 similar episodes of varying severity after eating different types of foods, but no consistent allergens could be identified. His most severe reaction occurred when he collapsed while strolling with his wife after he had eaten at a buffet. He was hospitalised for severe anaphylactic shock with hypotension, requiring intramuscular adrenaline, intravenous steroids and anti-histamines. This naturally caused the patient much anxiety and he was on a very restrictive diet. He had no follow-up for his suspected allergies and was never prescribed an adrenaline auto-injector, the therapy of choice for treating another potential life-threatening event. At his recent attendance, skin prick testing was positive to various wheat-containing foods and serum specific IgE levels to wheat and omega-5-gliadin were very high. He was

diagnosed with wheat-dependent exercise-induced anaphylaxis and was advised to avoid wheat and other co-factors. An emergency treatment plan and adrenaline auto-injector were prescribed. No further episodes of anaphylaxis have occurred since the appropriate avoidance measures were instituted.

Exercise induced anaphylaxis was first described 37 years ago.<sup>37</sup> If the patient had come under the care of an allergist when he first presented with his symptoms, he could have been saved a lot of anxiety, life-threatening events due to the lack of diagnosis and appropriate preventative education and treatment, and unnecessary visits to an over-stretched public healthcare system.

**Case 2:** A 15-year-old boy had been receiving monthly carboplatin treatment for his recurrent hypothalamic pilocytic astrocytoma at a public hospital. During his 5<sup>th</sup> maintenance cycle, and 90 minutes after taking aprepitant for prevention of vomiting, he was awakened by facial angioedema, conjunctival injection, upper body flushing, chest tightness and nasal congestion during the carboplatin infusion. Symptoms resolved after stopping the infusion and administration of inhaled salbutamol with intravenous chlorpheniramine and hydrocortisone. Serum tryptase measured one hour after the onset of allergic reaction rose to 5.3 microgram/L from 2.8 microgram/L, suggestive of drug-related anaphylaxis.<sup>38</sup>

The patient was initially labelled as having developed allergies to both carboplatin and aprepitant. Carboplatin, which was the first-line drug for his brain tumor, was stopped and a second-line chemotherapeutic regimen was considered.

Advice from the hospital’s paediatric allergy team was obtained and the patient underwent allergy testing to carboplatin, which showed no reactivity to skin prick (10 mg/mL) but had a 4-mm increase in wheal size and 20-mm flare from carboplatin intradermal injection (1 mg/mL).

The patient was desensitised to carboplatin in the Paediatric Intensive Care Unit according to a previously published 12-step protocol.<sup>39</sup> He initially tolerated the desensitization well, but during administration of the last infusion bag he developed similar (although less intense) symptoms as his first reaction. He was eventually successfully desensitised after his protocol was lengthened to 14 steps and he has been receiving the revised regimen safely in the Paediatric Ambulatory Care Unit on a monthly basis.

This case exemplifies the role that an allergist can play in the management of a patient with drug allergy. If the allergy team had not been involved in his care, the patient would not have received his anti-cancer treatment of choice.

**Case 3:** A 24-year-old man with atopic dermatitis and food allergies had attended the Paediatric Allergy Clinic in a public hospital since childhood. He originally had cow’s milk, egg and nut allergies. When his skin prick tests to milk and egg revealed reducing reactivity to both foods, he was subjected to oral food challenges which were negative and he has subsequently tolerated the foods. However, he continued to have severe allergies to peanut and tree nuts and experienced allergic reactions requiring the use of his adrenaline auto-injector after accidental ingestion. More recently, he had been having worsening eczema and frequent skin infections which would have been best managed by admission to the hospital to be treated following a well-established protocol.<sup>40</sup> However, the paediatric ward only accepts patients ≤18 years old and there is no adult allergy service,

so the patient has to receive suboptimal skin care in the outpatient setting.

The Paediatric Allergy team can no longer supervise his care as an adult and he does not have the resources to see a private adult allergist so he is left in limbo. This is a problem that arises only too frequently when there is no network, as in Hong Kong, to care for allergic children once they outgrow their paediatric health providers.

### **Recruitment from overseas**

One way to grow a discipline is to recruit specialists from overseas.<sup>1</sup> In 2016, a public hospital in Hong Kong recruited an American board certified general paediatrician and combined adult and paediatric allergist/immunologist from the United States. However, he has to spend another 4 to 5 more years fulfilling the requirements of the Hong Kong Medical Registration Ordinance and local specialty registration with a focus on non-allergy related clinical duties before he will be recognised as a specialist in Hong Kong. This delay creates a significant obstacle for recruitment from overseas and there is a well-justified debate on whether Hong Kong should have a mechanism to recognise the qualifications of these fully trained specialists more rapidly, especially those with expertise that can fulfill an unmet need.<sup>41-43</sup>

### **Realignment of resources**

It was also suggested earlier that it may be possible to create a new allergy service by realigning some resources,<sup>1</sup> as numerous studies have shown that the disease-modifying treatment of allergen-specific subcutaneous injection immunotherapy is associated with substantial cost savings in health care.<sup>44</sup> However, this strategy can be an obstacle because of competing priorities, so it is encouraging that in 2017 two public hospitals in Hong Kong have established new penicillin allergy testing services. One of the services has already made a significant impact. It was confirmed that beta-lactam drug allergies were often over-diagnosed; leading to unnecessary avoidance, increased medical costs and use of less effective antibiotic therapies. More than 90% of the patients tested so far for a beta-lactam allergy by skin and provocation testing did not have drug allergy and their false allergy labels could be removed.

The two new services are operated by a Respiratory Medicine specialist and the Immunology and Allergy trainee under supervision, respectively. They had both gained experience in testing for drug allergies at an Allergy Centre in a private hospital in Hong Kong supervised by an accredited Allergist. This example of collaboration between the private and public sectors could provide a template for future progress.

### **Training and academic developments**

In a long overdue development, Hong Kong now has its first trainee in adult Immunology and Allergy in 20 years, so there is a total of 5 trainees undergoing allergy training currently. There is also a specialist in Rheumatology who is interested in allergy and will spend a few months in the UK in 2017 to receive allergy training. While this bodes well for the future growth of the specialty, it is slow and more trainees are required, especially in adult allergy.

The trainee in adult Immunology and Allergy obtained permission from the Hong Kong College of Physicians (HKCP) to visit overseas Centres for parts of his training to maximise his exposure to Allergy as there were no approved allergy trainers in Hong Kong. The earliest time the current trainee can be considered as a trainer himself in Immunology and Allergy is 2021, when he will be Hong Kong's only formally approved adult allergy trainer if he continues to work in an approved Centre. While acknowledging there are significant advantages to be gained from overseas training, it would be ideal if Hong Kong could also develop mechanisms to enhance local opportunities to help train future allergists. This might include recruiting allergists in private practice to become official trainers. There is a precedent already as the Hong Kong College of Family Physicians enlists private General Practitioners to help deliver parts of their training programme.

Allergy is a major component of the training curriculum of both the specialties of Immunology and Allergy under the HKCP and PIID under the Hong Kong College of Paediatricians. As allergic patients comprise a large proportion of the daily clinical practice of PIID subspecialists, the subspecialty board of PIID has applied to change the name of the subspecialty to "Paediatric Immunology, Allergy and Infectious Diseases" (PIAID). The impact of this visionary decision, if approved, on securing the future of paediatric allergy as a discipline in Hong Kong cannot be overemphasized.

### **Professional societies and patient organisations**

The Hong Kong community is well supported by allergy related professional societies and patient organisations. These include the Hong Kong Institute of Allergy (HKIA), Hong Kong Society of Paediatric Respiriology and Allergy (HKSPRA), Hong Kong Society of Paediatric Immunology Allergy and Infectious Diseases (HKPIAID), Hong Kong Allergy Association (HKAA) and Hong Kong Asthma Society (HKAS). They all host allergy conventions, symposia and courses. They publish position papers and clinical allergy practice guidelines.<sup>3,45,46</sup> They have their own websites and some of them have social media platforms to engage the public. They publish regular newsletters. HKIA also offers scholarships for members to train overseas and provides travel grants to attend allergy conferences. A new competitive research grant scheme was launched in 2016.

Two patient organisations, namely HKAA and HKAS, are committed to making a difference to patient experience through peer-group support, patient and public education, advocacy, and organisation of social activities.

The HKAA was founded in 2008 by individuals with allergic disorders, their care-givers and medical professionals as a tax-exempted charity. It organises regular educational events for its members and peer-group sharing. The Association also offers out-reach programs to schools, teaching teachers how to use adrenaline auto-injectors. Funding was obtained from the government to employ a full-time staff member and rent a permanent premise at a relatively low cost. The premise was converted into an Education Centre and commenced its service in 2017.

The HKAS was established in 1989. This group provides services for asthma and allergy sufferers as well as their families so that they can better manage their condition. With the support of

the Hong Kong Thoracic Society and the HKSPRA, the HKAS also offers medical advice to its members.

## Discussion and recommendations

The creation of a flourishing allergy service has become increasingly pressing with the rising prevalence of allergic diseases and the strong emerging evidence that suggests there could be ways to prevent development of allergy, including the early introduction of allergenic foods to infants.<sup>3,4-6,16,17,47-52</sup>

However, the logistic difficulties and resource implications for implementing early introduction of peanut (let alone multiple foods) to prevent allergy are enormously challenging even for countries that are well served by allergists.<sup>53</sup> Hong Kong and many countries in the Asia Pacific are simply not equipped in terms of expertise, manpower and facilities to test the new hypotheses comprehensively or to take advantage of these major public health advances in allergy.

While it is encouraging to note that a few of the suggestions proposed in 2015<sup>1</sup> to improve allergy provision and training in Hong Kong have been accomplished, it is disappointing that the major proposal to establish Allergy Centres of Excellence is far from being achieved in any public hospital.

Centres of Excellence provide an integrated 'hub' of multi-professional allergy expertise. They have many advantages. They can provide much needed seamless transitional care to children with allergic diseases as they grow into adulthood. They conduct world leading research in the discipline. They can be a structured framework to plan staffing and resource allocation in the specialty. They coordinate teaching of allergy to undergraduates and postgraduates, and they develop transparent career paths and integrative exit strategies for trainees. They can provide outreach clinical services, train doctors and allied health professionals in primary and secondary care (the 'spokes') and raise the professional and public profile for the discipline.

Many patients with asthma, eczema and rhinosinusitis are currently being treated by specialists in Respiratory Medicine, Dermatology and Otolaryngology, respectively, and a Centre of Excellence in allergy is not intended to compete with specialists in these and other disciplines. To the contrary, successful Centres are trans-disciplinary and should be collaborating with colleagues to provide integrated and holistic care for complex patients who are often characterised by multisystem involvement.

Future interactions between Allergy Centres with other disciplines could be facilitated by defining the types of patients that would require specialised intervention and management by a Centre of Excellence in allergy. These criteria could be guided by those recommended by the UK's National Health Service Commissioning Clinical Reference Group<sup>54</sup> and include patients with increased risk of death because of the severity of their allergic condition (e.g. drug allergy, anaphylaxis, angioedema, brittle asthma), persisting poor quality of life with a major allergic component despite routine therapies, requirement for allergen immunotherapy, rare diseases leading to allergic symptoms requiring complex investigations and therapies (e.g. mastocytosis, hypereosinophilic disorders, hereditary angioedema) and diseases with allergic symptoms

but where the cause is unclear requiring input by specialists to make a specific diagnosis, identify triggers, optimise management and prevent further recurrences.

The hub-and-spoke model for Allergy Centres was adopted by the UK in 2007<sup>55</sup> and a pilot Centre was subsequently created. Each of the two medical schools with their partner hospitals in Hong Kong has the core of an allergy and immunology service already. It would not require a lot of resources to transform each of them into a Centre of Excellence. The imminent opening of a Children's Hospital also provides an opportunity to integrate various strands of paediatric allergy and immunology expertise into a Centre of Excellence. Before and after these Centres are created, service provision in allergy might be further improved by creatively establishing new clinics through realignment or sharing of resources.

An international survey<sup>2</sup> revealed that the ratio of allergists per head of population in countries that are best represented by allergists is 1 allergist: < 100,000 heads of population. Likewise in Hong Kong, most of the main medical specialties are also similarly serviced.<sup>33</sup> Thus, Hong Kong will require at least 70 allergists for a population of around 7 million if it is to approximate other medical disciplines locally and to be close to the best international standards. There are about 4.5 fold more adults than children in Hong Kong, so this equates to a minimum target of 16 (FTE) paediatric allergists and 54 (FTE) adult allergists.

The current lack of any adult allergy trainers in Hong Kong is an impediment to building a thriving clinical service. While acknowledging the benefits of gaining experience overseas, more consideration could be given on how to improve the local training environment, for example, by outreach to private allergists. Meanwhile, it will be essential to formulate a transparent exit strategy for the current batch of allergy trainees so that their career progression is an exemplar to other potential trainees who could be inspired to join the programme.

It would be helpful if a more expeditious mechanism for registration for overseas trained doctors in Hong Kong can be agreed so that they can be assimilated into Hong Kong's health care system more quickly in the face of ensuring high professional standards, especially those with expertise that can fulfill an unmet need such as allergy.

Finally, educating the profession and engaging the public about allergy and its prevention are urgently required. Introduction of changes, for example, to traditional infant feeding habits will require a seismic shift in professional practice as well as a change in societal culture and behaviour.

## Conclusion

The public and professional profile of allergy in Hong Kong has increased in the last few years. The community is well supported by professional societies and patient orientated patient organisations. The numbers of allergists have grown slightly. Two new drug allergy clinics have been established de novo in public hospitals. However, Hong Kong still has an unmet need for allergy specialists and is ill equipped to exploit recently discovered public health opportunities to prevent allergy.

The specialty can be helped to grow by recruiting suitably trained allergists from overseas and by realignment of resources



to create new services. Recruitment from overseas would be facilitated by shortening the process for the assimilation of recruits into Hong Kong's healthcare system.

It is our strong recommendation that at least two Centres of Excellence in allergy are established in Hong Kong, with their many clinical and academic advantages, to lead the drive towards reaching the goal of having one allergy specialist serving 100,000 heads of population.

Finally, engagement of the profession and the public about allergy is fundamental to any proposed strategy to develop the discipline.

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# Anaphylaxis to cutaneous exposure to bovine colostrum based cream

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## Abstract

Children who are highly sensitive to milk may also have severe allergic reactions after exposure to cow's milk proteins (CMP) through a different administration route than the oral one. We describe the case of a 16-year-old Caucasian boy with a clinical history of persistent cow's milk allergy (CMA), who developed one episode of anaphylaxis following cutaneous application of a bovine colostrum containing cream to a surgical wound. UniCAP testing showed a significant elevation in specific IgE antibodies to whey milk proteins. Until now, only three cases of anaphylaxis following cutaneous application of products containing milk proteins were available in the scientific literature.

**Key words:** anaphylaxis, asthma, bovine colostrum, cow's milk allergy, children.

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## Introduction

Cow's milk allergy (CMA) is the most common food allergy in pediatric patients and it affects 2–3% of children.<sup>1</sup> <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3604421/> - R2 Cow's milk contains more than 25 different proteins, but only whey proteins  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin, bovine serum albumin (BSA), and lactoferrin as well as four caseins have been identified as allergens.<sup>2</sup> In sensitized children, oral intake of cow's milk is responsible for IgE or non-IgE mediated reactions, even though skin contact and food inhalation may also trigger adverse reactions.<sup>3,4</sup> IgE-mediated food allergy can present with skin, gastrointestinal, respiratory, and cardiovascular symptoms, and sometimes may be life threatening and treatment takes place in the emergency setting.

We describe the case of a 16-year-old boy affected by CMA and mild chronic allergic asthma with anaphylaxis to cutaneous exposure to milk proteins in a colostrum based cream.

## Case Report

A Caucasian 16-year-old boy was affected by CMA since the age of 10 months. Family history was negative for atopy. He was exclusively breast-fed in the first months of life. The first adverse reaction was characterized by anaphylaxis after the ingestion of milk formula. CMA was confirmed by cutaneous and laboratory tests in the first year of life. Skin prick test (SPT) was conducted using both fresh milk and extracts of cow milk

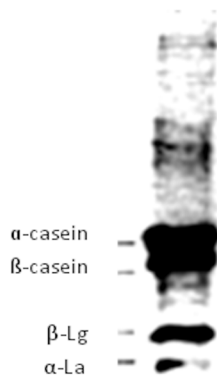
proteins including cow  $\beta$ -lactalbumin (Bos d 4),  $\beta$ -lactoglobulin (Bos d 5), and casein (Bos d 8) (Lofarma, Milan, Italy)—wheal diameters were 8 mm for milk, 13.5 mm for purified natural Bos d 4, 8 mm for Bos d 5, and 11 mm for Bos d 8. In addition, blood samples were drawn for measurement of total IgE (597 IU/mL), and milk-specific IgE levels (UniCAP, Phadia, Uppsala, Sweden) were 90 kUA/L for milk, 62.3 kUA/L for Bos d 8, 62.5 kUA/L for Bos d 4, and 36.6 kUA/L for Bos d 5. Therefore, an oral food challenge (OFC) with cow's milk (CM) formula was undertaken and within few minutes of formula administration, the patient developed generalized hives and wheezing, confirming the diagnosis of CMA. Avoidance of milk and dairy products was recommended, use of CM hydrolysate was suggested and auto-injectable epinephrine was prescribed as emergency medication for anaphylaxis.

Despite good compliance to an elimination diet, the patient experienced two episodes of anaphylaxis after the accidental ingestion of food products containing hidden milk. Every year the patient underwent OFC to CM in order to verify the appearance of spontaneous tolerance. However, the patient showed allergic symptoms also during the last OFC to CM performed when he was 15 years old. Furthermore, asthma symptoms occurred in preschool age and were associated with sensitization to the house dust mite (SPT 4 mm and sIgE 1.18 kUA/L for *Dermatophagoides pteronyssinus*, SPT 3 mm and sIgE 1.12 kUA/L for *Dermatophagoides farinae*), dog (SPT 4

mm and sIgE 1.0 kUA/L), and cat epithelium (SPT 5 mm and sIgE 5.36 kUA/L). Asthma control was obtained with a low dose of inhaled steroid (fluticasone propionate), and spirometry showed mild obstruction with FEV1 73% of predicted value, FVC 96% of predicted value, and FEV1/FVC ratio of 76%.

In addition, because a sacrococcygeal fistula was detected, surgical intervention was required when he was 15 years old. Six months after surgery, he applied a topical colostrum based cream (bovine colostrum, hyaluronic acid, sodium alginate, and Aloe barbadensis gel) to the surgical wound. Urticaria, itching, and wheezing occurred immediately within few minutes and symptoms disappeared after systemic epinephrine (Fastjekt 330 mcg), hydrocortisone (200 mg), and chlorpheniramine maleate (10 mg) administration. The last SPT tests with cow's milk proteins (CMPs) were performed six months before the evaluation in the emergency setting and a wheal diameter of 13 mm for Bos d 4, 12 mm for Bos d 8, and 8 mm for Bos d 5 confirmed CM sensitization.

Because no correlation with any food ingestion was detected, the suspicion of allergic reaction due to topical application of colostrum based cream was strong. The presence of potentially allergenic milk proteins in a sample of the implicated product was assessed by immunoblotting techniques. Three bands corresponding to the  $\alpha$ -lactalbumin ( $\alpha$ -La),  $\beta$ -lactoglobulin ( $\beta$ -Lg), and casein were detected in the bovine colostrum based cream incubated with patient sera (**Figure 1**).



**Figure 1. Immunoblotting of colostrum based cream incubated with patient sera ( $\alpha$ -casein,  $\beta$ -casein,  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin).**

## Discussion

IgE-mediated CMA is reported to be a major cause of anaphylaxis and mortality in children and requires compliance with strict avoidance of CM and dairy products, as well as self-carry of an epinephrine autoinjector.<sup>5</sup> Our case draws attention to potential problematic aspects related to the management of persistent IgE-mediated food allergy. Children who are highly sensitive to milk may have severe allergic reactions not only after ingestion or cutaneous contact of culprit food but also after cutaneous or intravenous exposure to CMPs contained in some drugs or preparations.

Allergic reactions due to cutaneous allergen contact are described in children with IgE-mediated CMA and atopic dermatitis, because the skin barrier impairment facilitates the epicutaneous penetration of molecules of food allergens<sup>6</sup>

(similar reactions are also described in children with non-eczematous skin).<sup>7</sup>

Sometimes, contact with food proteins without ingestion can cause severe systemic allergic reactions.<sup>3,8</sup> Tan et al. described five cases of children who experienced severe food allergic reactions by skin contact to milk and peanuts. Patients were characterized by strong family history of allergy, early age of onset, strong positive reactivity to foods by SPT, and multiple sensitizations to other foods.<sup>3</sup>

It is known that CMPs are present in a large variety of processed foods or drugs, but their presence may be hidden, as they are occasionally listed on the product label in a manner unrecognizable to the consumer. For this reason, the parents of more sensitive patients need to be instructed in the detection of these potential sources of hidden CMPs by carefully reading food and drug labels and in recognition of the sources; thus, avoiding possible adverse and severe reactions.

Bovine colostrum is mostly composed of  $\alpha$ -La (15%–20%) and  $\beta$ -Lg (55%–65%); the minor components include immunoglobulins (9%), bovine serum albumin (5.5%), lactoferrin, phospholipoproteins, bioactive factors, and enzymes.<sup>9</sup> It also contains endothelial and basic fibroblast growth factors and platelet-derived growth factor. The last one is believed to be an important mitogen for fibroblasts, which explains the use of bovine colostrum in products applied for wound healing.<sup>10</sup>

As previously described, our patient was highly sensitized to specific allergen components of CMPs as shown by SPT and sIgE that were significantly positive for purified natural Bos d 4 of  $\alpha$ -La and Bos d 8 of casein. Even if he hadn't had a positive family history of atopy and other sensitizations to food, he had early-onset allergy and strong positive reactivity to CMP by SPT, so the cutaneous exposition to the culprit allergen was sufficient to induce a severe allergic reaction requiring epinephrine administration.

To the best of our knowledge, only three cases of anaphylaxis following cutaneous application of products containing CMPs are available in the scientific literature. Anaphylaxis occurred in two children after cutaneous application of a casein containing ointment to an inflamed diaper area<sup>11,12</sup> and in an adult woman using a cosmetic that contained casein.<sup>13</sup>

## Conclusion

The effective management of food allergy includes avoidance of the food allergen and instructions on the use of epinephrine self-injection. Nevertheless, patients and their parents must be aware that other administration routes of allergen, like the cutaneous one, can cause serious reactions. Physicians must instruct patients and their parents more thoroughly about their allergies, above all specifying the relevant names of all allergens to which they are sensitized. In the future, may be the new active treatment of IgE-mediated food allergy, oral immunotherapy (OIT)<sup>14</sup> will provide a new effective therapy with the goal of increasing the threshold of reactivity and improving the quality of life of patients.

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## Author contributions

- Federica Porcaro collected data, drafted the manuscript, approved the final manuscript as submitted, and guarantees that every part of the work has been appropriately investigated and resolved.
- Lucia Caminiti contributed to data acquisition, managed the patients, critically revised the manuscript, approved the final manuscript as submitted, and guarantees that every part of the work has been appropriately investigated and resolved.
- Giuseppe Crisafulli contributed to data acquisition, managed the patients, critically revised the manuscript, approved the final manuscript as submitted, and guarantees that every part of the work has been appropriately investigated and resolved.
- Francesco Guglielmo contributed to data acquisition, managed the patients, critically revised the manuscript, approved the final manuscript as submitted, and guarantees that every part of the work has been appropriately investigated and resolved.
- Giovanni Battista Pajno coordinated and supervised data collection, critically revised the manuscript, approved the final manuscript as submitted, and guarantees that every part of the work has been appropriately investigated and resolved.

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# Cutaneous toxicities of epidermal growth factor receptor inhibitors: A prospective study in 60 Asian patients

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## Abstract

**Background:** Several prospective studies have been conducted in epidermal growth factor receptor (EGFR) inhibitor-related cutaneous reactions in Caucasian patients, but prospective studies in Asian populations are scarce.

**Objective:** To investigate the cutaneous side effects of EGFR inhibitors in Asian cancer patients and to assess tumor response to dermatologic manifestations.

**Methods:** Sixty patients with lung or colorectal cancer who were receiving EGFR inhibitors were prospectively followed for at least one year by oncologists and dermatologists.

**Results:** Of 60 patients (33 males, 27 females), 46 lung cancer patients received erlotinib (n = 29) and gefitinib (n = 17). Cetuximab was prescribed in 14 colorectal cancer patients. Fifty-eight patients (58/60, 96.7%) developed cutaneous reactions. The most common reactions were xerosis (82.8%), acne (79.3%), and skin desquamation (62.1%). Most reactions were mild and well-tolerated. Of 14 patients who had severe reactions, temporary treatment interruption was necessary in 3 patients and a decreasing dose was required in another 3 patients. There were no statistically significant differences in type, severity, or number of cutaneous reactions between responders (29/58) and non-responders (29/58) to EGFR inhibitors. At median follow-up time of  $11.92 \pm 1.08$  months, no patient died from cutaneous toxicities. Nine patients died from cancer and 11 patients lost to follow-up.

**Conclusion:** In this Asian population, almost all patients (96.7%) developed cutaneous toxicities of EGFR inhibitors. Xerosis, acne, and desquamation were common in Asian cancer patients. Most reactions were mild and well tolerated. Due to limited number of patients, this study did not show significant associations between cutaneous toxicities and tumor response.

**Key words:** epidermal growth factor receptor inhibitors, cutaneous toxicities, cetuximab, erlotinib, gefitinib

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## Introduction

Human epidermal growth factor receptors (EGFRs), which express in normal human skin tissue, are responsible for cell growth and the proliferation of keratinocytes, and the outer root sheath of hair follicles.<sup>1</sup> Overexpression of EGFRs may lead to uncontrolled cell growth, proliferation, angiogenesis, and metastasis. Previous studies showed that there is an overexpression of EGFRs in some solid cancers, such as non-small-cell lung cancer (NSCLC), colorectal cancer, and head and neck cancer.<sup>1,2</sup>

As a result of this underlying pathway in tumor proliferation, EGFR inhibitors were developed for the treatment of several chemotherapy-resistant solid cancers such as NSCLC, EGFR-positive colorectal cancer, and squamous cell carcinoma of head and neck.<sup>3</sup>

EGFR inhibitors are categorized into the following two groups: tyrosine kinase inhibitors and chimeric monoclonal antibodies.<sup>3,4</sup> Tyrosine kinase inhibitors bind to the tyrosine

kinase portion of EGFRs, which leads to a decrease in cell growth and differentiation of tumor cells. Chimeric monoclonal antibodies compete with EGFR ligands, resulting in down-regulation of transmembrane EGFRs.<sup>3-6</sup> Unlike conventional cytotoxic chemotherapies, anti-EGFR therapies do not usually cause myelosuppression, neuropathy, or significant nausea, because they have high specificity to EGFR. Common adverse effects of EGFR inhibitors are cutaneous toxicities, which have been reported in 45-100% of patients.<sup>1,4,6</sup> Roe, *et al.* investigated cutaneous side effects in 30 Caucasian patients during treatment with EGFR inhibitors. They found that acneiform eruption, followed by painful fissures in palms and soles and paronychia were the most common cutaneous reactions.<sup>1</sup> Another study that retrospectively evaluated 99 Asian patients found that the most common cutaneous event was xerosis (52.5%), followed by acneiform eruption (27.3%), erythematous maculopapular rash (11.1%), mucositis (6.7%), and paronychia (5.1%).<sup>7</sup> A systematic review and meta-analysis involving 6,798 patients reported that cutaneous eruptions were associated with longer progression-free survival and overall survival.<sup>8</sup> Several prospective studies have been conducted in EGFR inhibitor-related cutaneous reactions in Caucasian patients, but prospective studies in Asian populations are scarce.

The aim of this study was to investigate the cutaneous side effects of EGFR inhibitors in Asian cancer patients and to assess tumor response to dermatologic manifestations.

## Material and Methods

The protocol for this prospective study was approved by the Siriraj Institutional Review Board (SIRB), Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. Patients with stage IV cancer who were being started with EGFR inhibitors at the Oncology Unit, Department of Medicine, Siriraj Hospital were enrolled and followed during the 2013 to 2016 study period. All patients were followed by oncologists and dermatologists for at least one year. Demographic data, cancer type with background therapy, duration from drug initiation to the first cutaneous reaction, and therapy for cutaneous toxicities were recorded and analyzed. Cutaneous toxicities were graded as 1, 2, 3, 4, and 5 according to Common Terminology Criteria for Adverse Events (CTCAE) guideline version 3.0, United States Department of Health and Human Services, National Institutes of Health, National Cancer Institute.<sup>5,9</sup> Overall survival, progression-free survival, and tumor response evaluation were assessed using the Response Evaluation Criteria in Solid Tumors (RECIST) guideline. For tumor response evaluation, complete response (CR) was defined as disappearance of all target lesions. Partial response (PR) was defined as reduction of  $\geq 30\%$  in the sum of diameters of target lesions from baseline. Progressive disease (PD) was diagnosed if there was at least a 20% increase in the sum of diameters of target lesions. Change in the sum of diameters of target lesions that did not meet one of the above-mentioned criteria was defined as stable disease (SD).<sup>10</sup> In this study, patients who experienced SD, PR, or CR as the best response at some period of time after EGFR inhibitors treatment were defined as responders. Non-responders were defined as patients who met with PD criteria after EGFR

inhibitors treatment as the best response. Cutaneous reactions and severity between responders and non-responders were compared. Written informed consent was obtained from all study participants prior to their inclusion in this study.

## Statistical analysis

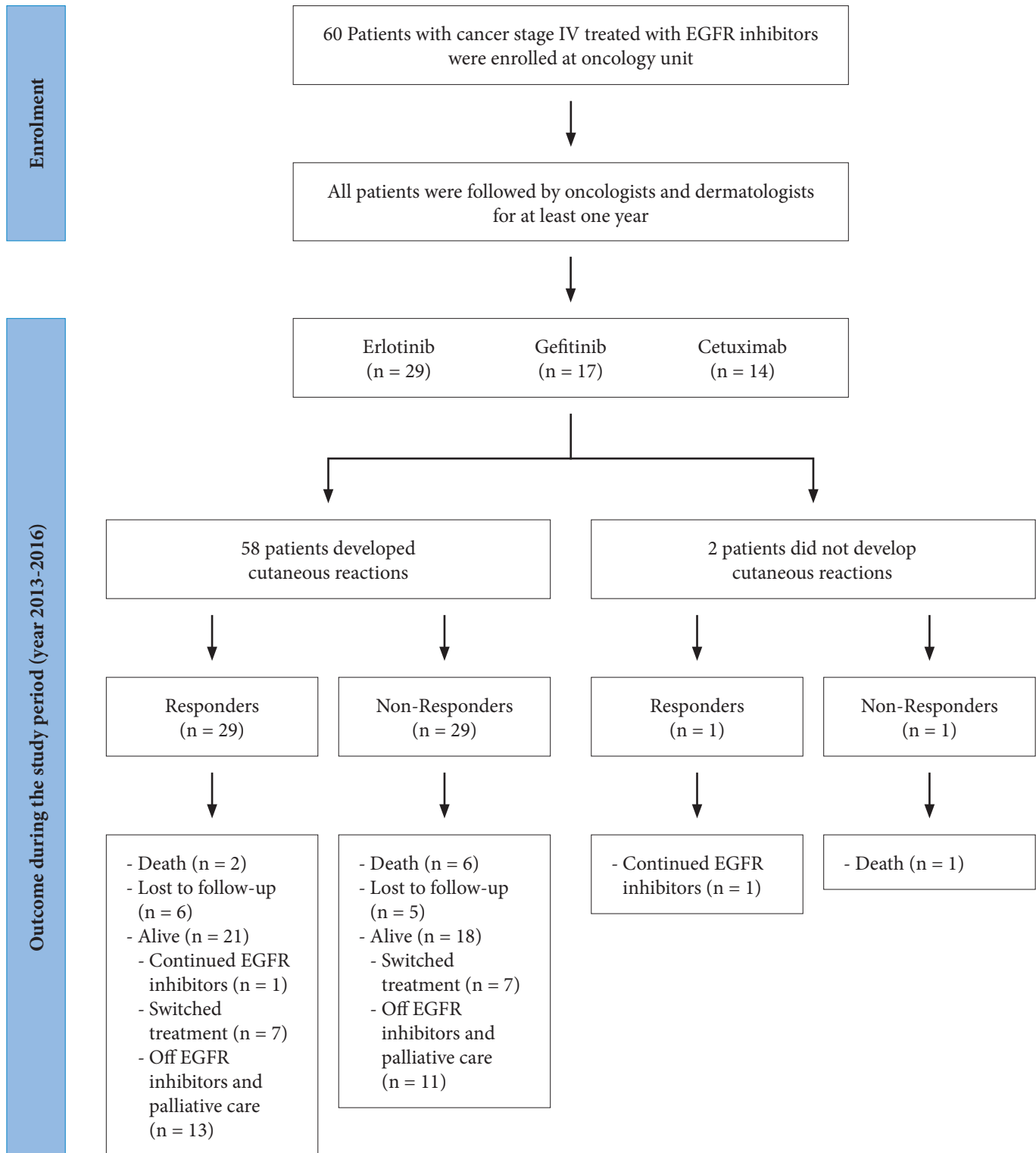
Data were analyzed using PASW Statistics version 18.0 (SPSS, Inc., Chicago, IL, USA). Descriptive statistics were used to describe demographic data, cancer type, and duration from drug initiation to the first cutaneous reaction. Associations between cutaneous toxicities and tumor response were determined using Chi-square test or Fishers's exact test. A *p*-value less than 0.05 indicated statistical significance.

## Results

A flow diagram of patient enrollment and patient outcome at the end of the study is shown in **Figure 1**. A total of 60 patients (33 males, 27 females) with a mean age of  $66.9 \pm 10.7$  years were included. Demographic data of our patients is shown in **Table 1**. All patients received EGFR inhibitor as a palliative treatment for metastatic lung or colorectal cancer. At the time of starting EGFR inhibitor, none of eligible patient experienced higher than grade 1 toxicity from previous cancer treatment. Forty-six patients had NSCLC and received tyrosine kinase inhibitors. Of those 46 patients, oral daily erlotinib 150 mg and oral daily gefitinib 250 mg were prescribed in 29 and 17 patients, respectively. Monoclonal antibodies (cetuximab) with an initial loading dose of 400 mg/m<sup>2</sup> was prescribed in 14 patients with colorectal cancer. All patients had stage IV cancer. Fifty-eight of 60 (96.7%) patients receiving EGFR inhibitor treatment developed cutaneous reactions. Number of responders was equal to that of non-responders. With the median follow up time of  $11.92 \pm 1.08$  months, 9 patients died

**Table 1. Demographic data of 60 patients treated with epidermal growth factor receptor (EGFR) inhibitors**

Demographic data	N/ 60 patients (%)
Males	33 (55)
Females	27 (45)
Underlying diseases	
Hypertension	28 (46.7)
Diabetes mellitus	13 (21.7)
Dyslipidemia	12 (20)
Types of cancer	
Non-small-cell lung cancer	46 (76.7)
Colorectal cancer	14 (23.3)
Previous treatments before EGFR inhibitors therapy	
Conventional chemotherapy	41 (68.3)
Radiotherapy	1 (1.7)
Conventional chemotherapies + Radiotherapy	6 (10)
Patients treated with tyrosine kinase inhibitors	
Erlotinib	29 (48.3)
Gefitinib	17 (28.3)
Patients treated with chimeric monoclonal antibodies	
Cetuximab	14 (23.3)
Concurrent treatments with EGFR inhibitors therapy	
None	38 (63.3)
Conventional chemotherapy	21 (35)
Radiotherapy	1 (1.7)



**Figure 1. Flow diagram of patient enrollment and outcome of epidermal growth factor receptor (EGFR) inhibitors**

from cancer and 11 patients lost to follow-up. Among 40 patients who were alive on the date of data cut-off, EGFR inhibitors were eventually discontinued in 38 patients because of progressive disease. Mean duration of EGFR inhibitor treatment was  $8.32 \pm 8.03$  months and  $5.83 \pm 4.28$  months in lung cancer and colorectal cancer patients, respectively.

The most common cutaneous toxicities in 58 patients were xerosis (48/58, 82.8%), acne/papulopustular rash (46/58, 79.3%),

and skin desquamation (36/58, 62.1%). Pruritus without skin xerosis and desquamation, mucositis, and trichomegaly/hypertrichosis were found in only 13.8% (8/58), 8.6% (5/58), and 5.2% (3/58) of patients, respectively. **Figure 2** demonstrates cutaneous toxicities from each EGFR inhibitor. Xerosis was more common in patients taking chimeric monoclonal antibodies (cetuximab) than in patients taking tyrosine kinase inhibitors. There were no statistically significant differences

in cutaneous toxicities between tyrosine kinase inhibitors and chimeric monoclonal antibodies. Type and time of onset of cutaneous reactions after initiation of EGFR inhibitors is shown in **Figure 3**. Acne/papulopustular eruption, xerosis, desquamation, and pruritus commonly occurred within one

month after starting treatment. Of 46 patients with acne, 54.3% developed papulopustular lesions within two weeks. The peak duration to the development of nail involvement (paronychia and/or painful fissures) was 6 to 8 weeks. Alopecia and trichomegaly occurred after 10 weeks of treatment.

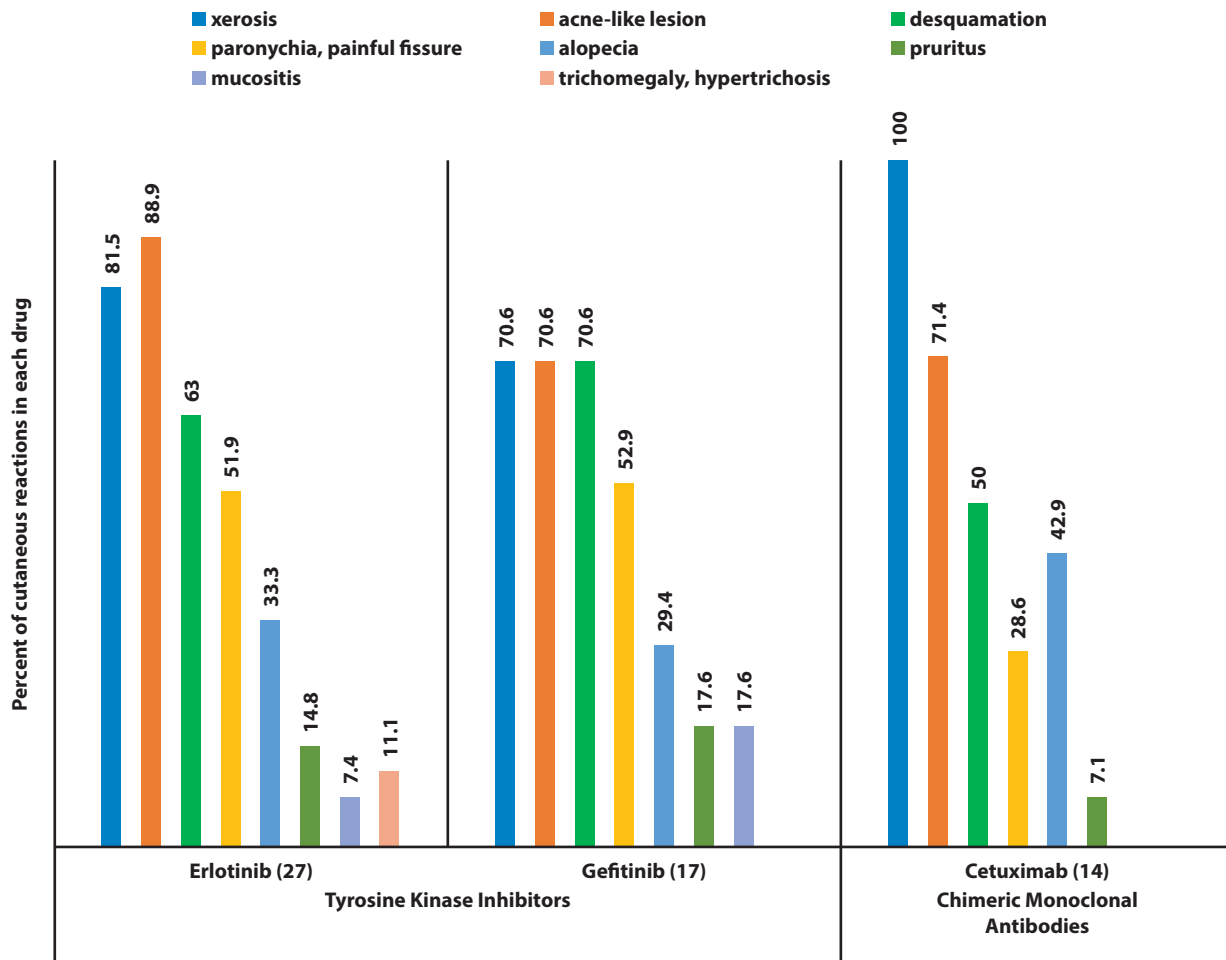


Figure 2. Cutaneous toxicities among types of epidermal growth factor receptor inhibitors



Figure 3a. Acne, xerosis, desquamation, and pruritus commonly occurred within one month after treatment. The peak duration of nail involvement (paronychia and/or painful fissures) was 6 to 8 weeks. Alopecia and trichomegaly occurred at week 10



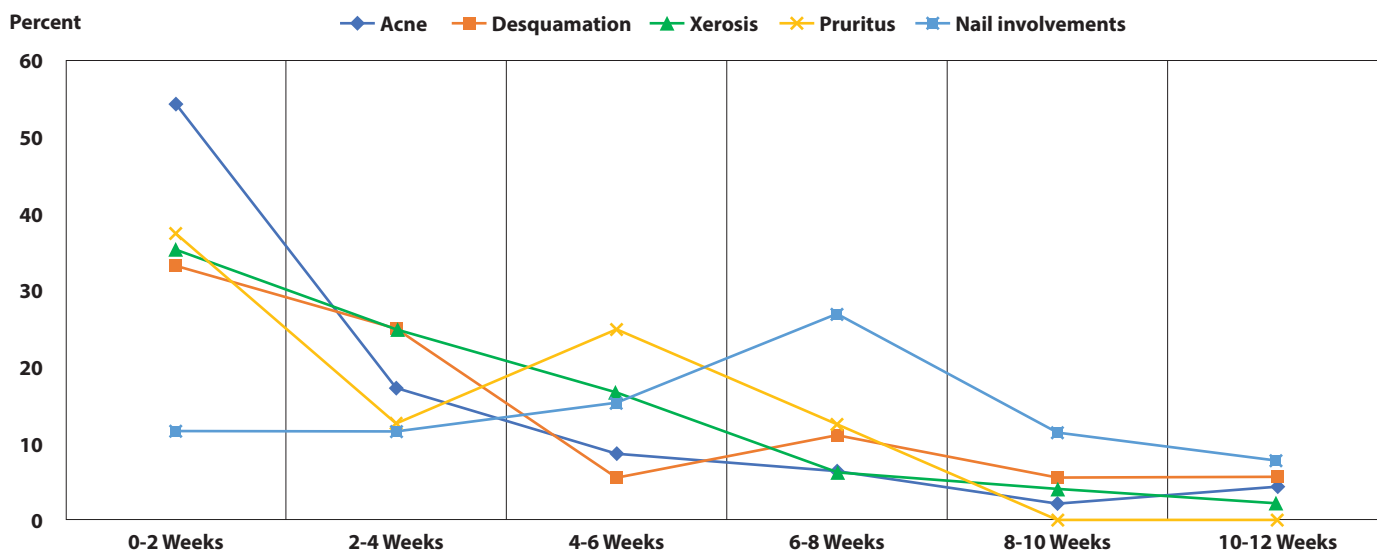


Figure 3b. The frequency of different clinical types of cutaneous reactions associated with duration of anti-EGFR treatment

Table 2. Grading severity of cutaneous reactions to epidermal growth factor receptor inhibitors

Cutaneous reactions	Overall	Grades 1-2	≥ Grade 3*
Xerosis, n (%)	48/58 (82.8)	45/48 (93.7)	3/48 (6.2)
Acne-like lesion, n (%)	46/58 (79.3)	41/46 (89.1)	5/46 (10.9)
Desquamation, n (%)	36/58 (62.1)	31/36 (86.1)	5/36 (13.9)
Paronychia/painful fissure, n (%)	27/58 (46.6)	27/27 (100)	0/27 (0)
Alopecia, n (%)	20/58 (34.5)	20/20 (100)	0/20 (0)
Pruritus**, n (%)	8/58 (13.8)	7/8 (87.5)	1/8 (12.5)

\* Grade 3 or higher indicates severe reactions, including those with pain, disfigurement, ulceration, or desquamation involving > 50% of the body surface area or that interfere with activities of daily living

\*\* Pruritus without skin xerosis and desquamation

Most patients were able to tolerate cutaneous reactions, because the level of severity of most reactions was not severe (Table 2). There was no fatal cutaneous toxicity founded in this cohort. Of 14 patients with ≥ grade 3 cutaneous reactions, 3 patients needed temporary interruption of EGFR treatment while another 3 patients required dose reduction. All patients were improved as a result of EGFR inhibitor modification with symptomatic treatment and then were able to resume the same treatment regimen at a later date. With regard to papulopustular reaction, bacterial cultures from pustular lesions from 11 patients showed positive for methicillin-sensitive *Staphylococcus aureus* in 10 patients (90.9%) and sterile pustule in 1 patient (9.1%). Treatment for acne-like lesions included topical antibiotics (clindamycin and erythromycin), 2% salicylic acid, and topical retinoids. Systemic

Table 3. Cutaneous reactions and severity between responders and non-responders

	Responders (n = 29)	Non-responders (n = 29)	p-value
<b>Cutaneous reactions</b>			
Acne, n (%)	24 (82.8%)	22 (75.9%)	0.52
Desquamation, n (%)	16 (55.2%)	20 (69.0%)	0.28
Xerosis, n (%)	23 (79.3%)	25 (86.2%)	0.49
Nail, n (%)	14 (48.3%)	13 (44.8%)	0.79
Alopecia, n (%)	13 (44.8%)	7 (24.1%)	0.10
<b>Acne severity</b>			
Intervention not indicated, n (%)	6 (20.7%)	6 (20.7%)	1.0
Intervention indicated, n (%)	15 (51.7%)	14 (48.3%)	0.80
Associated with pain, disfigurement, ulceration, and desquamation, n (%)	3 (10.3%)	2 (6.9%)	0.65
<b>Xerosis severity</b>			
Asymptomatic, n (%)	13 (44.8%)	18 (62.1%)	0.19
Symptomatic, not interfering with activities of daily living (ADL), n (%)	10 (34.5%)	4 (13.8%)	0.07
Symptomatic, interfering with activities of daily living (ADL), n (%)	0 (0%)	3 (10.3%)	0.08
<b>Desquamation severity</b>			
Lesions covering < 50% BSA with or without associated symptoms, n (%)	15 (51.7%)	16 (55.2%)	0.76
Lesions covering ≥ 50% BSA with or without associated symptoms, n (%)	1 (3.4%)	4 (13.8%)	0.16
<b>Number of skin reactions</b>			
1 type, n (%)	1 (3.4%)	4 (10.3%)	0.30
2 types, n (%)	6 (20.7%)	7 (24.1%)	0.76
≥ 3 types, n (%)	22 (75.9%)	19 (65.5%)	0.34

p-value < 0.05 indicates statistical significance; Responders were defined as patients with stable disease and partial response. Non-responders were defined as patients with progressive disease.

antibiotics (doxycycline and dicloxacillin) were used in patients with grade 3 severity. Low-dose systemic corticosteroids with a duration of less than 1 week were commenced in a few patients to rapidly reduce pain and skin inflammation. Moisturizers were recommended for use in all patients with xerosis and painful fissures on fingertips. Paronychia was treated with carbol-fuchsin in order to dry the wound and to prevent superinfection. There were no statistically significant differences in type, severity, or number of cutaneous reactions between responders and non-responders (Table 3).

## Discussion

The EGFR family is a group of receptor tyrosine kinases that consists of four members: ErbB1, ErbB2, ErbB3, and ErbB4. In normal human skin, ErbB1 is expressed in the basal layer of the epidermis, the outer root sheath of the hair follicle, and the sebaceous and eccrine epitheliums.<sup>3,11</sup> ErbB1 was also found to express in various types of cancers.<sup>3,11</sup> Cetuximab, erlotinib, and gefitinib target ErbB1, and these drugs are increasingly used in patients with metastatic cancer.<sup>11</sup> Due to their mechanisms of action, anti-EGFR therapies can cause both tumor suppression and cutaneous toxicities.

A retrospective study in Asians by Chanprapaph, *et al.* reported that 58.6% of patients taking erlotinib or gefitinib had skin reactions.<sup>7</sup> Our study found a higher rate of skin reactions (96.7%) from cetuximab, erlotinib, and gefitinib. Interestingly, both our study and the study by Chanprapaph, *et al.* were in Thai population. This difference may be due to one or more of the following factors: (i) the current study was a prospective study, (ii) skin examinations were performed by dermatologists, and (iii) skin reactions due to cetuximab were also investigated in our study. The incidence of xerosis due to EGFR inhibitor treatment was reported to vary from 4% to 100%.<sup>1,7,12-14</sup> This wide variation in incidence may be due to dissimilarities in xerosis definition, climate, and duration of drug administration among studies. However and similar to a previous study, we found xerosis to be the most common reaction among Thai patients.<sup>7</sup> Cetuximab was more likely to cause xerosis than erlotinib.<sup>9</sup>

Time duration to the development of each cutaneous reaction in Asian patients was comparable to the time durations found in Caucasian patients.<sup>1,7,14</sup> In this study, acne/papulopustular reaction, the most common reaction found in some other studies, developed between 1 and 3 weeks after the start of treatment. Paronychia and fissuring typically developed from 4 to 8 weeks after the start of treatment.<sup>14,18</sup> The pathogenesis of cutaneous reactions is not well understood. Disruption in normal EGFR functions, which include maintaining and controlling skin integrity and inflammation, is believed to be the main pathogenic factor.<sup>9,15,16</sup> For acne-like lesions, histopathology results showed 2 major patterns of reaction without sebaceous gland involvement, as follows: (i) suppurative folliculitis with associated rupture of epithelial lining; and, (ii) inflammatory cell infiltrate surrounding follicular infundibula, especially in the upper portion of the hair follicle.<sup>9,14,16</sup> Similar to our study, bacterial cultures from pustular lesions revealed sterile pustule or *Staphylococcus aureus* overgrowth.<sup>14-17</sup> Due to inconclusive pathogenesis

and superimposed bacterial infection, corticosteroids, antibiotics, retinoids, and immunomodulatory agents have been used empirically to treat acne-like reaction with varying response.<sup>14,16,18</sup>

A randomized phase III study evaluated the effect of prophylactic treatment with minocycline for erlotinib-induced skin rash in 150 NSCLC patients.<sup>19</sup> They found that prophylactic treatment did not decrease the incidence of skin reaction. Moreover, skin reaction was not self-limiting. Nevertheless, prophylactic treatment was able to reduce the severity of skin reaction, improve patient compliance, and did not interfere with the efficacy of erlotinib. Two meta-analyses involving 6,798 NSCLC patients treated with tyrosine kinase inhibitors (erlotinib and gefitinib) and 1,961 solid tumor patients treated with cetuximab reported that cutaneous reactions were found to be associated with better survival outcomes.<sup>8,20</sup> Chanprapaph, *et al.* studied 99 Thai patients, which is the same ethnicity as the population in this study, and found that skin rashes were significantly associated with tumor response.<sup>7</sup> We believe that our study was not able to demonstrate this same significant association due to the comparatively small size of our study population.

In conclusion, almost all patients in this study (96.7%) developed cutaneous toxicities of EGFR inhibitors. Xerosis, acne, and skin desquamation were common cutaneous reactions. Most reactions were mild and were well tolerated with supportive treatment. Although current evidence suggests that cutaneous reactions seem to associate with tumor response, our study was not able to demonstrate this association due to limited number of patients.

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## Conflict of interest declaration

The authors hereby declare no personal or professional conflicts of interest regarding any aspect of this study.

## Funding disclosure

This was an unfunded study.

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# Natural course and prognostic factors of chronic urticaria in Korean children: A single center experience

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## Abstract

**Background:** Chronic urticaria (CU) has an adverse effect on academic achievement and psychosocial development in children.

**Objective:** We aimed to investigate the natural course of CU and to identify relevant factors associated with a poor CU prognosis in Korean children.

**Methods:** We retrospectively analyzed 253 children with episodes of wheals or angioedema at least 3 times a week that persisted for at least 6 weeks. Clinical data and laboratory results were obtained from medical records and parental telephone interviews. Kaplan-Meier survival analysis and log rank tests were performed to assess the median time to remission of CU and prognostic factors.

**Results:** Median age at onset was 5.0 years (interquartile range, 2.5-9.1) and median follow-up period was 7.6 months (interquartile range, 3.9-19.7). Of 253 patients, 68.8% had chronic inducible urticaria and 31.2% had chronic spontaneous urticaria. Physical urticaria was the only cause of chronic inducible urticaria, and the most common physical urticaria was dermographism. Median duration to remission of CU was 10.2 months (95% confidence intervals, 8.0-12.5 months). Kaplan-Meier analysis revealed that 33.4%, 53.0%, and 71.2% of children were in remission at 6, 12, and 24 months, respectively, after the onset of CU. The presence of allergic sensitization was significantly associated with a poor CU prognosis in univariable and multivariable analyses ( $P = 0.010$  and  $P = 0.014$ , respectively).

**Conclusion:** Half of children with CU were in remission 10.2 months after onset. Allergic sensitization was a risk factor associated with longer duration CU.

**Key words:** Chronic urticaria; Etiology; Prognosis; Children; Korea

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## Introduction

Chronic urticaria (CU) is defined as wheals with recurrent episodes lasting for at least 6 weeks. The prevalence of CU has been reported to be 0.5-5.0% in the general population and about 0.1-0.3% in children.<sup>1</sup> It is known that children have a lower prevalence than adults.<sup>1</sup> Impairment of quality of life in patients with CU is comparable to that experienced by patients with other chronic diseases such as ischemic heart disease, cystic fibrosis, and epilepsy.<sup>2,3</sup> CU impairs physical activity and lowers social function, which can adversely affect academic achievement and psychosocial development in children and adolescents.<sup>3-5</sup>

Acute urticaria is frequently associated with identifiable causes; in contrast, it is difficult to identify the etiology and to predict the occurrence or exacerbation of symptoms in patients with CU.<sup>3,6</sup> CU is classified into chronic spontaneous urticaria (CSU) and chronic inducible urticaria (CIU) according to



whether symptoms occur spontaneously or are induced by specific triggers. Although its pathogenesis is poorly understood, mixed Th1/Th2 immune response, mast cell activation, and autoimmunity are considered to play a role in the pathogenic mechanisms of CU.<sup>7</sup> There are also several types of evidence supporting that autoantigen is synthesized by specific triggers leading to mast cell degranulation in patients with CIU.<sup>8,9</sup> In general, the duration of CSU is 1-5 years, but is likely to be longer in more severe cases.<sup>10</sup> A previous Korean study reported that the mean duration of CU was 3.76 years in 641 adults.<sup>11</sup> The co-existence of angioedema, severity, and the presence of anti-thyroid antibodies were associated with longer disease duration in adult patients with CU.<sup>12,13</sup> However, few studies have investigated the clinical course or identified prognostic factors of CU in children.

Providing correct information about the etiology and prognosis of CU can reduce the anxiety of pediatric patients and their parents and help establish a therapeutic plan. Therefore, our aims in this study were to investigate the clinical features and natural course of CU and to identify factors associated with a poor CU prognosis in Korean children.

## Methods

### Patients

We retrospectively reviewed the medical records of patients 18 years or younger that were diagnosed with CU in Samsung Medical Center from January 2007 to October 2015. The definition of CU was wheals or angioedema that had lasted for more than 6 weeks and that occurred repeatedly (more than 3 times a week).

Demographic and clinical data were collected, including gender, age at onset, duration and frequency of symptoms, extent of skin involvement, presence of angioedema, and personal and familial history of allergic or autoimmune diseases. A family history of allergic diseases was defined as the presence of one or more family members diagnosed with atopic dermatitis, asthma, or allergic rhinitis by a physician.

All patients were interviewed using a detailed questionnaire regarding trigger factors such as sunlight, cold, water, pressure, vibration, exercise, scratching, foods, and drugs. Food or drug allergy was defined as a convincing history of reproducible symptoms within two hours after exposure to a single food or drug. Physical urticaria was diagnosed according to the criteria described by the Joint Task Force on Practice Parameters.<sup>14</sup> In addition, medication use for CU (H1 receptor antagonists, H2 receptor antagonists, leukotriene antagonists, oral corticosteroids, immunosuppressants, and biologic agents) was investigated.

For patients who did not have data regarding remission of clinical symptoms on their medical chart, a telephone survey was utilized based on a preformulated questionnaire. Complete remission of CU was defined as the absence of urticaria for at least 4 weeks without any medication.<sup>13</sup> This study was approved by the Institutional Review Board (IRB) of Samsung Medical Center (IRB No.2015-06-188).

### Laboratory test

Eosinophils in peripheral blood, erythrocyte sedimentary

rate (ESR), thyroid stimulating hormone (TSH), free thyroxine (T4), triiodothyronine (T3), thyroid auto-antibodies (thyroglobulin antibody, antimicrosomal antibody, and TSH receptor antibody), antinuclear antibody (ANA), serum C-reactive protein (CRP) levels, and serum total immunoglobulin E (IgE) levels were measured. The ANA test was performed by using the indirect immunofluorescence method with sera (Fluoro Hepana Test, MBL, Nagoya, Japan). Skin prick tests (Allergopharma, Reinbek, Germany) on the back or measurement of specific IgE levels with the immunoCAP system (Thermo Fisher Scientific Inc., Waltham, MA, USA) were performed regarding the following common allergens: *Dermatophagoides pteronyssinus*, *D. farinae*, egg white, cow's milk, wheat, soybean, and peanut. Positive sensitization was defined as a wheal diameter  $\geq 3$  mm or specific IgE levels  $\geq 0.35$  kU/L. Eosinophilia was defined as when eosinophils comprised more than 4% of total leukocytes.

### Statistical analysis

Data for continuous variables were shown as median and interquartile range. Prevalence rates were shown as percentages. Cumulative survival curves were estimated by the Kaplan-Meier method, and relationships between the cumulative probability of complete remission of CU and prognostic factors were analyzed using a log-rank test. The influence of prognostic factors on remission was evaluated by univariable Cox regression analyses. The relative importance of multiple prognostic factors on CU remission was analyzed using multivariable analysis in conjunction with the Cox proportional regression model. Variables with a *P* value of less than 0.2 in univariable analysis were chosen for multivariable analysis. Candidate variables for adjustment included gender, age at onset ( $< 5$  years old or  $\geq 5$  years old), clinical symptoms (with or without angioedema), past history of allergic diseases (presence or absence), past history of autoimmune diseases (presence or absence), family history of allergic diseases (presence or absence), inducible factors (presence or absence), sensitization (negative or positive), total IgE ( $< 200$  kU/L or  $\geq 200$  kU/L), eosinophils ( $< 4\%$  or  $\geq 4\%$ ), ESR ( $< 20$  mm/hr or  $\geq 20$  mm/hr), CRP ( $< 0.5$  mg/dl or  $\geq 0.5$  mg/dl), and ANA (negative or positive). CRP and ANA were excluded from the multivariate analysis because data were available for only 80 and 88 cases, respectively. Statistical analyses were performed using SAS (version 9.3, SAS Institute, Inc., Cary, NC, USA). Statistical significance was defined as a *P* value  $< 0.05$ .

## Results

A total of 253 patients (142 boys and 111 girls) with CU were included in this study. Median age at onset was 5.0 years (interquartile range, 2.5-9.1) and median follow-up period was 7.6 months (interquartile range, 3.9-19.7 months) (Table 1). Overall, 29 (11.5%) patients had wheals accompanied with angioedema, while 224 (88.5%) patients had wheals only without angioedema. Of 253 patients, 68.8% (174/253) had CIU, while 31.2% (79/253) had CSU. The only cause of 174 patients with CIU was physical urticaria, and the most common physical urticaria was dermographism (128/174, 73.6%), followed by cholinergic urticaria (76/174, 43.7%), cold urticaria (15/174,

**Table 1. Subject characteristics and laboratory results (n = 253).**

Characteristic	Number (%)
Gender	
Male	142 (56.1)
Female	111 (43.9)
Age (years)*	5.0 (2.5-9.1)
< 1	15 (5.9)
1-4	111 (43.9)
5-9	78 (30.8)
≥ 10	49 (19.4)
Clinical symptoms	
Wheals only	224 (88.5)
Angioedema with wheals	29 (11.5)
Past medical history	
Atopic dermatitis	52 (20.1)
Allergic rhinitis	38 (15.0)
Asthma	24 (9.5)
Autoimmune disease	7 (2.8)
Family history of allergic diseases	
Atopic dermatitis	20 (7.9)
Allergic rhinitis	38 (15.0)
Asthma	8 (3.2)
Allergic sensitization**	90/219 (41.1)
Log(total IgE, kU/L)	2.0 ± 0.5

\*Data are presented as medians (interquartile range).

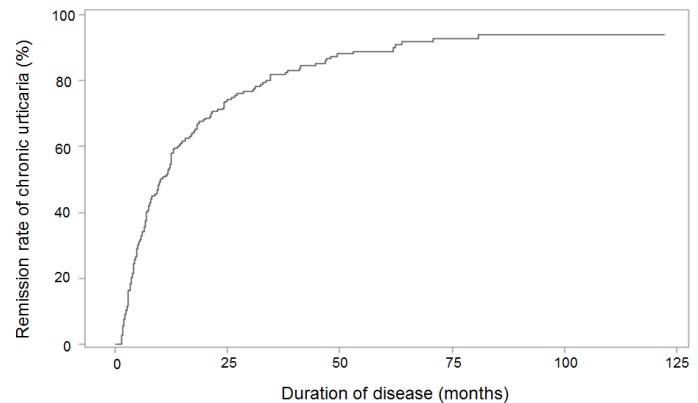
\*\* 219 patients underwent either a skin prick test or ImmunoCAP test.

8.6%), delayed pressure urticaria (9/174, 5.2%), solar urticaria (3/174, 1.7%), and aquagenic urticaria (2/174, 1.1%).

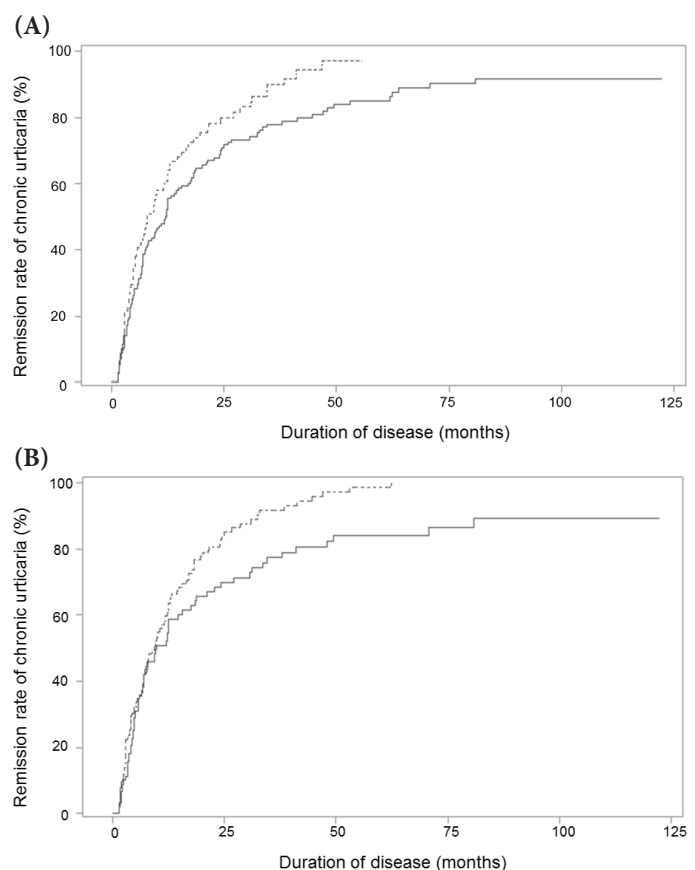
Twenty-two (25.0%) of 88 patients who underwent testing for ANA were positive. However, only one of these 22 patients had a history of autoimmune diseases such as systemic lupus erythematosus. TSH was elevated in only one of the 50 patients (2.0%) who underwent a thyroid function test, but her T3 and free T4 levels were normal.

During the observation period, 195 (77.0%) children had complete remission. In Kaplan-Meier survival analysis, 33.4%, 53.0%, and 71.2% of patients recovered at 6, 12, and 24 months after symptom onset, respectively (**Figure 1**). The median duration of CU was 10.2 months (95% confidence intervals, 8.0-12.5 months). A total of 190 patients (75.1%) were treated with H1 receptor antagonist alone, while 50 patients (19.8%) received two H1 receptor antagonists or more. In addition, nine patients (3.6%) were treated with both H1 and H2 receptor antagonists, and 21 patients (8.3%) were treated with an H1 receptor antagonist as well as a leukotriene receptor antagonist. No patients required systemic corticosteroids for ≥ 3 consecutive days or anti-IgE treatment.

Univariable analysis showed that patients who did not have inducible factors (CSU) or were not sensitized to common allergens had a better prognosis than those who had CIU or were sensitized to allergens ( $P = 0.021$  and  $P = 0.010$ , respectively) (**Table 2**). In Kaplan-Meier survival analysis, 40.7%, 60.9%, and 78.3% of CSU recovered at 6, 12, and 24 months after symptom onset, respectively. In addition, 29.4%, 49.4%, and 67.8% of CIU patients recovered at 6, 12, and 24 months,



**Figure 1. Kaplan-Meier survival analysis of children with chronic urticaria. Remission percentages at 6, 12, and 24 months after symptom onset were 33.4%, 53.0%, and 71.2%, respectively.**



**Figure 2. (A) Kaplan-Meier analysis of CIU (solid line) and CSU patients (dashed line). (B) Kaplan-Meier analysis of CU patients sensitized (solid line) and non-sensitized (dashed line) to allergens. CIU: chronic inducible urticaria, CSU: chronic spontaneous urticaria, CU: chronic urticaria.**

respectively (**Figure 2**). Median disease duration was 8.0 months for children with CSU and 12.1 months for those with CIU, and 9.4 months for non-sensitized patients and 9.8 months for sensitized patients. Median recovery time was 9.6 months for boys, 10.8 months for girls, 9.6 months for younger children (< 5 years old), and 12.0 months for older children (≥ 5 years

**Table 2. Predictors of remission of chronic urticaria according to univariable and multivariable Cox analyses.**

Variables	Univariable analysis			Multivariable analysis		
	Hazard ratio	95% CI	P Value	Hazard ratio	95% CI	P Value
Gender						
Female	1					
Male	0.945	(0.712-1.255)	0.697			
Age						
< 5 years	1			1		
≥ 5 years	1.276	(0.962-1.692)	0.092	1.006	(0.740-1.366)	0.970
Clinical symptoms						
Wheals only	1			1		
Angioedema with wheals	1.383	(0.861-2.222)	0.180	1.393	(0.837-2.320)	0.202
Past history of allergic diseases						
No	1					
Yes	1.171	(0.879-1.563)	0.281			
Past history of autoimmune diseases						
No	1			1		
Yes	2.451	(0.907-6.623)	0.077	1.698	(0.520-5.556)	0.381
Family history of allergic diseases						
No	1					
Yes	1.068	(0.770-1.479)	0.693			
Inducible factors						
No	1			1		
Yes	1.422	(1.054-1.919)	0.021	1.330	(0.965-1.835)	0.081
Sensitization						
Negative	1			1		
Positive	1.511	(1.104-2.070)	0.010	1.486	(1.083-2.037)	0.014
Total IgE						
< 200 kU/L	1					
≥ 200 kU/L	1.093	(0.791-1.511)	0.590			
Eosinophils						
< 4%	1					
≥ 4%	1.059	(0.756-1.486)	0.737			
ESR						
< 20 mm/h	1					
≥ 20 mm/h	0.745	(0.436-1.274)	0.282			
CRP						
< 0.5 mg/dl	1					
≥ 0.5 mg/dl	0.669	(0.398-1.122)	0.128			
ANA						
Negative	1					
Positive	1.733	(0.920-3.268)	0.089			

ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; ANA, anti-nuclear antibody.

old). However, there were no significant differences in disease duration with respect to gender or age ( $P = 0.697$  and  $P = 0.092$ , respectively) (Table 2). In addition, no differences were found in CU prognosis according to the presence of angioedema, past medical history, family history of allergic diseases, or laboratory findings such as total IgE level, eosinophilia, ESR, CRP level, or ANA status.

Multivariable analysis also showed that allergic sensitization was associated with the persistence of CU ( $P = 0.014$ ), and a trend for an association between the presence of inducible factors and remission was seen ( $P = 0.081$ ) (Table 2). There were no differences between patients who went into remission and

those that did not with respect to gender, age, the presence of angioedema, past medical history, family history of allergic diseases, or laboratory findings.

### Discussion

In our present study, we found that half of children with CU achieved remission at 10.2 months after disease onset. Overall, 33.4%, 53.0%, and 71.2% of patients achieved remission within 6, 12, and 24 months of disease onset. In addition, the symptoms of about 75% of subjects improved in response to avoiding inducible factors or taking a regular dose of H1 antihistamines, indicating a good response to conservative or

medical treatment. To our knowledge, this is the largest study to describe the natural course and prognostic factors of CU in Korean children. The percentages of patients in remission at different time points in the present study are inconsistent with those reported in previous studies.<sup>15-20</sup> Kang et al. reported a remission rate of 84.8% in Korean children with CU at the 1 year follow-up, which is a better prognosis than what we found in our study.<sup>15</sup> However, previous studies conducted in other countries reported poorer outcomes than those of our study and Kang et al.'s study. For example, a Turkish study reported that 16.5%, 38.8%, and 50.0% of children with CSU showed an improvement in symptoms within 1 year, 3 years, and 5 years after disease onset, respectively.<sup>20</sup> In Japanese and Thai studies, symptoms improved in 36.6% and 18.5% of patients, respectively, within 1 year.<sup>16,18</sup>

Disagreement among studies on the natural course of CU might be due to differences in the study population and the presence of inducible factors. In particular, a younger age is likely to be associated with a better prognosis.<sup>15,20</sup> In our study, the median age of patients was 5 years old, which is younger than the 8-9 years described in previous studies.<sup>18-20</sup> In addition, gender might affect the prognosis of CU, because sex hormones are involved in the action of mast cells, peripheral basophil cells, and dendritic cells.<sup>4,21</sup> In our study, the male to female ratio was 1.3: 1, which is a higher male predominance than in other studies.<sup>18,22</sup> According to previous reports, 50-80% of CU patients have CSU, and only 25-40% of CU patients have inducible factors, of which physical urticaria is the most common.<sup>22-24</sup> In our study, 31.2% of the patients did not have any cause of urticaria, despite detailed history taking and several tests. The only cause of CIU was physical urticaria, and dermatographism was the most common cause of physical urticaria, consistent with another domestic study that reported that 42.3% of CU patients have dermatographism.<sup>15</sup> In the present study, the proportion of CSU was lower than that reported in a previous study.<sup>15</sup> We should note that our study included dermatographism as an inducible CU factor, whereas previous studies did not.

Allergic sensitization was the only factor that was associated with a worse CU prognosis in our present study, although the importance of atopic status in the pathogenesis of CU is still a matter of debate. A previous Korean study showed that children with CU had a higher prevalence of sensitization against house dust mites and polysensitization compared to those with acute urticaria.<sup>25</sup> Several previous studies have reported a higher prevalence of sensitization to variable allergens in patients with CU than in normal healthy subjects,<sup>26-28</sup> while fewer studies have investigated the association between atopy and CU prognosis. Recently, Song et al. reported that house dust mite sensitization as well as a positive autologous serum skin test (ASST) result were related to more severe disease and a longer disease duration.<sup>26</sup> Furthermore, higher expression of CD63 was observed in basophils from patients with CSU, suggesting that spontaneous basophil activation may contribute to the relationship between CU and allergic sensitization.<sup>29</sup>

The presence of inducible factors and/or autoimmune antibodies have been considered poor prognostic factors in previous studies of patients with CU, although this is still controversial.<sup>15,22,23</sup> We found a trend for an association between

the presence of inducible factors and remission without statistical significance in the multivariable analysis. A previous study found that symptom duration was longer in autoimmune urticaria, which was defined in that study as positive ASST, than CSU.<sup>23</sup> In that study, 17% of patients with CU were ANA positive, and 16% and 12% of patients were positive for anti-thyroglobulin antibodies and anti-microsomal antibodies, respectively.<sup>23</sup> These autoantibodies could activate basophils and mast cells to induce histamine release by complement.<sup>30</sup> In our present study, the ANA-positive rate was 25.0%, which is higher than that reported previously for healthy children (6-14%).<sup>31,32</sup> However, we did not find an association between CU prognosis and autoimmune diseases, because the number of patients who received tests for autoantibodies was too small for robust statistical evaluation of this relationship. Further studies are required to identify additional prognostic factors in children with CU.

Our study had some limitations that must be considered. This study was performed in a single tertiary center, therefore selection bias might have occurred. Secondly, remission state was determined by telephone survey for some patients because of the retrospective nature of the study. There might be patients with persistent disease who were selectively missed, although the telephone survey was done with all caregivers whose children were lost to follow-up. In addition, the same laboratory tests were not performed in all patients. Therefore, some variables such as CRP and ANA were not included in the multivariable analysis. It also should be noted that the treatment in our present study followed Korean Expert Opinion Report on the Management of CU, not international guidelines.<sup>6</sup> Despite these limitations, we provide useful information regarding the natural course of CU in children, the response rates to medical treatment, and prognostic factors.

In conclusion, the median duration of CU in Korean children was 10.2 months, and half of the CU patients showed symptom improvement within a year, indicating that children with CU had a favorable outcome. Allergic sensitization was associated with a poor CU prognosis.

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# Assessment of small airway function and reversibility in symptom-controlled asthma in pediatric patients

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## Abstract

**Background:** The goals of asthma management aim to control the symptoms and minimize future risk. There is, however, an option to stop controller medication if the patient has been well-controlled for at least 6-12 months. To assess control, both clinical symptom assessment and lung function should be monitored periodically. In practical clinical practice of pediatric patients, lung function is not available at all health centers.

**Objectives:** to determine lung function with a focus on small airway function and the risk of reversibility among children who have been symptom-controlled.

**Methods:** Our participants were symptom-controlled asthmatic children according to GINA Guideline for at least 6 months with low dose inhaled corticosteroid. Written informed-consent was given by the parents and the children. They performed a self-evaluated symptom-controlled test (C-ACT) and a spirometric assessment. Abnormal lung function was defined as  $FEV_1 < 80\%$ ,  $FEV_1/FVC < 80\%$ , and  $FEF_{25-75} < 65\%$  predicted. Airway reversibility was determined by the change of  $FEV_1 > 12\%$  and  $FEF_{25-75} > 30\%$  post bronchodilator.

**Results:** Forty children (65% male) were enrolled. Age ranged between 6.7 and 15.0 years. The mean C-ACT score was  $25.2 \pm 1.7$ . Spirometry results were: mean  $FEV_1$  84.0%,  $FEV_1/FVC$  87.8%, and  $FEF_{25-75}$  85.5% predicted. Normal  $FEV_1$  was found among 72.5% of participants compared to normal  $FEF_{25-75}$  in 87.5%. Among the abnormal  $FEV_1$  and  $FEF_{25-75}$ , all were of mild severity as 10% retained airway reversibility.

**Conclusion:** Children with well-controlled asthma, based on their symptom assessment, may have persistent abnormal lung function. Spirometry should be performed before considering cessation of controller medication.

**Key words:** small airway function, symptom-controlled, asthma, pediatric, spirometry

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## Introduction

According to the terms of the Global Initiative for Asthma, the long-term goals for asthma management are to achieve good symptom control, and to minimize future risk of exacerbations, fixed airflow limitation, and side-effects of treatment. Symptom control is assessed using the frequency of daytime & night-time asthma symptoms, reliever use, and activity limitation. Poor symptom control is a risk factor for future exacerbations. Lung function, which mainly focuses on  $FEV_1$  and the  $FEV_1/FVC$  ratio, should be assessed as an indicator of future risk.<sup>1</sup>

It is well-established that inflammation in asthma involves not only the large airways, but also the small airways; the

complete airway tree.<sup>2-3</sup> Small airway inflammation is recognized as having an important role in controlling asthma. Increasing evidence suggests that small-airway dysfunction is associated with the clinical features of asthma: such as poor control of asthma and higher numbers of exacerbations.<sup>4</sup> In addition, significant bronchodilator reversibility in a patient taking controller treatment also suggests uncontrolled asthma.<sup>1</sup>

When asthma is well-controlled, complete cessation of inhaled corticosteroids (ICS) in adults is not advised as the risk of exacerbations is increased.<sup>1,5</sup> Nevertheless, a physician may consider stopping controller treatment only if there have

been no symptoms for 6-12 months and the patient has no risk factors.<sup>1</sup> By comparison, in clinical pediatric practice, routine lung function at each visit is difficult to perform. Moreover, spirometry is not available at all health care centers in our country (Thailand), so assessment of asthma symptom control is accomplished for reviewing the treatment and controller cessation. Even if children have clinical symptom control, the future risk of exacerbations and lung function (especially of the small airways) are unknown. We, thus, conducted the current study to determine lung function with a focus on small airway function as well as the risk of reversibility among children who have been assessed as symptom-controlled.

## Methods

We conducted a prospective, descriptive study in children diagnosed with asthma according to the GINA guideline, and who regularly attended the Pediatric Asthma Clinic at Srinagarind Hospital, Khon Kaen University, between August 2015 and August 2016. At each visit, the patients were assessed for symptoms and those who met the criteria for symptom-controlled were invited to participate in the study. This study was approved by the Ethics Committee for Human Research of Khon Kean University, Thailand (HE581314)

### Study populations

The enrolled asthmatic children were: 1) between 6 and 15 years of age and regularly attended the clinic; 2) symptom-controlled for at least 6 months [as per the GINA 2015 clinical assessment]; 3) regularly used low-dose inhaled controllers (Budesonide  $\leq$  200  $\mu$ g/day or Fluticasone  $\leq$  200  $\mu$ g/day plus LABA)<sup>1</sup>; and, 4) able to perform spirometry. Each child participant had informed, written parental consent as well as giving their own assent. Children who had had a respiratory infection in the previous 1 month and had a history of using bronchodilator before performing spirometry within 6 hours were excluded. The symptom control score for the last 1-month of symptoms was determined using the childhood asthma control test (C-ACT).<sup>6</sup> Parents and children who met all the eligibility criteria were asked to perform the C-ACT test before performing spirometry.

**Symptom-controlled (GINA clinical assessment)<sup>1</sup>** was defined as exhibiting none of the following: daytime symptoms more than twice/week; any night waking due to asthma; any reliever needed for symptoms more than twice/week; and, any activity limitation due to asthma in the past 4 weeks.

**Childhood asthma control test (C-ACT)<sup>6</sup>** is a seven-item assessment questionnaire, completed by the child and parent/caregiver, for assessing asthma control in children between 4 and 11 years of age in the preceding four weeks: the total score ranges between 0 and 27. The C-ACT test has been translated into Thai; however, it has not been validated in Thai children.

### Pulmonary and small airway function

Lung function was measured using a CHEST multifunctional spirometer HI – 801. Typical FEV<sub>1</sub> and FEV<sub>1</sub>/FVC were evaluated to determine large airway function, and FEF<sub>25-75</sub> —

the latter reflecting small airway function. Repeated spirometry was performed 15 minutes after giving inhaled Ventolin Evohaler® plus spacer to determine reversibility of the airway. Abnormal lung function was defined as FEV<sub>1</sub> < 80% predicted, FEV<sub>1</sub>/FVC < 80%, and FEF<sub>25-75</sub> < 65%. Post bronchodilator, the change of FEV<sub>1</sub> > 12% and FEF<sub>25-75</sub> > 30%, was positive for reversibility.<sup>7-8</sup> Spirometry was performed by the same trained nurse throughout the study. We documented the demographic characteristics, BMI, family history of atopy, co-morbidity, time of asthma diagnosis, and treatment.

### Study analysis

Statistical analyses were performed using SPSS version 19. Continuous data were presented as means  $\pm$  SD, medians, and ranges (minimum to maximum). Categorical data were presented as numbers and percentages of each group.

## Results

There were 40 patients who met the eligibility criteria. There were 26 (65%) males and overall the age ranged between 6.7 and 15.0 years (mean, 10.4  $\pm$  2.0). BMI ranged between 12.4 and 27.2 (mean, 18.3  $\pm$  3.8 kg/m<sup>2</sup>). No child was diagnosed as obese (i.e., BMI  $\geq$  95<sup>th</sup> percentile). Half of the patients had a co-morbidity with the allergic rhinitis. Sixteen (40%) had a family history of smoking by the father and/or grandfather. Other baseline characteristics included mean duration of diagnosed asthma (6.0  $\pm$  3.2 years) and mean duration of taking ICS (5.6  $\pm$  3.3 years). Half of the patients had had a history of admission due to asthma exacerbation prior to being symptom-controlled (average, 2 times). (Table 1) No child had severe

**Table 1. Demographic characteristics of the studied population**

Characteristic	Total N = 40
Sex, male (%)	26 (65.0)
Age, (years)	
mean $\pm$ SD	10.4 $\pm$ 2.0
median (min - max)	10.4 (6.7 - 15.0)
BMI, (kg/m <sup>2</sup> )	
mean $\pm$ SD	18.3 $\pm$ 3.8
median (min - max)	18.1 (12.4 - 27.7)
Birth weight, (g)	
mean $\pm$ SD	3,160.6 $\pm$ 337.7
median (min - max)	3,175.0 (2,100 - 3,750)
Breastfeeding, (months)	
mean $\pm$ SD	3.1 $\pm$ 2.0
median (min - max)	3.0 (1 - 8)
Family history of smoking, n (%)	16 (40.0)
History of allergic rhinitis, n (%)	20 (50.0)
History of admission prior to symptom-controlled, n (%)	20 (50.0)
mean number of admissions	2.1 $\pm$ 1.8
median (min - max)	1.0 (1 - 6)
History of severe exacerbation or admission in the past year	0

exacerbation or admission during the past year. The mean C-ACT score was  $25.2 \pm 1.7$ . The range of C-ACT for most children was 22 to 27. Only one child had a score as low as 19. Additional diagnoses and treatment are shown in **Table 2**.

Lung function outcomes were: mean of predicted  $FEV_1$   $84.0 \pm 8.1\%$ ,  $FEV_1/FVC$   $87.8 \pm 6.1\%$ , and  $FEF_{25-75}$  predicted  $85.5 \pm 20.0\%$ . Normal large airway function ( $FEV_1 \geq 80\%$ ) was found in only 72.5% of participants while normal small airway function ( $FEF_{25-75} \geq 65\%$ ) was found in 87.5%. Among the abnormal  $FEV_1$  and  $FEF_{25-75}$ , all were of mild severity ( $FEV_1 > 70\%$

**Table 2. Treatment and symptom control of asthma prior to enrollment**

Disease characteristic	
Onset of suspected asthma symptoms (years)	
mean $\pm$ SD	3.5 $\pm$ 3.2
median (min - max)	2.0 (0.1 - 12.0)
Age at asthma diagnosis (years)	
mean $\pm$ SD	4.3 $\pm$ 3.2
median (min - max)	4.0 (0.8 - 12.0)
Duration of asthma (years)	
mean $\pm$ SD	6.0 $\pm$ 3.2
median (min - max)	5.8 (0.8 - 11.2)
Duration of taking ICS (years)	
mean $\pm$ SD	5.6 $\pm$ 3.3
median (min - max)	5.1 (0.8 - 11.2)
Duration of controlled symptoms (years)	
mean $\pm$ SD	1.7 $\pm$ 2.3
median (min - max)	1.0 (0.5 - 10.0)
Maximum daily dose of controller prior to symptom-controlled, n (%)	
Budesonide > 400 $\mu$ g/day or Fluticasone > 250 $\mu$ g/day + LABA	27 (67.5)
Budesonide $\leq$ 400 $\mu$ g/day or Fluticasone $\leq$ 250 $\mu$ g/day + LABA	13 (32.5)
C-ACT score	
mean $\pm$ SD	25.2 $\pm$ 1.7
median (min - max)	25.0 (19 - 27)

**Table 3. Clinical characteristics of the subjects who had airway reversibility**

Characteristic	Subject 1	Subject 2	Subject 3	Subject 4
$FEV_1$ reversibility (% change > 12%)	Yes	No	Yes	No
$FEF_{25-75}$ reversibility (% change > 30%)	Yes	Yes	Yes	Yes
Age of asthma onset (year)	0.8	5	1.4	7
BMI (kg/m <sup>2</sup> )	16.6	21.1	13.3	17.6
Co-morbidity	No	Allergic rhinitis	No	Allergic rhinitis
Family history of asthma in 1 <sup>st</sup> degree relative	No	No	No	No
Passive smoker	Yes	No	Yes	No
History of severe exacerbation in the past year	No	No	No	No
Type of controller to achieve asthma control	Fluticasone + LABA	Fluticasone + LABA	Fluticasone + LABA	Budesonide
Maximum dose of controller needed to achieve asthma control ( $\mu$ g/day)	500	250	500	400
C-ACT score	26	22	26	25

predicted) (**Table 4**). All children who had an abnormal  $FEV_1$  and  $FEF_{25-75}$  had a C-ACT score between 22 and 27.

The post-bronchodilator results showed airway reversibility as follows:  $FEV_1$  returned to a normal predicted level in 34 patients (85%) while most of the abnormal  $FEF_{25-75}$  returned to a normal predicted level in 39 patients (97.5%). Among the children with normal lung function, 4 (10%) showed airway reversibility: 2 had both large airway ( $FEV_1 > 12\%$ ) and small airway reversibility ( $FEF_{25-75} > 30\%$ ) and another 2 had only small airway reversibility ( $FEF_{25-75} > 30\%$ ). (**Table 5**) The clinical characteristic of these 4 children are presented in **Table 3**. As for the risk of exacerbation, two had allergic rhinitis as co-morbidities with a history of smoking in the family. Three of them received the maximum dose of ICS at a medium level

**Table 4. Spirometric results**

Spirometric results	Pre BD	Post BD
$FEV_1$		
Mean $\pm$ SD	84.0 $\pm$ 8.1	86.5 $\pm$ 8.6
Median (min - max)	84.3 (69.1 - 108.1)	85.9 (61.2 - 105.9)
$\geq 80\%$ (normal), n (%)	29 (72.5)	34 (85)
$FEV_1/FVC$		
Mean $\pm$ SD	87.8 $\pm$ 6.1	90.6 $\pm$ 5.4
Median (min - max)	87.7 (71.4 - 100)	91.2 (76.4 - 100)
$\geq 80\%$ (normal), n (%)	37 (92.5)	38 (95)
$FEF_{25-75}$		
Mean $\pm$ SD	85.5 $\pm$ 20.0	99.7 $\pm$ 21.9
Median (min - max)	83.2 (46.8 - 152.2)	96.4 (58.1 - 162.9)
$\geq 65\%$ (normal), n (%)	35 (87.5)	39 (97.5)

**Table 5. Post bronchodilator spirometric results**

Lung function	$FEV_1$	Reversible N (%)	$FEF_{25-75}$	Reversible N (%)
Normal N (%)	29 (72.5)	2 (5)	35 (87.5)	4 (10)
Abnormal N (%)	11 (27.5)	0	5 (12.5)	0



but none experienced a severe exacerbation or admission in the past year nor were they activity-limited. All of them achieved asthma-control and the ICS could be stepped down to a low dose for more than 6 months, as required before enrollment.

In the abnormal lung function group, none showed any airway reversibility. One child had both mild fixed abnormal FEV<sub>1</sub> and FEF<sub>25-75</sub>. (Pre & Post BD FEV<sub>1</sub> = 71.4 & 75%, FEF<sub>25-75</sub> = 46.8 & 58% predicted, respectively). The child had a BMI of 27.3 kg/m<sup>2</sup> and had been diagnosed with asthma at 5. He had been on ICS for 5 years without any severe exacerbation, but was a passive smoker from his father since birth and his C-ACT score was 26.

## Discussion

This study was conducted to test the hypothesis that all asthmatic children who have achieved symptom controlled with low-dose ICS for at least 6 months have normal lung function of the entire airway but in particular of the small airway. As for asthma management, all of the patients not only have to achieve good symptom control, the future risk of exacerbations should be minimal. One of the important future risks is low FEV<sub>1</sub>. In addition, significant bronchodilator reversibility in a patient taking controller treatment suggests uncontrolled asthma.<sup>1</sup> The current study showed that some children who were supposed to be well-controlled according to their symptom assessment, continued to have both abnormal large and small airway function.

Several studies have been conducted to determine the correlation of symptom assessment and lung function during the treatment follow-up period. Munoz et al. demonstrated that eosinophilic or neutrophilic inflammation persisted in most well-controlled asthma patients despite their condition being controlled.<sup>9</sup> Recent studies, moreover, showed that small airway dysfunction is not only a feature of severe asthma but can also present in patients with mild asthma who have a low level of symptoms and normal FEV<sub>1</sub> values.<sup>10-13</sup> Huang et al. demonstrated that the majority of children with well-controlled asthma continued to have airway hyper-responsiveness and low small airway function as represented by FEF<sub>25-75</sub>.<sup>14</sup>

Many physiological and imaging techniques have been used to evaluate small airway function, including impulse oscillometry, exhaled nitric oxide, inert gas washout, high resolution computed tomography, and spirometry.<sup>15</sup> Spirometry is often readily available and used most frequently in routine clinical practice. Forced expiratory volume in one second (FEV<sub>1</sub>) and FEV<sub>1</sub>/forced vital capacity (FVC) mainly represent the larger airways, whereas forced expiratory flow between 25% and 75% of forced vital capacity (FEF<sub>25-75</sub>) reflects small airway function.<sup>15</sup> Rao et al showed that asthmatic children who had a low FEF<sub>25-75</sub> had nearly 3 times the odds (OR 2.8) of systemic corticosteroid use and 6 times the odds of asthma exacerbations (OR 6.3) compared with those who had normal spirometry. In addition, they also concluded that using the percent change in FEF<sub>25-75</sub> may be helpful in identifying bronchodilator responsiveness in asthmatic children with a normal FEV<sub>1</sub>.<sup>8</sup> Due to our limited resources, we used spirometric results (FEF<sub>25-75</sub> predicted) to represent small airway function and pre-post bronchodilator values to determine airway hyper-reactivity. We found that FEF<sub>25-75</sub> did not add more useful information for

the detection of abnormal lung function. Nevertheless, among children who had a normal FEV<sub>1</sub>, four (10%) continued to have airway reversibility especially of the small airway. Prior to being considered symptom-controlled, these four children were treated with ICS at a medium dose level. Regarding clinical assessment of future risk of exacerbation, they did not have any striking history, such as a history of severe exacerbation during the preceding year, no activity limitations or C-ACT score between 22-26. Two children had allergic rhinitis as a co-morbidity and environmental smoking exposure. Notwithstanding these clinical characteristics, they were similar to children with normal lung function. The finding of significant bronchodilator reversibility (i.e., an increase in FEV<sub>1</sub> > 12%, FEF<sub>25-75</sub> > 30%)<sup>16</sup> in a patient taking controller treatment suggests persistent uncontrolled asthma, which should be regarded as evidence for further continuing treatment.<sup>1</sup> Early controller cessation in these patients might lead to severe exacerbation and fixed airway obstruction in the future. This study confirmed the usefulness of assessment of small airway reversibility to determine asthma control status in children.<sup>8,16</sup>

The recent international practice guideline for asthma recommends that lung function—especially spirometry—is a useful indicator of exacerbation, so it should be monitored together with clinical assessment at the start of treatment, after 3-6 months of controller treatment, and periodically going forward.<sup>17-18</sup> Monitoring asthma management in children according to these guidelines is challenging. In general, symptom control assessment as provided by GINA is used routinely for control-based asthma management at most healthcare centers in our country. Nevertheless, several asthma control scores for children—i.e., Childhood Asthma Control Test (C-ACT)—have been developed to help in clinical assessment.<sup>6</sup> Medication adjustment as well as cessation of controller mainly depended on subjective symptom control assessments. The current study used the GINA assessment at the level of well-controlled as indicating symptom control.<sup>1</sup> We also compared the GINA assessment with the C-ACT score and found that most children who were well-controlled had a C-ACT score in the range of 22 to 27. Only one child whose lung function (both FEV<sub>1</sub> and FEF<sub>25-75</sub>) was normal had a C-ACT score of 19. The results of our study on symptom-control assessment are similar to the study by Koolen et al. who showed that a C-ACT score correlated well with the GINA criteria and that children who were well-controlled had a C-ACT score in the range of 23 to 27.<sup>19</sup> By comparison, Ito et al. showed that a C-ACT cut-off score of 23 was useful for identifying children with well-controlled asthma (sensitivity 78%; specificity 54%).<sup>20</sup> This C-ACT score has been translated into Thai but is not widely used possibly because it has not yet been validated.

The European Task Force recommends performing spirometry annually as a minimum, when monitoring asthma in children.<sup>18</sup> The results of our study confirmed the usefulness of objective monitoring in asthma patients especially spirometry; however, in our country, spirometry is not available at all levels of health care. The current study demonstrated that even if the clinical assessment (either GINA or C-ACT score) of asthmatic children indicates symptom controlled, their lung function might not be normal and they may even have airway reversibility. We, therefore, suggest that before considering

cessation of controller medication in children over 6 with well-controlled asthma, a lung function test be performed to demonstrate the actual status, if available.

The limitations of this study were that (a) symptom control assessments were done by several pediatricians caring for the patients, so there will be some variation in assessments; (b) no inter-rater variation was performed before enrollment; and (c) we had a small sample size. In addition, it was a cross-sectional study that might not be able to determine the exact future clinical outcomes. Further study with an adequate sample size and long term cohort should be performed to add more evidence to support the clinical practice guideline.

## Conclusion

The current study demonstrated that asthmatic children, who are considered symptom-controlled based on an assessment of their treatment and symptoms, may still have abnormal lung function. Spirometry should be performed before considering cessation of controller medication.

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# Beta-Expansin of Bermuda, Johnson, and Para grass pollens, is a major cross-reactive allergen for allergic rhinitis patients in subtropical climate

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## Abstract

**Background:** Subtropical grass pollens of Bermuda (BGP), Johnson (JGP), and Para or buffalo grass (PGP), are common causes of pollen allergies in warm climate area. Allergic rhinitis (AR) patients had positive skin prick test (SPT) to extract of these 3 grass pollens. However, no allergenic proteins of 3 grass pollens have never been studied.

**Objective:** To identify major allergens of BGP, JGP, and PGP in Thai grass pollen-allergic patients and to examine their sIgE cross-reactivity.

**Methods:** Serum of nine AR patients with positive SPT to at least 2 of 3 studied pollens were collected. Based on availability, only ImmunoCAP of BGP and JGP were available to determine a level of sIgE. Profiles of sIgE bound proteins from BGP, JGP, and PGP, were obtained by immunoblot. Major IgE bound protein was identified by liquid chromatography-tandem mass spectrophotometry (LC-MS/MS). Cross-reactivity of purified major allergen of the 3 grass pollens was determined by inhibition of sIgE in both ELISA and immunoblot.

**Results:** AR patients who have positive SPT to extract of BGP, JGP, and PGP, were 9, 8, and 6, respectively. Positive sIgE (> 0.35 kUA/L) to BGP and JGP were found in 9 and 8 patients, respectively. Eight profiles of IgE bound proteins of the 3 grass pollens showed 29-30 kDa pollen protein as major allergen and was identified as beta-expansin (ExpB). Moreover, purified ExpB of the 3 grass pollens cross-inhibited serum sIgE.

**Conclusion:** Approximately 30 kDa ExpB of BGP, JGP, and PGP, are major cross-reactive allergen for AR Thai patients.

**Key words:** Subtropical grass pollen allergens, pollen allergy, Allergic rhinitis, beta-expansin, allergic airway diseases, major airborne pollens

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## Introduction

Allergens from grass pollens (GP) are one of major causes of allergic airway diseases, especially allergic rhinitis (AR).<sup>1,2</sup> Subtropical grasses in Chloridoideae subfamily (Bermuda grass, B, or *Cynodon dactylon*) and in Panicoideae subfamily (Johnson grass, J, or *Sorghum halepense*, and Para or Buffalo grass, P, or *Brachiaria mutica*, also known as *Urochloa mutica*), are commonly distributed in warm regions such as parts of Africa, Asia, Australia, United States of America (California and Florida), Central and South America.<sup>2</sup> BGP, JGP, and PGP are reported as a source of allergenic proteins triggering allergic inflammation in upper airway. To date, 7 allergens of BGP and 2 allergens of JGP have been reported.<sup>2</sup> However, to date, allergens of PGP have not yet been identified. In Thailand, pollens of grasses are one of major airborne pollens found in a high count throughout a year and are highest count in May

and September for grass pollen  $\leq 40 \mu\text{m}$  and in September for grass pollen  $> 40 \mu\text{m}$ .<sup>3</sup> It has been reported that, based on skin prick test (SPT), 52.3% and 49.4% of 736 Thai patients attending the ENT Allergy Clinic at Siriraj Hospital, had reaction to protein extract of BGP and PGP, respectively.<sup>1</sup> Moreover, It was recently reported that, based on SPT results, 45.6% of 68 Thai volunteers with respiratory allergy had reaction to JGP extract.<sup>4</sup> These findings indicated that allergens of BGP, JGP, and PGP, are one of major causes for respiratory allergy in sensitized Thai patients. Despite these results, however, pollen allergens from these 3 pollens sensitized by Thai patients have not yet been identified. Therefore, this study aimed to identify major allergenic proteins of BGP, JGP, and PGP, sensitized by Thai pollen-allergic patients as well as examine their cross-reactivity.

## Methods

### Patients

The study was approved by the Institutional Review Board, Siriraj Hospital (SiEc 100/2012). Forty patients with history of AR were recruited. All patients signed an informed consent before SPT. Thirty-one of them were excluded due to mono-sensitization to grass allergen. Nine patients, aged 9-47 years old (mean  $26.3 \pm 4$  years), were included in this study based on positive SPT (mean wheal diameter,  $\text{MWD} \geq 3 \text{ mm}$ ) to protein extract from at least 2 of 3 BGP, JGP, and PGP. The SPT extracts were prepared as described previously.<sup>4</sup> Determination of level of serum sIgE against extract of BGP and JGP was carried out by ImmunoCAP (Phadia, Uppsala, Sweden). However, there is no available test for PGP.

### Pollen collection

Grass flowers were collected from various sites in Thailand. Flowers were dissected under a stereo-microscope and identified to species using identification key. Flowers were left for natural shedding for 1 day. Pollen grains were incubated for 24 h in a chamber containing silica gel before purified by passing through 120, 150, and 230 mesh-sieve. Only the pollen with 95% purity observed under the microscope were stored at  $-80^\circ\text{C}$  until used.

### Pollen crude extract preparation

Pollens were ground in liquid nitrogen and extracted in phosphate buffer saline (PBS) buffer containing 1 mM Phenyl-methylsulfonyl fluoride (PMSF) with continuous stirring at  $4^\circ\text{C}$  for 1 h. The extract was centrifuged at  $13,000 \times g$  for 15 min and supernatant was collected before filtered through 0.45 micron filter. Protein concentration was determined using Bradford's assay. The efficacy and safety of this extract has been previously reported.<sup>4</sup>

### Electro-elution of 29-30 kDa IgE bound pollen protein

Electro-elution was carried out using electro-eluter (Bio-Rad, USA). The preparative gels were carried in parallel with gels for immunoblot to ensure that correct protein bands were excised. The protocol was followed the instruction from the manufacturer. Twenty  $\mu\text{g}$  of pollen protein extract was loaded per well and resolved in 12% SDS-PAGE gel. This preparative

gel was prepared with the same percentage and running condition as the gels prepared for immunoblot. Coomassie G-250 stained protein band matched IgE bound protein band of immunoblot was excised and placed in glass tube connected with pre-soaked membrane cap (3.5 kDa MWCO) where eluted protein was retained. The assembled glass tube was filled with elution buffer (25 mM Tris-HCl pH 8.2, 192 mM glycine and 0.1% (w/v) SDS) and placed into a tube holder. The holder was placed in buffer tank of Mini Transblot Electrophoresis Transfer Cell (Bio-Rad, USA) and protein was eluted from gel at a constant current of 8 mA/glass tube for 3-4 h. Eluted protein retained in the membrane cap was transferred and stored at  $-20^\circ\text{C}$ . Analysis by SDS-PAGE gel and immunoblot were done to confirm its IgE binding activity before used in all IgE inhibition tests.

### Identification of Major IgE bound protein by Liquid Chromatography-Tandem Mass Spectrophotometry (LC-MS/MS)

The following protocol was done per gel piece of one protein band. An excised gel piece was submerged in buffer A [50% Acetonitrile (ACN), 50 mM  $\text{NH}_4\text{HCO}_3$ ] until colorless. The buffer A was replaced with 10 mM dithiothreitol (DTT) and incubated for 15 min at  $60^\circ\text{C}$  before was added with buffer B (55 mM iodoacetamide, 50 mM  $\text{NH}_4\text{HCO}_3$ ) and incubated for 30 min at room temperature in the dark. The 100% ACN was replaced to dry gel before adding trypsin solution [0.1mg/mL trypsin (Sigma-Aldrich, USA), 50 mM Ammonium bicarbonate] and incubated at  $37^\circ\text{C}$  overnight. The reaction was mixed with ACN at 1:1 (v/v) ratio and incubated for 20 min. All solution was transferred to a new tube and dried in centrifugal concentrator at  $45^\circ\text{C}$ . Resolved pellet in 0.1% formic acid was injected into an Ultimate 3000 nano-LC system (Dionex; Surrey, U.K.) coupled with Micro-ToF Q II mass spectrometer (Bruker; Bremen, Germany). The mass spectra data were acquired using Hystar software (Bruker Daltonics, Germany) and were converted by Compass Data-Analysis software (Bruker Daltonics, Germany). The converted files were analyzed with Mascot server (version 2.3.0, Matrix Science, USA) to search matched sequence in NCBI database with 95% confidence.

### Direct IgE binding and Inhibition of IgE ELISA

Pollen extracts were coated at 500 ng/well of a 96-well microtiter plate and incubated at  $4^\circ\text{C}$  overnight. The coated plate was washed and incubated with 1/4-1/20 diluted serum of SPT positive patients and control for 2 h and washed before incubated with HRP-labeled mouse IgG anti-human IgE for 1 h. HRP substrate TMB (3,3',5,5'-tetramethylbenzidine) was added and blue color reaction product was read at a wavelength of 650 nm.

For inhibition ELISA, 500 ng of eluted 30 kDa beta-expansin (ExpB) of BGP extract was coated per well. Pre-determined diluted sera of patients and control were incubated with 0.5, 1, 10, and 20  $\mu\text{g}/\text{ml}$  of eluted ExpB (29-30 kDa protein) of BGP (CD29), JGP (SH29), and PGP (BM30), for overnight at  $4^\circ\text{C}$  before was centrifuged at  $17,210 \times g$  for 10 min at room temperature (RT). The supernatant was added to coated wells and followed the mentioned direct ELISA protocol.



### Direct IgE Immunoblot and IgE inhibition immunoblot

Sera of patients and control were diluted at 1/10-1/50 in PBS containing 3% skimmed milk (buffer A) and used in the following tests.

For direct IgE immunoblot, 2 µg of pollen protein extract were resolved per well of 12% SDS-PAGE gel at constant current. Separated proteins in the gel were electro-transferred onto nitrocellulose membrane before incubated in buffer A for 1 h at RT. The membrane was washed with PBS containing 0.2% v/v tween-20 (buffer B) before incubated overnight at 4°C with diluted sera of patients and control in buffer A. The membrane was washed with buffer B and was incubated with 1:10,000 diluted horse-radish peroxidase (HRP) conjugated mouse IgG anti-human IgE antibody (KPL, MD, USA) in buffer A for 1 h. After washing, the membrane was incubated with HRP substrate (Millipore, MA, USA) and emitted signal was captured by x-ray film.

For IgE inhibition immunoblot, 1/50 diluted sera of patients and control were incubated with 20 µg of eluted ExpB for overnight at 4°C before was centrifuged at 17,210 × g for 10 min at RT. The supernatant was incubated with nitrocellulose membrane on which had transferred CD29, SH29, and BM30,

for 2 h at RT and the remained steps were the same as those of direct immunoblot.

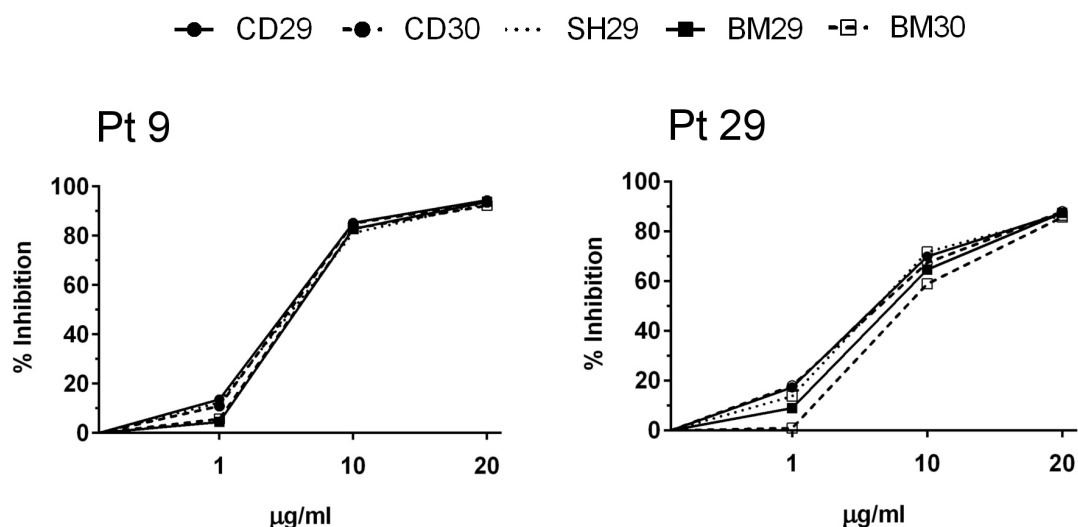
### Results

Nine patients with AR were included in this study based on positive SPT to at least 2 of 3 extracts (**Table 1**). The mean age of patients was 26.3 ± 12.3 years. The severity of allergic rhinitis was not different between patients who had low or high specific IgE. Thus, all included patients were classified as mild persistent allergic rhinitis. All patients had positive SPT to BGP extract while 8 of 9 had positive SPT to JGP extract (**Table 1**). However, only 6 of 9 patients had positive to PGP pollen extract. The MWD of SPT to extract of BGP, JGP, and PGP, were 5.8 mm, 3.8 mm, and 3 mm, respectively. The results of ImmunoCAP showed all patients had sIgE > 0.35 kUA/L to BGP extract while 8 patients had sIgE > 0.35 kUA/L to JPG extract (**Table 1**).

To further determine profiles of IgE bound pollen proteins extracted from 3 grasses of all included patients, the results showed that 8 profiles had IgE bound protein mobilized between 25 and 37 kDa markers (**Figure 1**). One profile (Pt33) could not be determined due to a lack of serum. Two profiles

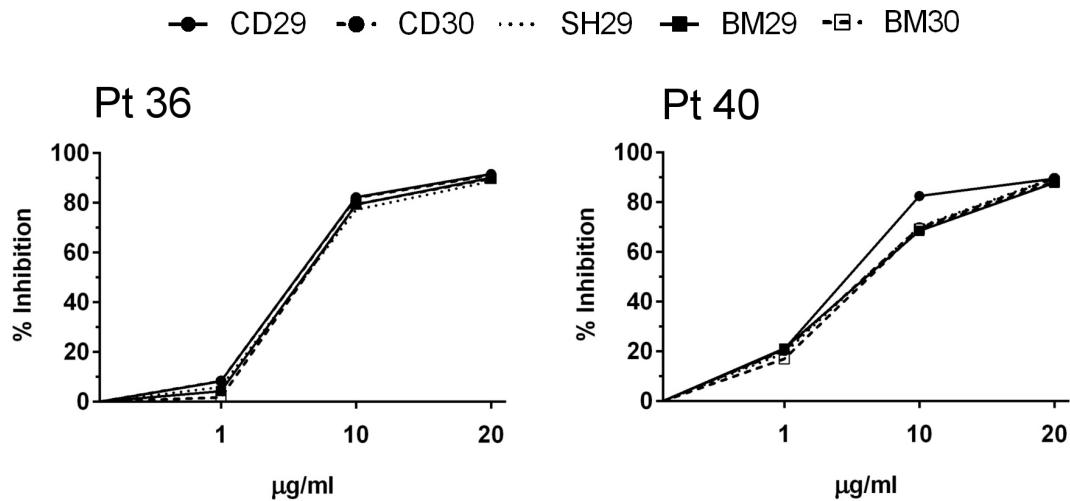
**Table 1. Demographic of recruited Allergic Rhinitis individuals of this study**

Pt. ID	sex	Age	SPT (mean wheal diameter, mm)			sIgE (kUA/L)	
			Bermuda	Johnson	Para	Bermuda	Johnson
9	F	46	9	5	3	30.3	34.7
17	M	30	4	4	2	4.16	5.19
29	M	21	5	3	2	4.02	3.05
33	M	10	8.5	4.5	3.5	>100	85.3
36	M	18	4	5	4.5	9.1	13.6
39	M	25	4	3.5	2	1.23	0.51
40	F	41	7.5	3.5	4.5	0.85	0.55
43	F	33	6.5	4	2.5	0.73	0.04
47	M	13	3.5	2	3	11.2	11.4



**Figure 1. Profiles of IgE bound pollen proteins extracted from BGP, JGP, and PGP.**

Note: total protein extract of grass pollens: 1 = BGP, 2 = JGP, and 3 = PGP.



**Figure 1. (Continued) Profiles of IgE bound pollen proteins extracted from BGP, JGP, and PGP.**

Note: total protein extract of grass pollens: 1 = BGP, 2 = JGP, and 3 = PGP.

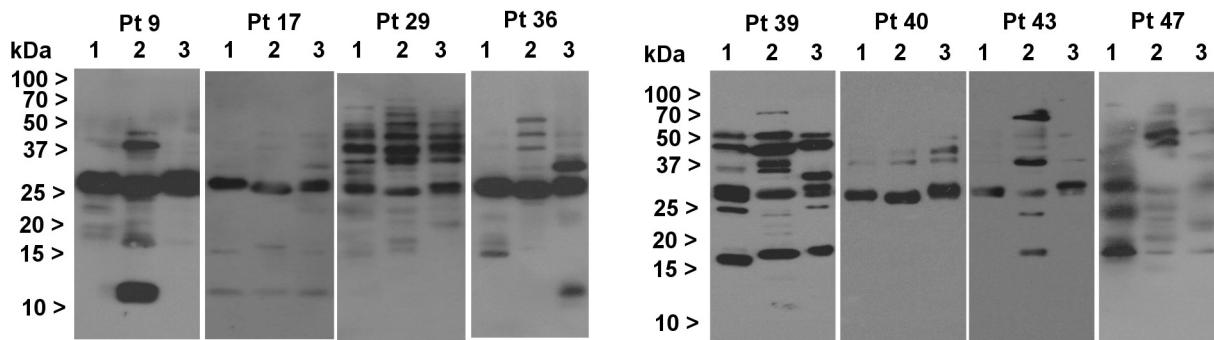
(Pt29 & Pt39) show IgE bound multiple proteins of 3 grass mobilized 37-50 kDa markers, while other five profiles show IgE bound multiple proteins of 1 grass mobilized 37-50 kDa markers (**Figure 1**). Moreover, three profiles (Pt17, Pt39 & Pt47) showed IgE bound multiple proteins of 3 grass mobilized 10-20 kDa markers (**Figure 1**). Based on these IgE bound protein profiles, protein mobilized between 25-37 kDa appears to be a major allergen among the 3 grass and was subjected for LC-MS to identify its identity.

The results of LC-MS/MS showed that 2 excised proteins (CD29, 30) of BGP extract matched 5-7 peptides of ExpB or Cyn d 1 (**Table 2**). Excised protein of JGP (SH29) and PGP (BM29, 30) extract matched 2-5 peptides of expansin B1 of corn (*Zea mays*) (**Table 2**). Moreover, the sequence of BM30 also matched 4 peptides of expansin B9 of Japanese rice (*Oryza sativa subsp. japonica*) (**Table 2**). These results suggested that a protein of ExpB family is the major allergen of BGP, JGP, and PGP, sensitized by Thai AR patients. To confirm

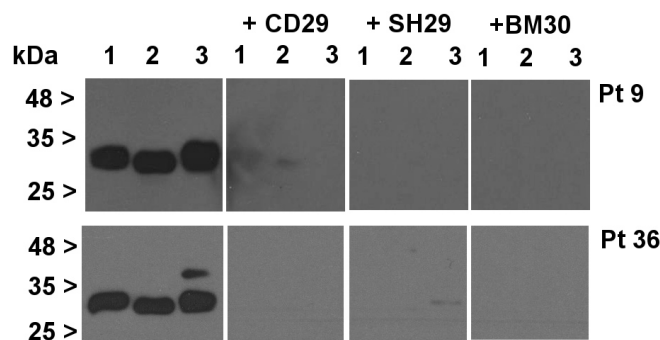
**Table 2. LC-MS identified IgE bound protein of 3 grass pollens**

ID	protein	mass	Protein score	peptide sequence
CD29	Cyn d 1	26872	153	SSWGAIWR ATFYGSNPR AGELTLQFR KPLKGPFSIR KAGELTLQFR CKEPVECSGEPVLVK
CD30	Cyn d 1	26872	140	SSWGAIWR ATFYGSNPR AGELTLQFR DSDEFIPMK KPLKGPFSIR
SH29	Expansin-B1 <i>Zea mays</i>	29066	137	GCGSCYEVR AFGLAKPGLNDK AFGLAKPGLNDKIR NVNLPYSGMTACGNVPIFK NVNLPYSGMTACGNVPIFKDGK
BM29	Expansin-B1 <i>Zea mays</i>	29066	65	LSWGAIWR ALKGPFSIR AFGLAKPGLNDK AFGLAKPGLNDKIR
BM30	Expansin-B1 <i>Zea mays</i>	29066	42	LSWGAIWR AFGLAKPGLNDKIR
	Expansin-B9 <i>Oryza sativa subsp. japonica</i>	28966	34	LSWGAIWR AGIIDMQFR KAGIIDMQFR AGIIDMQFR

Note: Matching of amino acid sequence using Mascot server (version 2.3.0, Matrix Science, USA) with 95% confidence.



**Figure 2. Cross-inhibition of specific IgE binding to coated CD29 with CD30, SH29, BM29, and BM30.**  
 Note: serum of 4 individuals: Pt9, Pt29, Pt36, and Pt40; specific IgE binding was inhibited at an average  $IC_{50}$  of 6  $\mu$ g/ml.



**Figure 3. Inhibition of specific IgE to ExpB of 3 grass pollens with eluted ExpB.**  
 Note: serum specific IgE of Pt9 and Pt36; 1 = eluted CD30; 2 = eluted SH29; 3 = eluted BM30; +CD29, +SH29, and +BM30 = incubated serum with eluted CD29, SH29, and BM30, respectively.

IgE cross-reactivity of ExpB of the 3 grass pollens, inhibition of sIgE binding to CD29 by CD30, SH29, BM29, and BM30, were determined. The results of inhibition ELISA showed all 5 proteins were able to cross-inhibit sIgE binding to coated CD29 at an average  $IC_{50}$  of 6  $\mu$ g/ml (Figure 2). Moreover, the results of inhibition immunoblot also showed complete inhibition of IgE binding to CD29, SH29, and BM30 by the same 3 proteins (Figure 3).

### Discussion

This is the first study to report that ExpB of BGP, JGP, and PGP, is major IgE cross-reactive allergen for grass pollen allergic Thai AR patients. It has been reported that ExpB or Cyn d 1 of BGP appears to be major grass pollen allergen in many subtropical areas.<sup>5</sup> The Cyn d 1 cross-inhibited sIgE binding to ExpB of subtropical Bahia grass (Pas n 1) with strong affinity while to that of temperate Ryegrass (Lol p 1) with moderate affinity.<sup>5,6</sup> The difference in IgE affinity to group 1 allergen of subtropical grass and temperate grass may be caused by different subfamily. In subtropical grasses, Bermuda is in Chloridoideae subfamily while both Johnson and Para are in Panicoideae subfamily. Both subfamilies are from the same lineage (PACMAD clade) in phylogenetic tree and ExpB of the 2 subfamilies shared 65% identity of amino acid sequence. Based on the LC-MS results, identical amino acid sequences between ExpB of BGP and PGP are WGAIWR and LKGPFSIR, located in the C-terminal domain of Cyn d 1.<sup>8,9</sup> Moreover, the peptide, WGAIWR (aa194-199) was showed

to be one of major IgG4 binding sites on Cyn d 1.<sup>9</sup> Despite a moderate sequence identity, ExpB of these 3 grass pollens could cross-inhibit sIgE to ExpB with strong affinity ( $IC_{50}$  of 6  $\mu$ g/ml). It has been reported that IgE cross-reactivity between group 1 of subtropical grass and that of temperate grass could also trigger allergic reaction in patients from either subtropical or temperate regions.<sup>10,11</sup> Based on these results, thus, ExpB of one grass species, such as Cyn d 1, could be a good allergen used in diagnosis and immunotherapy of AR patients who sensitize to subtropical grass pollens from grass of PACMAD clade. Moreover, several agricultural crops, such as maize, sorghum, sugar cane, and pearl millet, are belonged to the same Panicoideae subfamily as Johnson and Para grass. Thus, investigation of IgE cross-reactivity of ExpB in pollens from mentioned agricultural crops would be essential because they are economically important crops for countries in warm regions and many field workers may likely suffer pollen-caused AR.<sup>2</sup>

Results of SPT and ImmunoCAP appear to correlate well as 7 of 9 patients had a value of sIgE against BGP and JGP grass pollen  $\geq 0.35$  kUA/L while had  $\geq 3$  mm MWD in SPT. However, the other 2 patients did not have all positive results from both testes, for instance, Pt8 had SPT positive to both BGP and JGP but sIgE to only BGP or, for Pt9, SPT positive to BGP but sIgE to both BGP and JGP (Table 1). Although values of sIgE and SPT of 7 patients indicated they are allergic to proteins of BGP and JGP, however, values of these 2 tests may not predict results of one another. For instance, Pt4 had SPT value of 8.5 mm to BGP and 4.5 mm to JGP,

while sIgE values were > 100 and 85.3 kUA/L to BGP and JGP, respectively. In contrast, Pt7 had SPT value of 7.5 mm to BGP and 3.5 mm to JGP, while sIgE values were 0.85 and 0.55 kUA/L to BGP and JGP, respectively. Based on these results, it appears that SPT could be sufficient test to diagnose pollen allergy as also reported by others.<sup>1,4-7,10,11</sup> Pollen extract in this report was prepared from 95% purified pollens from identified grass species.<sup>4</sup> Therefore, results from SPT and IgE reactivity assays, such as ELISA, would be a result from allergens interacting sIgE.<sup>1,4-7,10,11</sup>

In conclusion, ExpB of Bermuda, Johnson, and Para grass pollens is the major cross-reactive allergen for Thai patients with AR and could be one of essential components for diagnosis of pollen allergy.

### Acknowledgement

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# *Lactobacillus plantarum* induces genomic DNA-dependent and TLR9-mediated elafin secretion from Caco-2 cells

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## Abstract

**Background:** Lactobacilli show anti-inflammatory effects in the human intestine, and their genomic DNA was identified as one of the anti-inflammatory components. Increased levels of the natural protease inhibitor elafin in the intestine plays an important role in protection against intestinal inflammation. However, there have been no previous reports regarding whether lactobacilli increase elafin levels.

**Objective:** This study was performed to investigate whether *Lactobacillus plantarum* induces elafin secretion from the human epithelial colorectal adenocarcinoma cell line, Caco-2. Moreover, we examined the roles of bacterial genomic DNA and toll-like receptor 9 (TLR9), a specific receptor of bacterial DNA, in this effect.

**Methods:** Elafin secretion from Caco-2 cells by live and heat-killed *L. plantarum* was measured. The analysis was also performed using DNase-treated *L. plantarum* and genomic DNA extracted from *L. plantarum*. We examined the role of TLR9 in elafin secretion by *L. plantarum* and its genomic DNA by suppressing TLR9 expression using RNAi in Caco-2 cells.

**Results:** Heat-killed *L. plantarum* time- and dose-dependently increased elafin secretion, whereas live *L. plantarum* had no such effect. The elafin secretion by heat-killed *L. plantarum* was partially abolished by DNase treatment of the bacterium. In addition, *L. plantarum* genomic DNA also increased elafin secretion. Furthermore, suppression of TLR9 expression partially or completely abolished elafin secretion by heat-killed *L. plantarum* and its genomic DNA.

**Conclusion:** Our results indicated that heat-killed *L. plantarum* induced genomic DNA-dependent and TLR9-mediated elafin secretion. The anti-inflammatory effects of lactobacilli may be mediated by increases in the levels of elafin in the intestine.

**Key words:** elafin, *Lactobacillus plantarum*, genomic DNA, toll-like receptor 9, anti-inflammatory effect

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## Introduction

Lactobacilli, which are normal components of the human gut microflora, have been used as both foods and medicines because of their beneficial effects on the health of the host. For example, treatment with lactobacilli has been reported to ameliorate human intestinal inflammation in patients with inflammatory bowel disease (IBD).<sup>1</sup> The genomic DNA, double-stranded RNA, and cell wall components of lactobacilli have been identified as components responsible for the anti-inflammatory effects.<sup>2-5</sup> Specifically, there have been a number of

reports regarding the anti-inflammatory effects of genomic DNA from lactobacilli.<sup>5-9</sup> Rachmilewitz et al. reported that intragastric administration of probiotic medical food VSL#3<sup>®</sup> (VSL Pharmaceuticals, Inc.), which consists of eight strains of lactic acid bacteria including lactobacilli, ameliorated the severity of colitis in a dextran sodium sulfate (DSS)-induced mouse model of IBD, whereas DNase-treated VSL#3<sup>®</sup> had no effect.<sup>5</sup> They also demonstrated that VSL#3<sup>®</sup> had no effect on the severity of DSS-induced colitis in mice deficient for

toll-like receptor 9 (TLR9), which is a specific receptor of bacterial DNA<sup>10</sup> and has been reported to play a role in one of the major pathways responsible for the anti-inflammatory effects of genomic DNA from lactobacilli.<sup>6,8</sup> Taken together, these observations suggest that the anti-inflammatory effects of lactobacilli are mediated by their genomic DNA, and TLR9 signaling is essential for these anti-inflammatory effects.

IBD, including Crohn's disease and ulcerative colitis, are chronic inflammatory disorders of the intestine. Although the etiology of IBD has yet to be determined, a number of mediators seem to play prominent roles in the pathogenesis of chronic inflammatory disorders. Recent studies have highlighted the roles of proteases and their endogenous inhibitors in the pathogenesis of enteritis.<sup>11-14</sup> Elevated proteolytic activities of several proteases have been detected in the intestinal tissues of IBD patients.<sup>11</sup> This increased proteolytic activity may result from decreased efficacy and expression of endogenous protease inhibitors.<sup>12</sup> These results suggest that the shift of protease/anti-protease balance toward a more proteolytic environment causes chronic inflammation in the intestine. Among the endogenous protease inhibitors, elafin is a natural inhibitor of the human neutrophil proteases, elastase and proteinase 3.<sup>15,16</sup> Elafin is mainly secreted from the epithelial cells of various tissues, including the intestine,<sup>17</sup> and exerts anti-inflammatory effects by protease inhibition and by restoring barrier function to damaged epithelial cells in the intestine.<sup>13</sup> The expression of elafin was downregulated in the intestinal tissues of IBD patients, and this was associated with increased elastase activity.<sup>14</sup> In addition, a previous study using transgenic mice expressing human elafin demonstrated that an increased level of human elafin in the intestine re-equilibrates the protease/anti-protease balance and prevents intestinal inflammation in DSS- and trinitrobenzene sulfonic acid (TNBS)-induced mouse models of IBD.<sup>13</sup> These observations suggest that an increased level of elafin in the intestine may be useful in the treatment of IBD.

In the present study, we hypothesized that lactobacilli may increase elafin secretion from intestinal epithelial cells, as both lactobacilli and increased levels of elafin are involved in intestinal anti-inflammatory effects. In this context, Motta et al. engineered recombinant human elafin-expressing lactic acid bacteria, including lactobacilli, and demonstrated that oral administration of these recombinant bacteria ameliorates the symptoms of inflammation in DSS-induced murine colitis.<sup>14</sup> However, the increase in elafin by lactobacilli in the intestine has not been characterized in rodent studies, because a rodent homolog of human elafin has not been identified. Therefore, we examined elafin secretion from the human epithelial colorectal adenocarcinoma cell line, Caco-2, a model of human intestinal epithelial cells, induced by *Lactobacillus plantarum*, which is widely used in the food industry due to its metabolic versatility, and it has been reported to show anti-inflammatory effects both *in vitro* and *in vivo*.<sup>18,19</sup> To elucidate the molecular basis underlying the upregulation of elafin secretion by *L. plantarum*, we examined the roles of the bacterial genomic DNA and TLR9 in this effect.

## Methods

### **Bacterial strain and culture conditions**

*L. plantarum* strain D2905 was used in this study.<sup>20</sup> The bacterium was grown at 27 °C, the optimal temperature for its culture, in MRS medium under static conditions. To produce heat-killed *L. plantarum*, an overnight culture was inoculated into MRS medium to an optical density at 600 nm (OD<sub>600</sub>) of 0.05 and grown for 8 h. After centrifugation (7,000 × g, 15 min, 4 °C) and two washes with PBS, the bacterium was incubated for 10 min at 65 °C. The bacterium was killed by this incubation (bacterial viability: 0.001%). This heat-killed bacterial preparation was used without centrifugation and washing in the following experiments. Furthermore, DNase-treated heat-killed *L. plantarum* was prepared by incubation with 5 µg/mL DNase I (Roche) for 16 h at 37 °C.

### **Preparation of genomic DNA from *L. plantarum***

Genomic DNA was extracted from an overnight culture of *L. plantarum* using a Genra Puregene Yeast/Bact. Kit (Qiagen) according to the manufacturer's protocol. The concentration and purity of genomic DNA preparation were confirmed by measuring OD<sub>260</sub>, and OD<sub>260/280</sub> and OD<sub>260/230</sub> ratios, respectively. Only genomic DNA with OD<sub>260/280</sub> > 1.8 and OD<sub>260/230</sub> > 2.0 was used for the experiments. Purified genomic DNA was tested for endotoxin contamination using Limulus Amebocyte Lysate QCL-1000 (Lonza).

### **Measurement of elafin secretion by *L. plantarum***

Caco-2 cells were used as described previously.<sup>6</sup> Aliquots of 2 × 10<sup>5</sup> Caco-2 cells were plated in each well of 24-well plates (Nunc). Cells were treated with three concentrations of live or heat-killed *L. plantarum* (1, 2, and 5 × 10<sup>9</sup> CFU/mL) for 12, 24, and 48 h. After incubation, cell supernatants were centrifuged at 15,000 × g for 5 min at 4 °C. The concentration of elafin in the cell supernatants was determined using a human elafin ELISA kit (R & D Systems).

### **Measurement of elafin secretion by transfected *L. plantarum* genomic DNA**

Aliquots of 2 × 10<sup>5</sup> Caco-2 cells were plated in each well of 24-well plates. Subsequently, 5 µL of *L. plantarum* genomic DNA (0.35, 0.7, and 1.7 mg/mL) was added to 245 µL of minimum essential medium (Invitrogen) containing 1% fetal bovine serum (Biowest). The genomic DNA solution was then added to 250 µL of medium containing 1.25 µL of siLentFect Lipid Reagent (Bio-Rad Laboratories) and incubated for 30 min at room temperature to produce the transfection mix. Caco-2 cells were transfected with the transfection mix for 12, 24, and 48 h. After incubation, elafin secretion was measured by ELISA as described above. As the genome size of *L. plantarum* is approximately 3 Mbp, 1, 2, and 5 × 10<sup>9</sup> CFU/mL of *L. plantarum* contain approximately 3.5, 7, and 17 µg/mL of genomic DNA, respectively. Therefore, we used these three concentrations of genomic DNA (3.5, 7, and 17 µg/mL) in this experiment.

### **Transfection of Caco-2 cells with TLR9-siRNA**

TLR9 expression in Caco-2 cells was suppressed by RNAi, as described previously.<sup>21</sup> Briefly, aliquots of 2 × 10<sup>5</sup> Caco-2 cells plated in 24-well plates were transfected with 50 nM TLR9

-siRNA or scramble TLR9-siRNA (Bonac Corp.) as a negative control using siLentFect Lipid Reagent. After incubation for 24 h, the cells were treated with heat-killed *L. plantarum* ( $5 \times 10^9$  CFU/ml) or transfected with *L. plantarum* genomic DNA (17  $\mu$ g/ml) for 48 h as described above. The cell supernatants were collected, and the cellular protein extracts from Caco-2 cells were prepared with PRO-PREP Protein Extraction Solution (iNtRON Biotechnology) according to the manufacturer's protocol. The concentration of elafin in the cell supernatants was determined by ELISA. Suppression of TLR9 expression in the protein extracts of cells transfected with TLR9-siRNA, but not scramble TLR9-siRNA, was confirmed by Western blotting using an antibody to TLR9 as described previously.<sup>6</sup>

**Detection of intracellular translocated genomic DNA**

Aliquots of  $2 \times 10^5$  Caco-2 cells were plated in each well of 24-well plates and treated with heat-killed *L. plantarum* ( $5 \times 10^9$  CFU/ml) or *L. plantarum* genomic DNA (17  $\mu$ g/ml) for various times (0, 0.5, 1, 3, 6, 24, and 48 h). After treatment, Caco-2 cells were trypsinized using trypsin-EDTA (Invitrogen) at 37 °C for 10 min. The cells were then collected and centrifuged at  $1,000 \times g$  for 5 min and washed five times with PBS. DNA was extracted from Caco-2 cells using ISOGEN (Nippon Gene) according to the manufacturer's protocol. Detection of intracellular genomic DNA was performed by PCR based on the *L. plantarum*-specific 16S rRNA gene.<sup>6</sup> PCR was performed using KOD FX Neo (Toyobo) in 30- $\mu$ L reaction mixtures containing 1  $\mu$ g of DNA preparation and the following primers: sense 5'-TGGTATTGATTGGTGCTTGCA-3' and anti-sense 5'-CCACCTTCCTCCGGTTTGTC-3'. PCR products were subjected to 1.5% agarose gel electrophoresis.

**Statistical analysis**

Data are presented as means  $\pm$  standard deviations. Statistical analyses were performed using Origin Pro 8.1 (OriginLab). In all analyses,  $P < 0.05$  was taken to indicate significance.

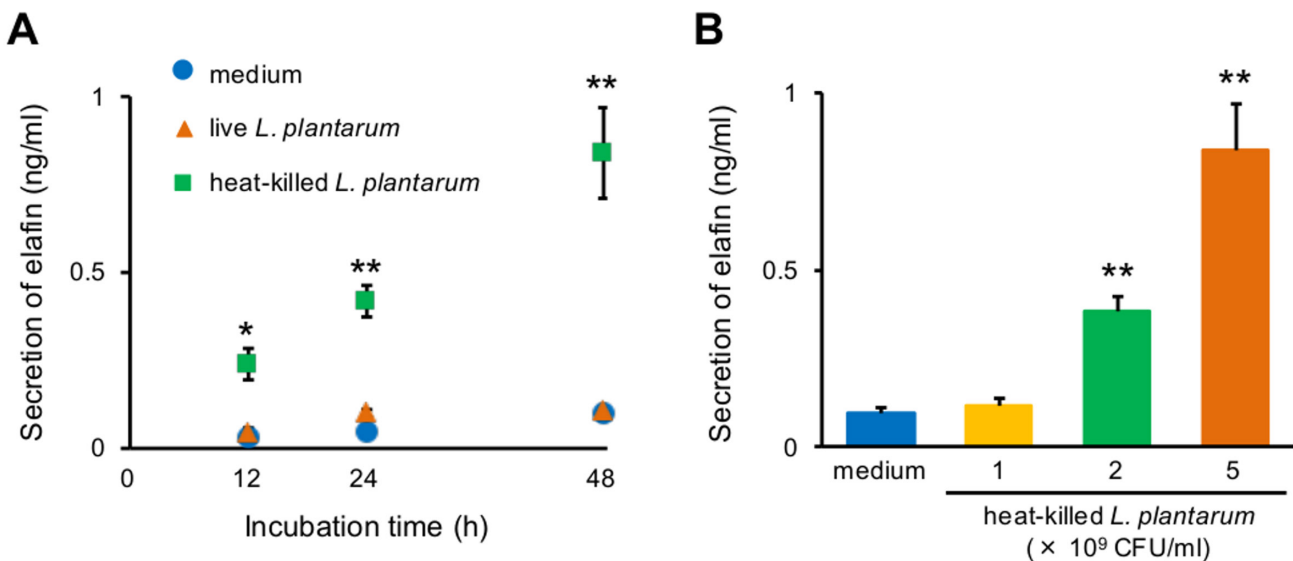
**Results**

**Increase in elafin secretion by heat-killed *L. plantarum***

Treatment with heat-killed *L. plantarum* ( $5 \times 10^9$  CFU/ml) significantly increased elafin secretion from Caco-2 cells, reaching 0.84 ng/ml after 48 h of treatment ( $P < 0.01$  vs. medium alone by two-way ANOVA followed by Tukey's test) (Figure 1A). In contrast, elafin secretion remained close to the background level after 48 h of treatment with live *L. plantarum*. Therefore, heat-killed *L. plantarum* was used in the following experiments. As shown in Figure 1B, treatment for 48 h with heat-killed *L. plantarum* at 2 and  $5 \times 10^9$  CFU/ml significantly increased elafin secretion from Caco-2 cells compared to medium alone (0.38 and 0.84 ng/ml vs. 0.10 ng/ml, respectively;  $P < 0.01$ , one-way ANOVA followed by Tukey's test). In contrast, there was no significant difference in elafin secretion between cells treated with  $1 \times 10^9$  CFU/ml heat-killed *L. plantarum* and medium alone. These results indicated that heat-killed *L. plantarum* increased elafin secretion in a time- and dose-dependent manner.

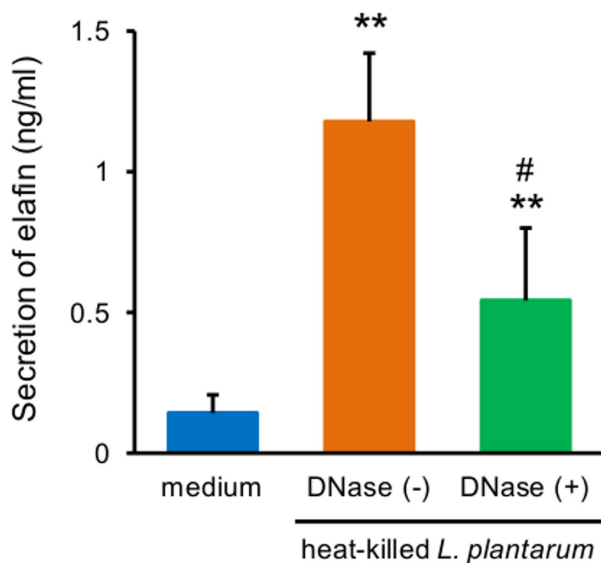
**Decrease in heat-killed *L. plantarum*-induced elafin secretion by DNase treatment**

Treatment with DNase-treated and non-treated heat-killed *L. plantarum* significantly increased the level of elafin secretion from Caco-2 cells compared with medium alone (1.18 and 0.54 ng/ml vs. 0.20 ng/ml, respectively;  $P < 0.01$ , one-way ANOVA followed by Tukey's test). However, the level of elafin secretion in cells treated with DNase-treated heat-killed *L. plantarum* was significantly lower than that in cells treated with non-treated heat-killed *L. plantarum* ( $P < 0.05$ ) (Figure 2). These observations indicated that DNase treatment partially abolished the increase in elafin secretion by heat-killed *L. plantarum*, although we could not exclude the possibility that DNase caused the decrease in elafin secretion by heat-killed *L. plantarum* regardless of the DNase activity.



**Figure 1. Time- and dose-dependent increases in elafin secretion by heat-killed *Lactobacillus plantarum*.**

Caco-2 cells were treated with live or heat-killed *L. plantarum* ( $5 \times 10^9$  CFU/ml) for 12, 24, and 48 h (A), or with three concentrations of the heat-killed *L. plantarum* (1, 2, and  $5 \times 10^9$  CFU/ml) for 48 h (B). The elafin concentrations in the cell supernatants were determined by ELISA. Data are expressed as ng/ml and represent the means  $\pm$  standard deviations of three separate experiments performed in triplicate. \* $P < 0.05$ , \*\* $P < 0.01$  compared with medium alone.



**Figure 2. Influence of DNase treatment on elafin secretion by heat-killed *Lactobacillus plantarum*.**

Caco-2 cells were treated with DNase-treated (DNase (+)) or non-treated (DNase (-)) heat-killed *L. plantarum* ( $5 \times 10^9$  CFU/ml) for 48 h. The elafin concentrations in the cell supernatants were determined by ELISA. Data are expressed as ng/ml and represent the means  $\pm$  standard deviations of three separate experiments performed in triplicate. \*\* $P < 0.01$  compared with medium alone, # $P < 0.05$  compared with DNase non-treated heat-killed *L. plantarum*.

#### Increase in elafin secretion by *L. plantarum* genomic DNA

Transfection with *L. plantarum* genomic DNA (17  $\mu$ g/ml) significantly increased elafin secretion from Caco-2 cells, reaching 0.33 ng/ml after 48 h of treatment ( $P < 0.01$  vs. medium alone, two-way ANOVA followed by Tukey's test) (Figure 3A). As shown in Figure 3B, transfection with *L. plantarum* genomic DNA at 7 and 17  $\mu$ g/ml for 48 h significantly increased elafin

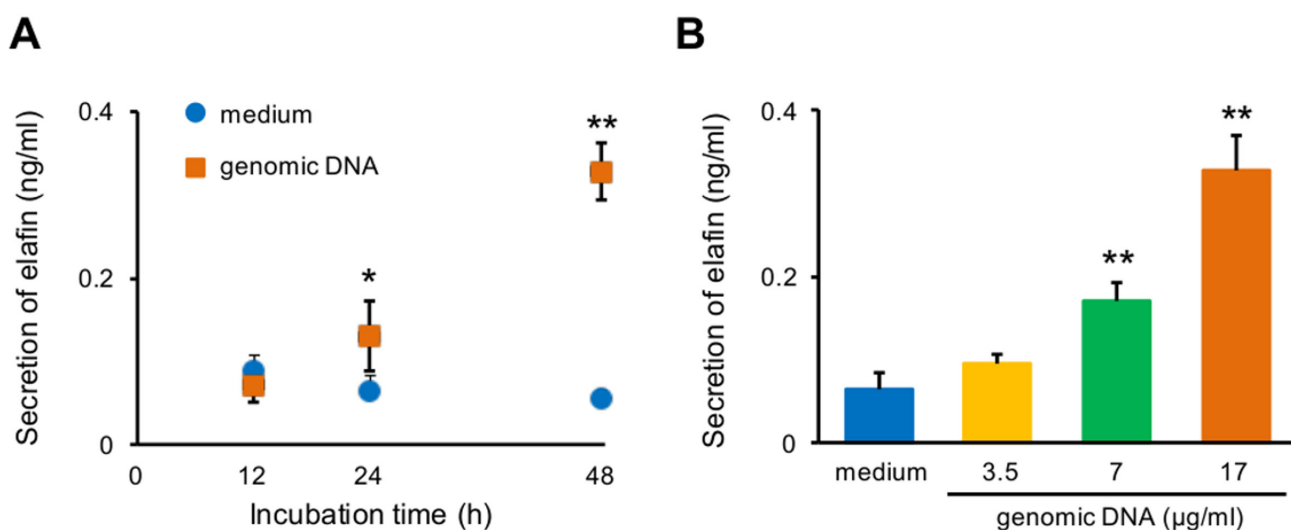
secretion from Caco-2 cells compared with medium alone (0.17 and 0.33 ng/ml vs. 0.07 ng/ml, respectively;  $P < 0.01$ , one-way ANOVA followed by Tukey's test). In contrast, no significant change in elafin secretion was observed in those transfected with 3.5  $\mu$ g/ml of genomic DNA compared to controls. These results indicated that *L. plantarum* genomic DNA increased elafin secretion in a time- and dose-dependent manner.

#### Mediation of TLR9 on elafin secretion by heat-killed *L. plantarum* and its genomic DNA

TLR9-siRNA-transfected Caco-2 cells showed decreased TLR9 expression compared to siRNA non-transfected and scramble TLR9-siRNA-transfected cells as a negative control (Figure 4A). This observation confirmed that TLR9-siRNA suppressed TLR9 expression in Caco-2 cells. Treatment with heat-killed *L. plantarum* significantly increased the level of elafin secretion in non-transfected cells, scramble TLR9-siRNA-transfected cells, and TLR9-siRNA-transfected cells (0.74, 0.80, and 0.39 ng/ml, respectively). On the other hand, treatment with *L. plantarum* genomic DNA significantly increased elafin secretion in non-transfected cells and scramble TLR9-siRNA-transfected cells (0.33 and 0.35 ng/ml, respectively), but the genomic DNA had no such effect in TLR9-siRNA-transfected cells (0.09 ng/ml). In addition, the levels of elafin secretion in TLR9-siRNA-transfected cells treated with heat-killed *L. plantarum* and *L. plantarum* genomic DNA were significantly lower than those in non-transfected cells and scramble TLR9-siRNA-transfected cells ( $P < 0.05$  and  $P < 0.01$ , respectively; two-way ANOVA followed by Tukey's test) (Figure 4B). These observations indicated that suppression of TLR9 expression partially or completely abolished elafin secretion by heat-killed *L. plantarum* and its genomic DNA, respectively.

#### Intracellular translocation of *L. plantarum* genomic DNA

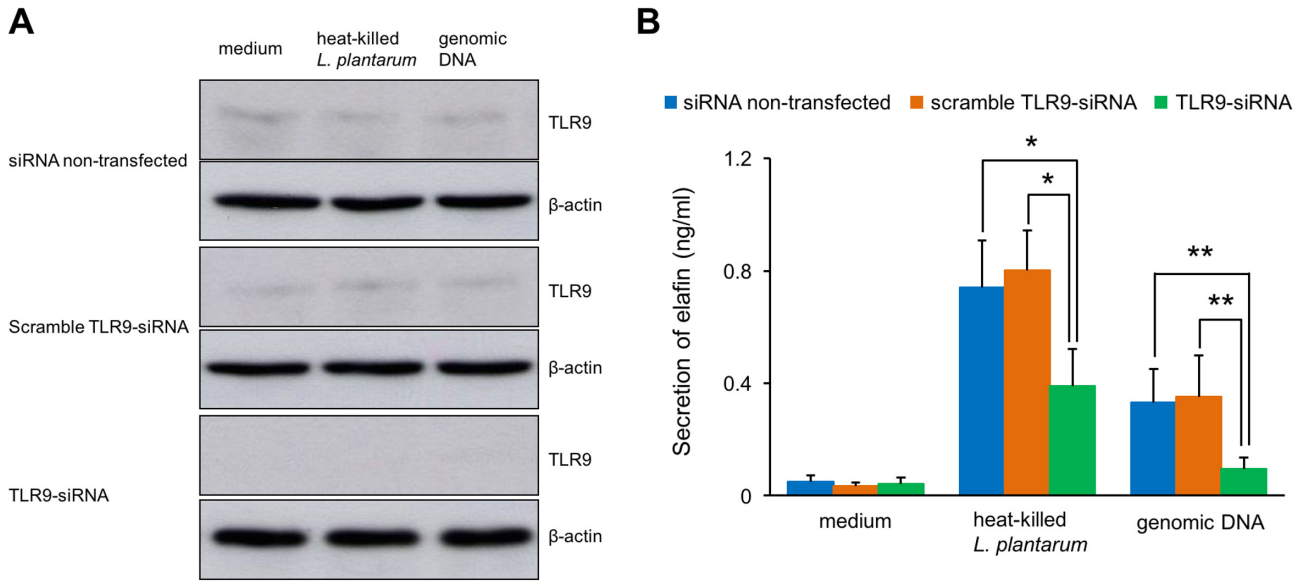
Intracellular translocation of the genomic DNA was a prerequisite for recognition by TLR9, because TLR9 is localized in



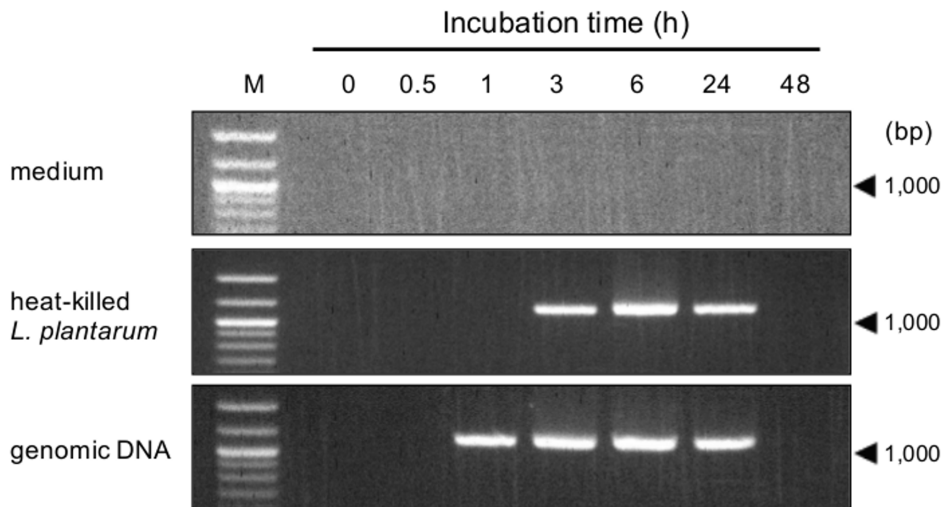
**Figure 3. Time- and dose-dependent elafin secretion by genomic DNA from *Lactobacillus plantarum*.**

Caco-2 cells were transfected with *L. plantarum* genomic DNA (17  $\mu$ g/ml) for 12, 24, or 48 h (A) or with three concentrations of the genomic DNA (3.5, 7, and 17  $\mu$ g/ml) for 48 h (B). The elafin concentrations in the cell supernatants were determined by ELISA. Data are expressed as ng/ml and represent the means  $\pm$  standard deviations of three separate experiments performed in triplicate. \* $P < 0.05$ , \*\* $P < 0.01$  compared with medium alone.





**Figure 4. Effects of TLR9 suppression on elafin secretion by heat-killed *Lactobacillus plantarum* and its genomic DNA.** TLR9-siRNA- and scramble TLR9-siRNA-transfected Caco-2 cells were treated with heat-killed *L. plantarum* ( $5 \times 10^9$  CFU/ml) or transfected with *L. plantarum* genomic DNA (17  $\mu$ g/ml) for 48 h. (A) TLR9 expression in cellular protein extract was confirmed by Western blotting using anti-TLR9 antibody.  $\beta$ -actin was used as an internal control. Data are representative of three separate experiments. (B) The elafin concentrations in the cell supernatants were determined by ELISA. Data are expressed as ng/ml and represent the means  $\pm$  standard deviations of three separate experiments performed in triplicate. \* $P < 0.05$ , \*\* $P < 0.01$ .



**Figure 5. Intracellular translocation of genomic DNA from *Lactobacillus plantarum* in Caco-2 cells.** Caco-2 cells were treated with heat-killed *L. plantarum* ( $5 \times 10^9$  CFU/ml) or *L. plantarum* genomic DNA (17  $\mu$ g/ml) for various times (0 – 48 h). Intracellular translocation of the genomic DNA was detected by PCR based on the coding sequence of the *L. plantarum*-specific 16S rRNA gene. Data are representative of three separate experiments. M; 100 bp DNA ladder marker.

the endosome and lysosome.<sup>22</sup> Therefore, we examined whether *L. plantarum* genomic DNA can be translocated into Caco-2 cells. *L. plantarum* genomic DNA was detected within Caco-2 cells at 3–24 h after treatment with heat-killed *L. plantarum*, and the genomic DNA was also detected within cells at 1–24 h after treatment (Figure 5). No *L. plantarum* genomic DNA was detected within cells following treatment with medium alone.

## Discussion

The results of the present study indicated that treatment

with heat-killed *L. plantarum* increased the level of elafin secretion from Caco-2 cells, which was partially dependent on the bacterial genomic DNA. Moreover, TLR9 was shown to be the primary mediator of elafin secretion by heat-killed *L. plantarum* and its genomic DNA. To our knowledge, this is the first report regarding induction of elafin secretion from intestinal epithelial cells by lactobacilli and the molecular mechanisms underlying this effect.

Over the past several decades, lactobacilli have been reported to show anti-inflammatory effects and have been used

to treat patients with IBD.<sup>1</sup> Increased elafin levels have been shown to play an important role in intestinal anti-inflammatory effects.<sup>13,14</sup> However, there have been no previous reports of increases in elafin levels induced by lactobacilli in the intestine. As elafin production has not been characterized in rodents, we examined whether lactobacilli could increase the level of elafin in the intestine using Caco-2 cells, and our results indicated that heat-killed *L. plantarum* increased elafin secretion from Caco-2 cells (**Figure 1**). These observations suggested that orally administered lactobacilli may increase elafin levels in the intestines of IBD patients. As the protease/anti-protease imbalance due to downregulation of elafin was shown to be involved in chronic inflammation in the intestine of IBD patients,<sup>11-14</sup> the increase in elafin levels by lactobacilli in the intestine may lead to re-equilibration of the protease/anti-protease balance and subsequently ameliorate inflammation in these patients. On the other hand, live *L. plantarum* had no effect on elafin secretion (**Figure 1**), although live lactobacilli therapy has been reported to ameliorate human IBD.<sup>1</sup> In general, orally administered live lactobacilli cannot survive in the low pH gastric environment, and the secretion of lysozymes, bile acid, and pancreatic fluid in the duodenum also influence the viability of the lactobacilli.<sup>23,24</sup> Consequently, the lactobacilli would be killed by these conditions prior to reaching the intestine. Thus, it is possible that orally administered live lactobacilli are killed, and then increase elafin secretion from human intestinal epithelial cells.

Genomic DNA has been identified as the principle anti-inflammatory component of lactobacilli.<sup>5-9</sup> Therefore, we hypothesized that the increase in elafin secretion by heat-killed *L. plantarum* depends on its genomic DNA. In the present study, DNase treatment partially abolished the increase in elafin secretion by heat-killed *L. plantarum* (**Figure 2**). Moreover, *L. plantarum* genomic DNA increased elafin secretion from Caco-2 cells, although to a lesser extent than heat-killed *L. plantarum* (**Figures 1 and 3**). These results indicated that the increase in elafin secretion by *L. plantarum* is partially mediated by its own genomic DNA and suggested that lactobacilli may exert anti-inflammatory effects through genomic DNA-dependent elafin secretion from epithelial cells in the human intestine. These observations also suggested that bacterial components other than genomic DNA may also be involved in the increase in elafin secretion by heat-killed *L. plantarum*. Previous studies showed that dsRNA and cell wall components are also anti-inflammatory components of lactobacilli.<sup>2-4</sup> Therefore, the increase in elafin secretion by heat-killed *L. plantarum* may be related not only genomic DNA but also to these other bacterial components.

The anti-inflammatory effects of lactobacilli genomic DNA are primarily mediated by TLR9 signaling.<sup>5,6</sup> Consistent with this, suppression of TLR9 completely abolished the increase in elafin secretion by *L. plantarum* genomic DNA in the present study (**Figure 4**). These observations indicated that TLR9 signaling is a major pathway responsible for the induction of elafin secretion by *L. plantarum* genomic DNA. However, suppression of TLR9 only partially abolished the increase in elafin secretion by heat-killed *L. plantarum*, suggesting that TLR9 is one, but not the only, mediator of elafin secretion by heat-killed *L. plantarum*. As bacterial components other than genomic DNA,

including dsRNA and cell wall components, may be involved in the upregulation of elafin secretion by heat-killed *L. plantarum*, the receptors for these bacterial components (i.e., TLR3 and TLR2)<sup>25,26</sup> may also partially regulate these effects. Further studies are required to fully elucidate the molecular mechanism of elafin secretion by heat-killed *L. plantarum*.

Overproduction of inflammatory cytokines results in the development of inflammation in the intestine. In fact, the levels of the inflammatory cytokine interleukin (IL)-8 have been shown to be increased in the intestine of IBD patients.<sup>27</sup> Therefore, it is important to examine IL-8 secretion in assessment of the level of inflammation in the intestine. Our previous study indicated that genomic DNA of lactobacilli decreased H<sub>2</sub>O<sub>2</sub>-induced IL-8 secretion from Caco-2 cells.<sup>6</sup> In addition, we identified an oligodeoxynucleotide (ODN), which markedly decreased H<sub>2</sub>O<sub>2</sub>-induced IL-8 secretion, from the genomic DNA of *L. casei*, and demonstrated that oral administration of this anti-inflammatory ODN to mice ameliorated DSS-induced murine colitis.<sup>21</sup> Therefore, suppression of IL-8 secretion *in vitro* is an important factor for ameliorating colitis in this *in vivo* model of IBD. In our preliminary study, we found that heat-killed *L. plantarum* and its genomic DNA decreased H<sub>2</sub>O<sub>2</sub>-induced IL-8 secretion from Caco-2 cells without inducing a pro-inflammatory response (data not shown). These observations suggested the possibility of an anti-inflammatory effect in an *in vivo* model of IBD by heat-killed *L. plantarum* and its genomic DNA. On the other hand, we also showed previously that suppression of TLR9 expression by RNAi abolished the decrease in H<sub>2</sub>O<sub>2</sub>-induced IL-8 secretion by lactobacilli genomic DNA and the anti-inflammatory ODN.<sup>6,21</sup> These observations indicated that the TLR9 signaling pathway plays a major role in mediating the decrease in IL-8 secretion. However, at present, we cannot explain the mechanism by which activation of the TLR9 signaling pathway results in the observed decrease in IL-8 secretion. The results of the present study indicated that *L. plantarum* genomic DNA increased elafin secretion from Caco-2 cells through TLR9 (**Figures 3 and 4**). Therefore, the increase in elafin levels may play important roles in the reduction of IL-8 secretion by lactobacilli genomic DNA, likely due to inhibition of pro-inflammatory proteases and restoration of barrier function to damaged epithelial cells.<sup>13</sup> Further studies of the increase in elafin levels may elucidate the mechanism by which the TLR9 signaling pathway induces the observed anti-inflammatory effects.

TLR9 is expressed in human epithelial cells, including Caco-2 cells, and is localized in the endosome and lysosome.<sup>22</sup> The bacterial genomic DNA must undergo translocation into Caco-2 cells to be recognized by TLR9. However, bacterial genomic DNA cannot translocate into mammalian cells because of its high molecular weight and hydrophilicity. Therefore, translocation of DNA into eukaryotic cells has generally been performed using transfection reagents, such as liposomes. In the present study, we confirmed that *L. plantarum* genomic DNA translocated into Caco-2 cells in the presence (**Figure 5**), but not in the absence (data not shown), of transfection reagent. However, translocation of genomic DNA was also observed in Caco-2 cells treated with heat-killed *L. plantarum*. At present, we cannot explain these observations. Previous

studies showed that *L. plantarum* produces a major polyamine, putrescine,<sup>28</sup> and that polyamines form a complex with and stabilize the DNA. This complex subsequently forms nanoparticles and is subsequently readily translocated into eukaryotic cells.<sup>29,30</sup> One possible explanation for the translocation of genomic DNA into Caco-2 cells treated with heat-killed *L. plantarum* is that the genomic DNA and polyamine contained in heat-killed *L. plantarum* may form such a complex, thus facilitating translocation of the genomic DNA into Caco-2 cells due to the transfection effect of the polyamine.

## Conclusion

The results of the present study indicated that heat-killed *L. plantarum* increased the level of elafin secretion from Caco-2 cells, and the bacterial genomic DNA and TLR9 play crucial roles in this effect. Lactobacilli therapy is useful for the treatment of IBD, although the underlying mechanisms are not fully understood. Our observations suggest that one possible mechanism for the efficacy of lactobacilli therapy may involve an increase in elafin levels in the intestine. Further studies of the elafin induction associated with lactobacilli and their genomic DNA may lead to the development of novel therapeutic approaches to ameliorate intestinal inflammation.

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# Common antibody dependent cell mediated cytotoxicity (ADCC) antibody epitopes of HIV-1 CRF01\_AE Env and Gag in early HIV-1 infected individuals

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## Abstract

**Background:** There have been a few studies aimed at identifying epitopes of ADCC-inducing antibodies when compared to those of neutralizing antibodies and cytotoxic T lymphocytes against a variety of HIV-1 clades.

**Objective:** To map the common ADCC epitopes of HIV-1 CRF01\_AE.

**Methods:** We screened 65 sera of confirmed early HIV-1 CRF01\_AE infected individuals for ADCC antibody against gp120 utilizing an EGFP-CEM-NK<sup>r</sup> flow cytometric assay. Sera with high ADCC antibody were then examined against ADCC epitopes using the complete HIV-1 CRF01\_AE gp160- and subtype A Gag-overlapping peptide sets which were divided into 7 pools:E1-E7 and 5 pools:G1-G5, respectively. Each positive peptide pool was further investigated for fine ADCC epitope mapping using matrix formats.

**Results:** Twenty, 25 and 20 sera demonstrated the high-, medium- and low-ADCC antibody activities against gp120, respectively. Interestingly, 11 Env- and 6 Gag-peptides of pools E3, E4, E7 and pools G1, G2, G4 with high ADCC responses were also responded by at least 20%, 12% and 5%, 10% of medium- and low-ADCC antibody sera, respectively. These eleven common Env ADCC epitopes were localized at C2-V3-C3-V4 regions of gp120 and cytoplasmic tail of gp41 while six common Gag ADCC epitopes were localized at p17-p24-p2 regions.

**Conclusions:** Although the degree of ADCC antibody responses to the gp120 protein varied from high to low, there were certain consensus Env and Gag peptides that could induce the ADCC antibody responses of 21.54-58.46% and 23.08-41.54%, respectively of the early infected individuals. This epitope information should be useful as the new antibody-based vaccine immunogens.

**Key words:** ADCC antibody epitopes; HIV-1 CRF01\_AE; Env; Gag; early infection

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## Introduction

The development of effective HIV-1 vaccines is urgently required for controlling future spread of HIV-1.<sup>1</sup> Current prophylaxis HIV-1 vaccine trials are based on both arms of immune responses, i.e., humoral and cellular immunity. However, each arm has not yet been successful.<sup>2</sup> Antibody dependent cell mediated cytotoxicity (ADCC) is a cytolytic

mechanism of natural killer (NK) cells against virus-infected cells by combining of the innate and adaptive immunity. The mechanism of ADCC can be stimulated by the interaction of specific antibody bound to HIV-1 antigens on the infected cells and Fc receptor CD16 (FcγRIIIa) expressed on the surface of NK cells. Activation of NK cells lead to lysis of target cells.<sup>3</sup>



ADCC responses have been reported to correlate with the control or limit viremia during acute HIV-1 infection and help to slow the progression of disease in both HIV-1 infected individuals and SIV infected macaques.<sup>4,5</sup> Of the six HIV-1 efficacy trials, the RV144 prime-boost regimen phase III trial has provided evidence of vaccine-induced 31.2% protection.<sup>2</sup> Vaccinees who showed significantly low risk of HIV-1 infection demonstrated the high level of ADCC antibody responses.<sup>6</sup>

In the clinical course of HIV-1 infection, the acute phase of infection reveals an initial high peak of plasma viremia and decreased number of CD4<sup>+</sup> T lymphocytes.<sup>7</sup> In addition, expansion of inefficient HIV-1-specific CD8<sup>+</sup> T cells during acute infection has recently been reported.<sup>8</sup> Finding of the early effective immune responses against transmitted virus in acute HIV-1 infection may give a chance to control initial viremia, prolong HIV-1 disease progression, decrease immune cell damage and prevent transmission.<sup>9</sup> Identification of the common epitopes of HIV-1 that could respond to ADCC antibody of sera with low-, medium- or high-ADCC against envelope protein at the early phase would be an important new information that can be utilized for vaccine development. Here we have identified common ADCC antibody epitopes of Env and Gag of HIV-1 CRF01\_AE using sera of confirmed early HIV-1 infected individuals.

## Methods

### Volunteer samples

All sera samples from young Thai men who were enrolled to the conscripts during year 2001-2013 were obtained with written informed consents following approval from Institutional Review Board (IRB) of Royal Thai Army Medical Department (Code: S034b/57) and IRB of the Faculty of Medicine Siriraj Hospital (COA: Si609/2014) prior to initiation of this study. The sera samples obtained had no individual identifiers. These sera were confirmed as early HIV-1 infection by using the Aware™ BED™ EIA HIV-1 Incidence Test (Calypte Co., Portland, OR, USA).

### Cell line

The NK cell resistant, EGFP-CEM-NK<sup>r</sup> cells were cultured and prepared for the assay as previously described.<sup>10</sup>

### Peptides

The gp120 of HIV-1 CRF01\_AE CM243 (Protein Sciences, Meriden, CT, USA) was used to pulse the EGFP-CEM-NK<sup>r</sup> cells. In addition, the complete peptide set based on the HIV-1 CRF01\_AE Env TH023/CM240 sequence consists of 168 peptides (122 peptides of TH023 gp120 and 46 peptides of TH023/CM240 gp41) each containing 15 amino acids (aa) in length, overlapping by 11 aa. The 143 peptides of TH023 were kindly provided by the Natural and Medical Sciences Institute (University of Tübingen, Germany) and 25 peptides of CM240 were commercially synthesized (GenScript, Piscataway, NJ, USA). These Env peptides were divided into seven pools:E1-E7, each containing 24 peptides. To further identify the reactive peptide, each informative peptide pool was again divided into ten small pools according to the 5 by 5 matrix formats. Moreover, Gag peptide set of HIV-1 subtype A sequence

consisting of 49 peptides each containing 20 aa in length, overlapping by 10 aa was kindly provided by the National Institutes of Health (NIH) AIDS reagent repository (catalog #3991). These Gag peptides were divided into five pools:G1-G5, each containing 9-10 peptides. Each pool was again divided into three small pools as 3 by 3 matrix formats. Each peptide pool was also used to pulse the EGFP-CEM-NK<sup>r</sup> cells.

### Preparation of sera

All sera were from individuals infected with HIV-1 CRF01\_AE as determined by C2-V4 nucleotide sequencing as described elsewhere.<sup>11</sup> A HIV-1 sero-negative pooled serum and a sero-positive serum with high ADCC antibody activity were used as controls with each assay. All sera were inactivated at 56 °C for 30 min. prior to use and used at dilutions of 1:250, 1:2,500 and 1:25,000.

### Target cells

The gp120- or peptide pool-pulsed EGFP-CEM-NK<sup>r</sup> target cells were prepared by the addition of either 0.1 µg gp120 of HIV-1 CRF01\_AE CM243 or 1 µg of each peptide pool to 1 × 10<sup>5</sup> target cells. The peptide and target cells were incubated for 1 h at room temperature. The gp120 pulsed cells were washed three times with phosphate buffered saline. The target cells were then re-suspended in growth medium containing RPMI 1640 (Gibco, Grand Island, NY) supplemented with 2 mM l-glutamine, 100 µg/ml streptomycin, 100 IU/ml penicillin, and 10% heat-inactivated fetal calf serum (Gibco) at a concentration of 1 × 10<sup>5</sup> cells/ml.

### Effector cells

Cryopreserved peripheral blood mononuclear cells (PBMC) were prepared from leukopaks of a single HIV-1 seronegative blood donor as previously described.<sup>10</sup> Cells were later thawed and overlaid overnight at 1 × 10<sup>6</sup> cells/ml in growth medium at 37 °C with 5% CO<sub>2</sub>. The cells were then re-suspended in growth media at a concentration of 2.5 × 10<sup>6</sup> cells/ml and used as effector cells.

### EGFP-ADCC flow cytometric assay

The EGFP-ADCC flow cytometric assay was based on a method previously described.<sup>10</sup> Lysis of target cells by ADCC activity was measured and analyzed with a FACScan flow cytometer (Becton Dickinson Biosciences {BDB}, San Jose, CA, USA). The actual percent lysis of target cells at each serum dilution was calculated as previously reported.<sup>10</sup> The cut-off for positive results using gp120- and peptide pool-pulsed target cells are 7.21% lysis and 5.46% lysis, respectively.<sup>12</sup>

## Results

### Screening for high ADCC antibody activity against gp120 pulsed target cells

Sixty five confirmed early HIV-1 CRF01\_AE infected sera were investigated for ADCC antibody activity against gp120 pulsed target cells. The percent lysis of target cells ranged from 9.15 % to 56.12 % with mean and SD values of 24.08 and 10.72, respectively. Twenty, 25 and 20 sera demonstrated the high (> 29.44% target cell lysis, > mean + 0.5SD), medium

(29.44-18.72% lysis, mean  $\pm$  0.5SD) and low (< 18.72% lysis, < mean-0.5SD) ADCC antibody activities, respectively.

#### Identification of HIV-1 CRF01\_AE gp160- and Gag-epitopes that are the target of ADCC reactive antibodies

Twenty sera with relatively high ADCC antibody activity were further examined against each of the seven peptide pools: E1-E7 of the HIV-1 CRF01\_AE Env peptide set and each of the five peptide pools: G1-G5 of HIV-1 subtype A Gag formulated as described in the methods section. These 20 sera demonstrated positive ADCC antibody responses against a number of Env peptide pools while 18 sera responded against Gag peptide pools as shown in **Table 1**. For Env peptide pools, whereas sixteen (80%), fourteen (70%), and twelve (60%) sera demonstrated significantly higher frequency of ADCC responses against pools E7, E3 and E4, respectively, six to ten (30-50%) of these sera showed significant but somewhat lower frequency of ADCC responses to peptide pools E2, E1, E6 and E5. Moreover, 9/20 (45%) of tested sera demonstrated the responses to all three peptide pools: E3, E4, E7. In addition, all tested sera responded to at least one of these three peptide pools. Thus, a 5 by 5 matrix of each of the 3 peptide pools E3, E4 and E7 was selected to perform the fine epitope mapping of ADCC responses. The potential ADCC antibody epitopes were identified by verifying the common peptides from this

matrix. The common peptides within pools E3, E4 and E7 that responded to ADCC activity of each of the 20 tested sera are shown in **Table 2**. Twelve potential ADCC antibody epitopes with  $\geq$  50% responses of all tested sera in each pool were identified. For the parent E3 peptide pool, 9/14 (64.29%) and 7/14 (50%) sera showed ADCC activity against peptides (78, 83) and 71, respectively. For the parent E4 pool, 8/12 (66.67%), 7/12 (58.33%) and 6/12 (50%) of sera showed ADCC activity against peptides 87, (84, 85, 95, 97) and (89, 92), respectively. Moreover, 8/16 (50.00%) sera demonstrated ADCC activity against peptides 190 and 191 from the parent E7 pool.

For Gag peptide pools, whereas fourteen (70%), twelve (60%) and ten (50%) sera demonstrated significantly higher frequency of ADCC antibody responses against pools G4, G2 and G1, respectively, three to seven (15-35%) of these sera showed significant but somewhat lower frequency of ADCC responses to pools G3 and G5 (**Table 1**). In addition, 6/20 (30%) of tested sera recognized all three peptide pools: G1, G2, G4. However, two sera (10%) did not respond to any pool of Gag peptide. Thus, a 3 by 3 matrix of each of the 3 peptide pools G1, G2 and G4 was selected to perform the fine epitope mapping of ADCC responses. The common peptides within pools G1, G2 and G4 that responded to ADCC activity of each of the 18 sera tested are shown in **Table 3**. Six potential ADCC antibody epitopes with  $\geq$  50% responses of all tested sera in each pool

**Table 1. Identification of peptide pools that are the targets of antibody dependent cell mediated cytotoxicity (ADCC) antibody responses in high ADCC antibody activity sera.**

Serum No.	Obtained year	# Env peptide pools (E1-E7) response to ADCC antibody							# Gag peptide pools (G1-G5) response to ADCC antibody				
11	2001												
4	2007	1	2			5	6	7		1			4
14	2007	1	2					7		2			4
10	2008	1					6	7					4
15	2009	1		3	4	5		7		2	3		4
3	2011			3				6					-
11	2011	1	2	3		5		7		1	2		4
15	2011			3	4			7		2			5
16	2011			3	4			7		1	2		5
4	2012		2		4	5		7		1	2	3	4
5	2012			3	4	5	6	7		2			5
7	2012			3	4	5	6	7		1	2		4
9	2012				4			7		1	2	3	
12	2012			3		5				1	2		4
17	2012	1		3	4			6	7	1	2		4
5	2013			3				7					4
6	2013		2	3	4	5	6	7					4
7	2013		2		4	5	6						4
8	2013	1		3	4	5		7		1	2		4
9	2013			3	4			6	7	1			4
<b>Total</b>		<b>7</b>	<b>6</b>	<b>14</b>	<b>12</b>	<b>10</b>	<b>9</b>	<b>16</b>		<b>10</b>	<b>12</b>	<b>3</b>	<b>14</b>

High ADCC antibody activity against HIV-1 gp120 pulsed target cells of twenty HIV-1 seropositive sera were analyzed for their individual responses to target cells pulsing with either each of 7 peptide pools (E1-E7) comprising the complete HIV-1 subtype CRF01\_AE TH023/CM240 gp160 peptide set consisting of 168 peptides 15 amino acids in length or each of 5 peptide pools (G1-G5) comprising the complete HIV-1 subtype A Gag peptide set consisting of 49 peptides 20 amino acids in length. Each pool of Env- and Gag-peptides contains 24 and 9-10 peptides, respectively. Sera years of HIV-1 infection are also shown.

**Table 2. Peptide mapping of high ADCC antibody sera against Env peptide pools E3, E4 and E7.**

Serum		Single peptide number of Env pool responses to ADCC antibody			Total peptide responses
No.	Obtained year	Env pool 3 (E3)	Env pool 4 (E4)	Env pool 7 (E7)	
11	2001	<b>78, 83</b>	-	-	2
4	2007	-	-	<b>188, 190, 191</b>	3
14	2007	-	-	176, 181, 186	3
10	2008	-	-	175, 176, 180, 181, 185, 186, <b>190, 191</b>	8
15	2009	62, 65, 66, <b>83</b>	<b>87, 88, 92, 93, 97, 98</b>	177, 179, 186-189, <b>190, 191</b>	18
3	2011	<b>78</b>	-	-	1
11	2011	<b>70, 71, 75, 77, 78, 82, 83</b>	-	<b>181, 191</b>	9
15	2011	63, 65, 66, 68, 70, <b>71, 75, 77, 78, 80, 81, 83</b>	90, <b>92, 94, 95, 97, 99</b>	179, 180	20
16	2011	<b>70, 71, 77, 78, 82, 83</b>	<b>84, 85, 86, 87, 88, 90, 91, 92, 93, 95, 96, 97, 98</b>	178-180, 188, 189, <b>190</b>	25
4	2012	-	91, <b>92, 96, 97, 101, 102</b>	177, 178, 181, 187, 188, <b>191</b>	12
5	2012	56, 57, 61, 62, 66, 67, <b>71, 74, 78, 79, 83</b>	<b>86, 87, 89</b>	168, 173, 178, 180, 188, <b>190</b>	20
7	2012	62, 65, 66, 67, 70, <b>71, 74, 77, 78, 79, 82, 83</b>	<b>84, 85, 86, 87, 88</b>	168-170, 173-175, 183-185	26
9	2012	-	<b>84, 85, 87, 89</b>	172-175, 187-189, <b>190</b>	12
12	2012	<b>70, 71</b>	-	-	2
17	2012	56-59, 62-64, 67-69, <b>71</b>	<b>84, 85, 87, 89, 95, 97, 99, 100, 102, 104</b>	169, 171, 179, 181, 184, 186, 189, <b>190, 191</b>	29
5	2013	<b>78</b>	-	<b>191</b>	2
6	2013	56, 57, 67, 79	<b>87, 90, 92, 95, 97</b>	167-169, 171, 182-184, 186	17
7	2013	-	<b>84, 85, 86, 89, 95, 96, 99, 105, 106, 109</b>	-	10
8	2013	<b>71, 74, 77, 78, 79, 82, 83</b>	<b>84, 85, 88, 89, 90, 93, 94, 95, 98-100, 103, 104</b>	169, 170, 174, 175	24
9	2013	63, 66, 80, <b>83</b>	<b>84, 85, 87, 89, 90, 92, 94, 95, 97, 99, 100, 102, 104</b>	167, 169-172, 174-177, 179-182, 184-187, 189, <b>190, 191</b>	37

The peptide epitopes that showed  $\geq 50\%$  of ADCC antibody responses of all tested sera against each pool are shown in bold.

**Table 3. Peptide mapping of high ADCC antibody sera against Gag peptide pools G1, G2, and G4.**

Serum		Single peptide number of Gag pool responses to ADCC antibody			Total peptide responses
No.	Obtained year	Gag pool 1 (G1)	Gag pool 2 (G2)	Gag pool 4 (G4)	
4	2007	3778	-	3797-8, 3803-4, 3806	6
14	2007	-	<b>3789, 3790</b>	3798-9, 3804-5	6
10	2008	-	-	3798-9, 3801, <b>3802</b> , 3804-5	6
15	2009	-	3784, <b>3789, 3790, 3742</b>	<b>3800, 3801</b>	6
11	2011	3775	3784, <b>3790</b>	<b>3800, 3802</b>	5
15	2011	-	3784, <b>3790</b>	-	2
16	2011	<b>3776, 3779</b>	<b>3789, 3790, 3742</b>	-	5
4	2012	3778, <b>3779, 3780</b>	3788, <b>3789, 3790</b>	<b>3800, 3802, 3803</b>	9
5	2012	-	3788, <b>3789</b>	-	2
7	2012	<b>3776, 3779</b>	3786, <b>3789</b>	<b>3800, 3803</b>	6
9	2012	<b>3776, 3779</b>	3784, <b>3789, 3790, 3742</b>	-	6
12	2012	<b>3776, 3779</b>	3784, 3786-7, <b>3789, 3790, 3742</b>	3797, <b>3800, 3806</b>	11
17	2012	3775, <b>3776, 3777-8, 3779, 3780</b>	3782-7, 3742	3803	14
5	2013	-	-	<b>3800, 3802</b>	2
6	2013	-	-	3797, 3799, <b>3800, 3802, 3806</b>	5

The peptide epitopes that showed  $\geq 50\%$  of ADCC antibody responses of all tested sera against each pool are shown in bold.

**Table 3. (Continued) Peptide mapping of high ADCC antibody sera against Gag peptide pools G1, G2, and G4.**

Serum		Single peptide number of Gag pool responses to ADCC antibody			Total peptide responses
No.	Obtained year	Gag pool 1 (G1)	Gag pool 2 (G2)	Gag pool 4 (G4)	
7	2013	-	-	<b>3800</b>	1
8	2013	3772-5, <b>3776</b> , 3777, 3781	3782-8, <b>3789</b> , <b>3790</b> , 3742	<b>3802</b>	18
9	2013	3775, <b>3776</b> , 3777-8, <b>3779</b> , 3780	-	3801, <b>3802</b>	8

The peptide epitopes that showed  $\geq 50\%$  of ADCC antibody responses of all tested sera against each pool are shown in bold.

**Table 4. Sequences of potential ADCC antibody epitopes of HIV-1 CRF01\_AE Env TH023/CM240**

Peptide Pool - No.	Potential ADCC Ab epitopes of HIV-1 CRF01_AE gp160	Amino acid #	Number of sera respond to ADCC epitopes of group			Total sera responses (%)	Glycoprotein location
			High responses (%)	Medium responses (%)	Low responses (%)		
3 - 71	TNNAK TIIVH LNKSV	280-294	8/20 (40)	7/25 (28)	3/20 (15)	18/65 (27.69)	gp120 (C2)
3 - 78	SINIG PGQVF YRTGD	308-322	9/20 (45)	18/25 (72)	11/20 (55)	38/65 (58.46)	gp120 (V3)
3 - 83	RKAYC EINGT KWNEV	328-342	9/20 (45)	6/25 (24)	2/20 (10)	17/65 (26.15)	gp120 (V3-C3)*
4 - 84	CEING TKWNE VLKKV	332-346	7/20 (35)	6/25 (24)	9/20 (45)	22/65 (33.85)	gp120 (C3)
4 - 85	GTKWN EVLKK VTKKL	336-350	7/20 (35)	11/25 (44)	10/20 (50)	28/65 (43.08)	gp120 (C3)
4 - 87	KKVTK KLKEH FNNKT	344-358	8/20 (40)	7/25 (28)	6/20 (30)	21/65 (32.31)	gp120 (C3)
4 - 89	EHFNN KTIIF QPPSG	352-366	6/20 (30)	7/25 (28)	11/20 (55)	23/65 (35.39)	gp120 (C3)
4 - 92	PSGGD LEITM HHFNC	364-378	6/20 (30)	11/25 (44)	15/20 (75)	32/65 (49.23)	gp120 (C3)
4 - 95	FNCRG EFFYC NTTRL	376-390	7/20 (35)	8/25 (32)	2/20 (10)	17/65 (26.15)	gp120 (C3-V4)**
4 - 97	YCNTT RLFNN TCMEN	384-398	7/20 (35)	0/25 (0)	0/20 (0)	7/65 (10.77)	gp120 (V4)
7 - 190	LSFQT PSHHQ REPDR	708-722	8/20 (40)	5/25 (20)	1/20 (5)	14/65 (21.54)	gp41 (CT)
7 - 191	TPSHH QREPD RPEGI	712-726	8/20 (40)	5/25 (20)	5/20 (25)	18/65 (27.69)	gp41 (CT)

C = constant region; V = variable region; CT = cytoplasmic tail

\*V3 (aa 328-331) - C3 (aa 332-342); \*\*C3 (aa 376-384) - V4 (aa 385-390)

**Table 5. Sequences of potential ADCC antibody epitopes of HIV-1 CRF01\_AE Gag**

Peptide Pool - No.	Potential ADCC Ab epitopes of HIV-1 CRF01_AE Gag	Amino acid #	Number of sera respond to ADCC epitopes of group			Total sera responses (%)	Protein location
			High responses (%)	Medium responses (%)	Low responses (%)		
1 - 3776	LERFA LNP SL LETTE GCQOI	41-60	7/20 (35)	6/25 (24)	2/20 (10)	15/65 (23.08)	p17
1 - 3779	GTEEL RSLYN TVATL YCVHQ	71-90	7/20 (35)	8/25 (32)	7/20 (35)	22/65 (33.85)	p17
2 - 3789	MFSAL SEGAT PQDLN MMLNI	171-190	9/20 (45)	3/25 (12)	6/20 (30)	18/65 (27.69)	p24
2 - 3790	PQDLN MMLNI VGGHQ AAMQM	181-200	9/20 (45)	5/25 (20)	7/20 (35)	21/65 (32.31)	p24
4 - 3800	CKSIL RALGA GATLE EMMTA	330-350	6/20 (30)	8/25 (32)	9/20 (45)	23/65 (35.39)	p24
4 - 3802	CQGVG GPGHK ARVLA EAMSQV	350-370	6/20 (30)	13/25 (52)	8/20 (40)	27/65 (41.54)	p24-p2*

p17 = MA (matrix); p24 = CA (capsid)

\*p24 (aa 350-363) - p2 (aa 364-370)

were identified. For the parent G1 peptide pool, 7/10 (70.00%) sera showed ADCC activity against peptides 3776 and 3779. For the parent G2 and G4 pools, 9/12 (75.00%) and 7/14 (50.00%) of the sera showed ADCC activity against peptides (3789, 3790) and (3800, 3802), respectively.

**The responses of medium- and low-ADCC antibody sera against the common ADCC antibody epitopes of HIV-1 CRF01\_AE Env and Gag that were identified in high ADCC antibody sera**

Twenty five medium- and 20 low-ADCC antibody sera were examined for their responses to the 12 and 6 potential common

ADCC antibody epitopes of HIV-1 Env and Gag, respectively. For Env protein, the range of 5/25 (20%) - 18/25 (72%) and 1/20 (5%) - 15/20 (75%) of medium- and low-ADCC antibody sera, respectively demonstrated the responses to 11 common ADCC epitopes (peptides 71, 78, 83, 84, 85, 87, 89, 92, 95, 190, 191); i.e., except peptide 97 (Table 4). The overall responses of 65 tested sera against these peptides were  $> 20\%$ , i.e., 21.54% - 58.46%. These 11 common epitopes located within the C2, V3, C3 and V4 of gp120 and the cytoplasmic tail (CT) of gp41 region.

For Gag protein, the range of 3/25 (12%) - 13/25 (52%) and 2/20 (10%) - 9/20 (45%) of medium- and low-ADCC antibody sera, respectively, demonstrated the responses to 6 common



ADCC epitopes (peptides 3776, 3779, 3789, 3790, 3800, 3802) (Table 5). The overall responses of 65 tested sera against these peptides were > 20%, i.e., 23.08% - 41.54%. These 6 common epitopes are localized within the p17, p24 and p2 regions of Gag protein.

### Discussion

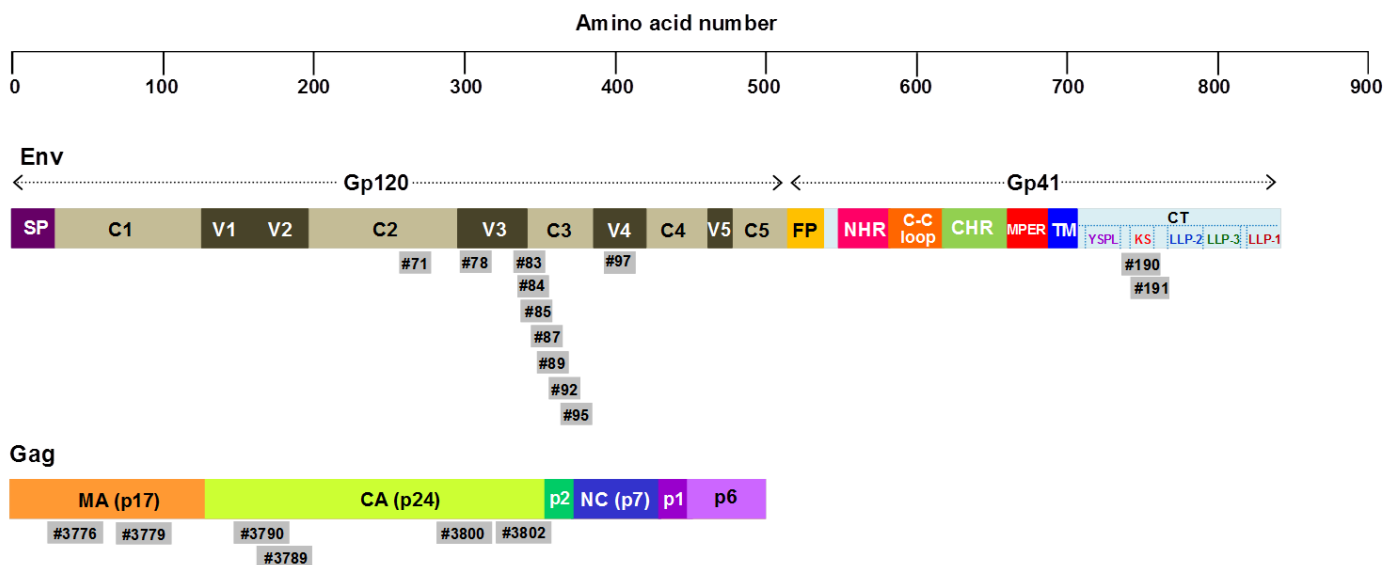
In this study, sixty five sera of young Thai men who enrolled to the conscripts were confirmed as sera from early HIV-1 CRF01\_AE infected individuals using the Aware™ BED™ EIA HIV-1 Incidence Test (data not shown). Estimation of the mean duration of recent infection (MDRI) was 198.4 days for BED.<sup>13</sup> All sera were screened for high-, medium- and low-ADCC antibody activity against gp120 pulsed target cells utilizing an EGFP-CEM-NK<sup>r</sup> flow cytometric assay. The gp120 was used instead of gp160 because only HIV-1 CRF01\_AE gp120 is commercially available. Our assay measures the number of target cells that are killed.<sup>10</sup>

Identification of potential ADCC antibody epitopes using a matrix format led to the finding that the spectrum of peptide specific reactivity varied for each serum tested. All tested sera showed reactivity against a total of 1-37, and 1-18 peptides for Env and Gag proteins, respectively (Tables 2, 3). Studies on Env protein further showed that three epitopes (peptides 71, 78, 83) of pool E3, seven epitopes (peptides 84, 85, 87, 89, 92, 95, 97) of pool E4 and two epitopes (peptides 190, 191) of pool E7 were identified as being targets of ADCC antibodies from at least 50.00% of the tested sera (Table 2). Interestingly, all these potential epitopes except peptide 97 could be able to gain the responses from sera of medium- and low-ADCC activities for at least 20% and 5%, respectively which made the overall total responses to the range of 21.54-58.46% (Table 4). The aa sequences of the 12 potential ADCC epitopes of HIV-1 CRF01\_AE gp160 are also shown in Table 4. The common peptides 71, 78, and 83 are localized within the C2, V3, and V3-C3 regions of gp120, respectively while the common peptides (84, 85, 87,

89, 92), 95 and 97 are localized within the C3, C3-V4 and V4 of gp120, respectively (Figure 1). In addition, the common peptides 190 and 191 are in the cytoplasmic tail region of gp41. Kijak et al. studied the molecular evolution of the HIV-1 epidemic among volunteers of RV144 vaccine trial from 2003 to 2009.<sup>14</sup> They found that CRF01\_AE strains were 31% more diverse than the ones from the 1990s Thai epidemic. Thus, HIV-1 CRF01\_AE strains that infected individuals in our study should also be diverse since the sera were obtained during 2001-2013. Interestingly, sera from these individuals could be able to demonstrate ADCC antibody responses to these common potent epitopes. This indicates that these ADCC epitopes were quite conserved and immunodominant.

Moreover, peptides 78, 85 and 92 showed the responses to ADCC activity at a larger number of sera of medium- and low-ADCC response groups than those of a high-ADCC response group, i.e., (72%, 55% vs 45%), (44%, 50% vs 35%), (44%, 75% vs 30%), respectively. This revealed the importance of these peptide sequences as the prominent ADCC epitopes for induction of ADCC responses against Env peptides of early HIV-1 infected individuals, i.e., 58.46%, 43.08% and 49.23%, respectively, of total 65 tested sera (Table 4).

Kulkarni et al. reported that 5 out of 10 Indian long-term non-progressors (LTNPs) demonstrated ADCC activity against the target epitopes in Env-C V3 region (aa 288-330: LNESVEI VCTRPNNNTRKSIRIGPGQGTGDIIGDIRQAHC).<sup>15</sup> They proposed the potential role of this anti-V3 ADCC response in viral control which may contribute to the slower disease progressors. Their epitope sequences included our full epitope peptide 78 (aa 308-322: SINIGPGQVFYRTGD) and partial of epitope peptide 71 (aa 290-294:...LNKSV). The V3 region is one of the most immunogenic epitopes in the envelope which the V3 crown composed of the highly conserved aa I307, I309 and F317 that can induce specific antibody in > 90% of HIV infected individuals.<sup>16,17</sup> V3-specific Abs from RV144 vaccinees were shown to capture infectious virions.<sup>18</sup> Moreover,



**Figure 1. Epitope distribution across the Env gp160 and Gag of HIV-1.** ADCC antibody epitopes of HIV-1 CRF01\_AE identified in this study are shown. The epitope sizes are not drawn to scale and the amino acid locations of the epitopes are aligned as close as possible with the true alignment. C = constant region; V = variable region; CT = cytoplasmic tail; KS = Kennedy sequence; MA = matrix; CA = capsid

Montefiori et al. demonstrated the high Nt antibody activity against V3 peptide of HIV-1 CRF01\_AE TH023 (aa 302-326: SNNTRTSINIGPGQVFYRTGDIIGD) from volunteer sera of RV144 phase III trial.<sup>19</sup> The initial ADCC antibody response to the V3 epitopes in our study may occur against the founder virus in most of the early HIV-1 infected individuals. However, the later consequence may depend on the efficiency of each individual immune response. If one can control the virus as in LTNP, the ADCC antibody against these epitopes should be detectable for a longer time period. On the other hand, the virus may mutate the V3 sequence to evade the immune responses<sup>20</sup> and later ADCC antibody may be further adapted to recognize the coming mutated V3 epitopes. Thus, the ADCC antibody at the non-early phase would not be able to respond to these original V3 epitopes as we previously reported on ADCC epitopes of HIV-1 in asymptomatic infected individuals.<sup>12</sup> Our finding of peptide 85 (aa 336-350: GTKWNEVLKKVTKKL) as dominant ADCC epitope was also correspondent to the data of Kulkarni et al.<sup>15</sup> They demonstrated the recognition of novel antigenic ADCC epitope in sera of LTNPs at the C3 region (aa 331-360: CNISEEKWNKTLQRVSEKLKEHFPNKTIKF).

The CD4-bound conformation of Env was recently shown to be a major target of ADCC-mediating antibodies present in the sera of HIV-1-infected individuals.<sup>21</sup> Prévost et al. reported the influence of the envelope gp120 Phe 43 cavity on HIV-1 sensitivity to ADCC responses.<sup>22</sup> While residue S375 in the gp120 Phe 43 cavity is well conserved in the majority of group M HIV-1 isolates, CRF01\_AE strains have a naturally occurring histidine at this position (H375). They demonstrated that the H375 increases Env propensity to sample the CD4-bound conformation, thus increasing susceptibility of HIV-1 infected cells to ADCC. They raise the intriguing possibility that the presence of His 375 in the circulating CRF01\_AE strain in Thailand might have contributed to the efficacy of the trial by spontaneously exposing epitopes recognized by ADCC-mediating antibodies elicited by the RV144 vaccine regimen. Our finding on peptide 92 (aa 364-378: PSGGDLEITMHHFNC) with His 375 emphasized the immunodominant property of this peptide as ADCC epitope since 49.23% of tested sera showed the ADCC responses.

Tomaras et al. reported that the first anti-HIV-1 antibody was the antibody against gp41 and appeared 13 days after the appearance of plasma virus.<sup>23</sup> In our study, the common peptides 190 (aa 708-722: LSFQTPSHHQREPDR) and 191 (aa 712-726: TPSHHQREPDRPEGI) which are located in the Kennedy sequence (KS) of cytoplasmic tail (CT) region of gp41 showed the ability to induce ADCC antibody with the total responses of 21.54% and 27.69%, respectively, of 65 tested sera (**Table 4**). This confirmed the study of Dimmock et al. on the alternative topological model of CT sequence: external localization.<sup>24</sup> Moreover, anti-KE (Kennedy epitope) serum that could specifically neutralize HIV in vitro implied the localization of the KE on the outer surface of the virion.<sup>25</sup> Abacioglu et al. revealed that the KS epitope PDRPEG was a conformational epitope which could induce non-Nt antibody (Chessie 8) binding to HIV-infected cells.<sup>26</sup> This indicated the property of ADCC antibody inducible of our peptides as we demonstrated in our study.

Identification of ADCC antibodies targeting conserved non-Env proteins such as Gag may reveal more potent ADCC activities. The appearance of HIV-core antigens on the surface of infected cells has been shown for p17 and p24.<sup>27,28</sup> Moreover, the monoclonal antibodies (mAbs) to p24-core protein of HIV-1 have been demonstrated to mediate ADCC activity to destroy infected cells.<sup>29</sup> Our studies on Gag protein further demonstrated that two epitopes of each peptide pools G1 (peptides 3776, 3779), G2 (peptide 3789, 3790) and G4 (peptides 3800, 3802) were identified as being targets of ADCC antibodies from at least 50.00% of the sera tested (**Table 3**). Interestingly, all these 6 epitopes could be able to gain the responses from sera of medium- and low-ADCC activities for at least 12% and 10%, respectively which made the overall responses to the range of 23.08-41.54% (**Table 5**). The aa sequences of the 6 potential ADCC epitopes of HIV-1 CRF01\_AE Gag are also shown in **Table 5**. The common peptides 3776 and 3779 are localized within the p17 (MA, matrix) region while the common peptides 3789, 3790 and 3800, 3802 are localized within the p24 (CA, capsid) region (**Figure 1**). Our finding of peptide 3789 (aa 171-190: MFSALSEGATPQDLNMLNI) was correspondent to that of Grunow et al.<sup>29</sup> They showed the recognition of p24-ADCC epitope (aa 178-187: GATPQDLNIM) expressed on the HIV-infected T cells by mAbs. Interestingly, peptides 3779, 3800 and 3802 demonstrated the responses to ADCC activity at an almost equal/higher number of sera of medium- and low-ADCC response groups than those of a high-ADCC response group, i.e., (32%, 35% vs 35%), (32%, 45% vs 30%) and (52%, 40% vs 30%), respectively. This revealed the important of these peptide sequences as the dominant ADCC epitopes for induction of ADCC responses against Gag peptides of early HIV-1 infected individuals, i.e., 33.85%, 35.39% and 41.54%, respectively, of total 65 tested sera. Genetic data support a major role for the cytoplasmic tail of gp41 and the matrix domain of Gag in Env glycoprotein incorporation.<sup>30</sup> Both Gag and Env display properties of being associated with lipid rafts at the plasma membrane. Gag-Env interactions affect their co-recruitment to the virological synapse and subsequent early events in the next round of viral replication. The ADCC antibody against epitopes on matrix domain of Gag demonstrated in our study may have an effect on the new coming viral progeny at the Env incorporation process.

In conclusion, although the degree of ADCC antibody responses to the gp120 protein varied from high to low, there were certain potent consensus Env and Gag peptide epitopes that could induce the ADCC antibody responses in a majority of early infected individuals as shown in this study. This information should be useful for further design of the antibody epitope based vaccine. The pre-existing potent antibody induced by the vaccine should be able to limit the transmitted/founder viruses, delay acquisition, control viral replication and prevent the establishment of infection. Since cell-to-cell transmission appears to be more efficient than that of free virion for HIV-1 infection,<sup>31</sup> the ADCC responses would be a very important mechanism to eliminate the small number of virus-infected cells from an infected partner before the establishment of infection. Although, Nt antibodies have

no effect on cell-to-cell transmission of HIV-1,<sup>32</sup> their responses would be very crucial for eliminating free virions that may enter simultaneously with the viral infected cells during viral transmission. This warrants further study whether these ADCC epitopes also act as Nt antibody epitopes. Our studies are limited by the use of peptide/protein pulsed target cells instead of HIV-1 infected cells. The 3-dimensional structure of Env or Gag on the target cell surface may be not the same as those on the infected cells.

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# Lymphocyte subsets and natural killer cell cytotoxicity in intravenous drug users with HIV-1 infection among Thai population

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## Abstract

**Background:** Intravenous drug users (IVDUs) are among the high-risk groups who are most vulnerable to HIV infection. Several illicit drugs alter host immune function with increased incidence of infections including that of HIV. Many studies of the immune response of NK cells in HIV-1 seronegative IVDUs and HIV-1 seropositive IVDUs have been published from the Western countries and yet no data is available from Thailand.

**Objective:** To determine natural killer cell cytotoxicity and lymphocyte subsets in Thai HIV-1 infected intravenous drug users.

**Methods:** The NK cell cytotoxic function was determined using our well-established EGFP-K562 flow cytometric assay in 30 IVDUs with HIV-1 infection (IVH) comparing with those from the same number of non-infected IVDUs (IVX), HIV-1 seropositive individuals (HIV-1+ve) and healthy controls. The percentage and the absolute number of NK cells, helper CD4<sup>+</sup> T cells and cytotoxic CD8<sup>+</sup> T cells were also investigated.

**Results:** Among the study groups, IVH showed not only the lowest percentage of lytic activity by NK cells, but also a decline in the percentage and absolute count of NK cells. A decline in helper CD4<sup>+</sup> T cells and an increase of cytotoxic CD8<sup>+</sup> T cells of IVH group when compared to those of other 3 groups were also demonstrated.

**Conclusions:** The failure of innate immune NK cell function and their number in IVH may support the involvement of additional components of the immune system in the control of HIV-1 disease.

**Key words:** NK cells; NK cell cytotoxicity; CD4<sup>+</sup> T cells; CD8<sup>+</sup> T cells; HIV-1; intravenous drug users

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## Introduction

Human immunodeficiency virus (HIV)/AIDS is still a major global health problem with an estimated 35.3 million people are living with HIV.<sup>1</sup> In Thailand, more than 1 million of HIV-1 infected people were reported in 2015.<sup>2</sup> Of the estimated 440,000 people living with HIV, the relatively high levels of prevalence were in men who have sex with men, transgender people, male/female sex workers and their partners, migrant workers and intravenous drug users (IVDUs).

Interestingly, the HIV-1 prevalence among the Thai IVDUs has the highest HIV-1 prevalence (about 30-50%) comparing to other risk groups of HIV-1 infection<sup>3</sup> with predominantly a CRF01\_AE subtype.<sup>4-6</sup> It has been known that illicit drugs alter immune function and decrease host resistance to microbes with increased incidence of infections or immune disorders in humans, including infection with HIV and disease progression to AIDS.<sup>7,8</sup>



Human natural killer (NK) cells are large granular lymphocytes of the innate immune system.<sup>9</sup> NK cells are recognized as a subset of cytotoxic innate lymphoid cells which play a role in the killing of tumor and virus-infected cells and participate in shaping the adaptive immunity by secretion of cytokines.<sup>10</sup> NK cells are cytotoxic with small granules in their cytoplasm contain proteins such as perforin and proteases known as granzymes, these proteins are released when they encounter with an infected cell by inducing either apoptosis or osmotic cell lysis.<sup>11,12</sup> NK cells can also recognize and lyse target cells by antibody dependent cell-mediated cytotoxicity (ADCC). The increased risk for the disease progression in HIV-infected individuals are associated not only with the low number of the NK cells but also with the defect in their cytotoxic activity<sup>11</sup> and also with decline of helper CD4<sup>+</sup> T cells and the accumulation of cytotoxic CD8<sup>+</sup> T cells.<sup>13,14</sup>

Although there are many studies of the innate immune response of NK cells in HIV-1 seronegative IVDUs and HIV-1 seropositive IVDUs,<sup>15-18</sup> the study of NK cells due to the HIV-1 infection and/or intravenous drug abuse in Thais has not been conducted. In this study, we used our well-established enhanced green fluorescent protein (EGFP)-K562 flow cytometric method<sup>19</sup> for measuring the NK cell function in IVDUs with HIV-1 infection (IVH). Results were also compared with those from HIV-1 infected individuals (HIV-1+ve) and non-infected IVDUs (IVX) as well as healthy individuals.

## Methods

### Study population and blood samples

Thirty HIV-1 uninfected IVDUs, 22 males and 8 females, and 30 HIV-1 infected IVDUs, 24 males and 6 females, at Public Health Center 3 (Bang Sue), Health Department, Bangkok Metropolitan Administration were enrolled in this study with written informed consents. All volunteers were documented to have treatment for drug addiction. All 30 HIV-1 infected IVDUs had previously tested once for HIV-1 antigen in saliva at this center. Fresh venipuncture Acid citrate dextrose solution B (ACD, Becton Dickinson Biosciences {BDB}, CA, USA) blood samples and Tripotassiummethylenediamine tetra-acetate (K<sub>3</sub>EDTA, BDB) blood samples from each subject were collected. Complete blood counts (CBC) of each blood sample was performed at the laboratory of Department of Clinical Pathology, Faculty of Medicine Siriraj Hospital by using automate hematological analyzer Sysmex XE-5000 (Sysmex Corporation, Kobe, Japan).

Leuko-pak and K<sub>3</sub>EDTA blood from 30 asymptomatic HIV-1 infected blood donors, 22 males and 8 females, were kindly provided by the Department of Transfusion Medicine, Faculty of Medicine Siriraj Hospital and Thai Red Cross. Thirty healthy HIV-1 seronegative blood subjects, 23 males and 7 females were also recruited from the blood bank donors. All samples obtained had no donor identifiers. There were no differences in the base line characteristics of all 4 study groups, i.e., age, sex ratio.

This study was approved by Ethics Committee of the Bangkok Metropolitan Administration for IVDUs subjects (No. 147-2009) and Institutional Review Board, Faculty of Medicine Siriraj Hospital (COA: Si031/2007) for all samples.

### Immunophenotyping staining of peripheral blood samples

Percentages and absolute cell numbers of NK cells (CD3<sup>+</sup>CD16<sup>+</sup>CD56<sup>+</sup>), helper CD4<sup>+</sup>T cells (CD3<sup>+</sup>CD4<sup>+</sup>) and cytotoxic CD8<sup>+</sup>T cells (CD3<sup>+</sup>CD8<sup>+</sup>) were determined by standard TriT-ESTmethod<sup>20,21</sup> using BD Tritest™ monoclonal antibody (mAb) reagents. Briefly, ten µl of TriT-EST 3-color mAb reagents were mixed with 50 µl of EDTA-anticoagulated whole blood and incubated for 20 min at room temperature in the dark before adding 450 µl of FACS Lysing Solution (BDB). After an incubation time of 15 min, the cells were washed at 1,400 rpm for 5 min. The cell pellet was resuspended in 300 µl of 1% paraformaldehyde and kept at 2-8°C until analysis by FACSCalibur flow cytometer (BDB). Percentages of NK cells, helper CD4<sup>+</sup> T cells and cytotoxic CD8<sup>+</sup> T cells were obtained by using CellQuest™ Analysis software (BDB). The absolute cell numbers of these lymphocyte subsets were determined by multiplying the percentage of each subset with the absolute lymphocyte counts from the CBC.

### Preparation of effector cells

Peripheral blood mononuclear cells (PBMCs) from each ACD blood sample were separated by standard ficoll-hypaque density gradient centrifugation, washed three times with 0.2 M phosphate buffered saline (PBS) and resuspended in freezing medium containing 60% RPMI 1640 (Gibco Laboratories, Grand Island, NY, USA), 30% fetal bovine serum (FBS, Gibco Laboratories) and 10% dimethyl sulfoxide (Sigma-Aldrich Corp, MO, USA). Aliquots of PBMCs at a density of 5x10<sup>6</sup> cells/ml were cryopreserved in liquid nitrogen until use. One day prior to the experiment, PBMCs were thawed and added to 100 mm polystyrene tissue culture plates (Griener Bio-One GmbH, Frickenhausen, Germany) and incubated overnight at 37°C with 5% CO<sub>2</sub> to deplete adherent monocytes. The non-adherent lymphocytes were collected and resuspended at a density of 1x10<sup>6</sup> for use as effector cells in the NK cell cytotoxicity assay.

### Preparation of target cells

The EGFP-K562 cell line was prepared as previously described<sup>19</sup> and used as targets for measuring NK cell cytotoxicity by flow cytometry. This cell line was cultured in growth medium containing RPMI 1640 supplemented with 2 mM L-glutamine (Gibco Laboratories), 100 µg/ml streptomycin (Gibco Laboratories), 100 IU/ml penicillin (Gibco Laboratories), and 10% heat inactivated FBS (Gibco Laboratories) and 400 µg/ml of neomycin analogue G-418 (Roche Diagnostics, IN, USA). Prior to the experiment, the target cells were resuspended in growth medium at a density of 1 × 10<sup>5</sup> cells/ml.

### NK cytotoxicity flow cytometry assay

The EGFP-K562 flow cytometric assay for measuring NK cytotoxicity was based on a method previously described.<sup>19</sup> Briefly, four hundred microliters of PBMCs as effector cells at a density of 1 × 10<sup>6</sup> cells/ml were added into individual round bottom 12 × 75 mm polystyrene (Falcon) tubes (BDB) with 2-fold serial diluted in 10% RPMI 1640 growth medium. Fifty microliters of EGFP-K562 target cells as a density of 1 × 10<sup>5</sup> cells/ml were added into each tube to yield the effectors per

target cells (E/T) ratios of 80:1 and 40:1. All sample tubes were mixed and incubated at 37°C with 5% CO<sub>2</sub> for 4 hours. After incubation, propidium iodide (PI) (Sigma-Aldrich Corp) solution at a concentration of 100 µg/ml was added into the tubes to stain compromised or dead cells and then the tubes were incubated at room temperature for 10 minutes. The cytotoxic activity of NK cells to lyse target cells was measured by using a FACSCalibur flow cytometer (BDB). For measuring the percent lysis of NK cytotoxicity, exactly  $2 \times 10^4$  events per test sample were examined and no gating was used for acquisition. The threshold for side scatter (SSC) was set on the linear scale to discriminate the debris and dead cells. Two parameters of the forward and side scatter (FSC/SSC) dot plots signals were analyzed by using CELLQuest™ software (BDB) (**Figure 1A**). The EGFP versus PI data were obtained to evaluate live and dead target cells using log scale of green fluorescent (FL1) or EGFP in the X axis and the log scale of red fluorescent (FL2) or PI in the Y axis (**Figure 1B**). The strong green fluorescent at the lower right (LR) quadrant were identified as the live target cells whereas the red and green fluorescent on the upper right (UR) quadrant were identified as compromised or dead target cells. The red fluorescence on the upper left (UL) quadrant were defined as the autodamaged or potentially lysed effector cells or target cells with decreased EGFP whereas the lower left (LL) were considered to be the living effector cells or living

target cells with weak green fluorescence. Effector cells alone and target cells alone stained with PI were used as controls. The percentage of target cell lysis was calculated by using the following formula: % Lysis = (UR/UR + LR) × 100.

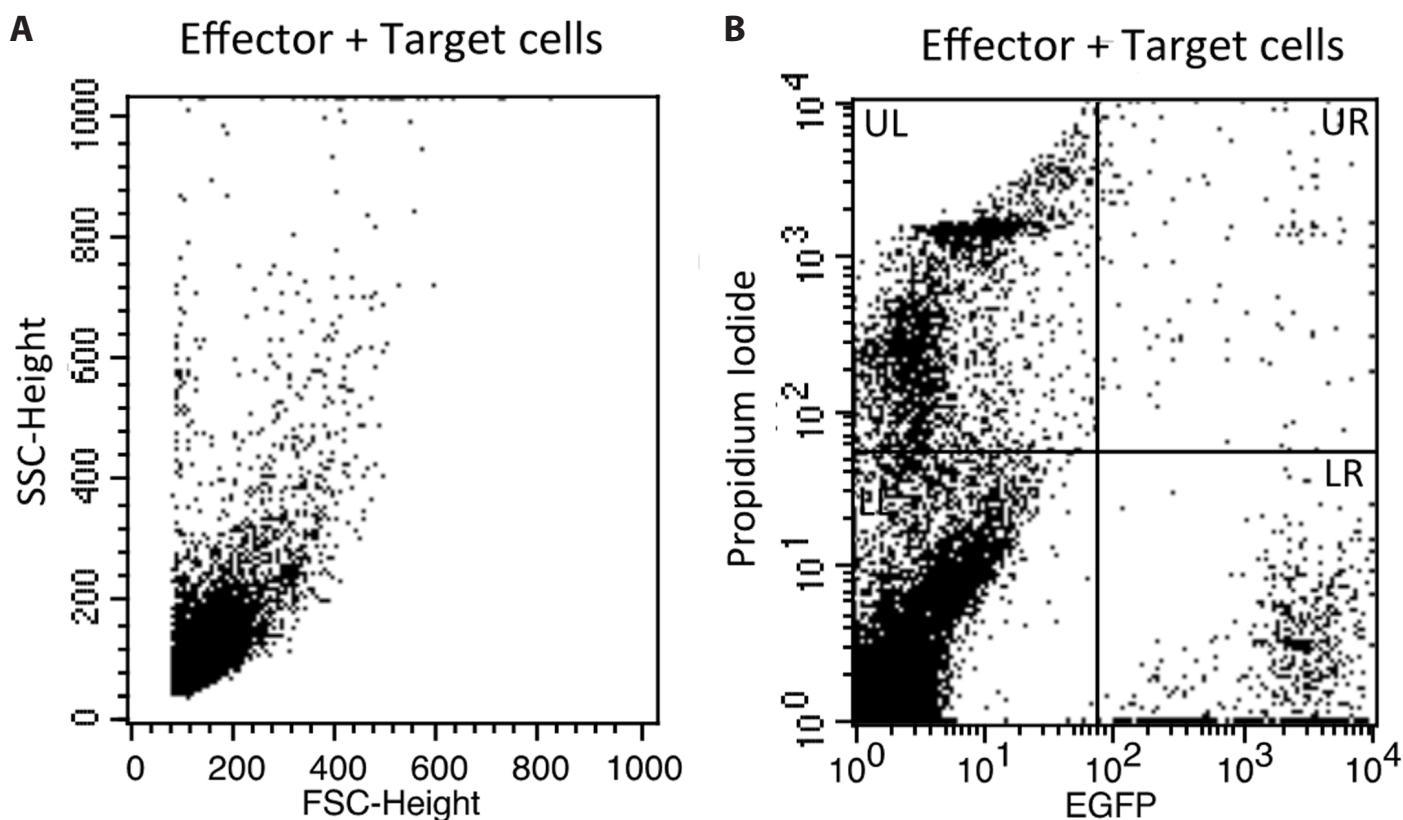
### Statistical analysis

Statistical analysis in this study was conducted on SPSS software version 11.5 (SPSS Inc., NY, USA). Statistical significance within group was analyzed by Shapiro-Wilk test and statistical significance between groups was analyzed with one-way ANOVA test. The significant level of each test between groups was adjusted by multiple testing using Bonferroni and Tamhane correction for homogeneity and heterogeneity of variance, respectively. The significant threshold of four comparisons of each test was set at p value < 0.05.

## Results

### Characteristics of study populations

Characteristics of Thai HIV-1 seronegative individuals (Healthy), HIV-1 seronegative IVDUs (IVX), HIV-1 seropositive individuals (HIV-1+ve) and HIV-1 seropositive IVDUs (IVH) in this study were shown in **Table 1**. Healthy group did not have any vaccination at least one year prior to this study. In IVX group, there was only one participant with history of Hepatitis B virus infection. All subjects in IVH group had



**Figure 1.** Representative flow cytometric two-parameter dot-plots on NK cytotoxicity test showing Forward Scatter (FSC) vs. Side Scatter (SSC) of NK (effector) cells and EGFP-K562 (target) cells **A** and logarithmic EGFP vs. propidium iodide expression of live and dead effector vs. target cells **B**. Cells at the lower right (LR) quadrant are lived target cells whereas the red and green fluorescent on the upper right (UR) quadrant are dead target cells. Cells at the upper left (UL) quadrant are defined as the autodamaged or potentially lysed effector cells or target cells with decreased EGFP whereas the lower left (LL) quadrant represents the living effector cells or living target cells with weak green fluorescence.

**Table 1. Demographics and characteristics of the study populations.**

Group	Subjects	Age (yrs) Mean ± SD	Gender		Injecting drug (%)			Inhalation drug (%)	
			Male	Female	Heroin	Methamphetamine	Domicum	Methamphetamine	Cannabis
Healthy	30	30 ± 6.7	23	7	-	-	-	-	-
IVX	30	39 ± 6.9	22	8	66.6	52.4	28.6	68.8	56.3
HIV-1+ve	30 <sup>a</sup>	31 ± 9.1	22	8	-	-	-	-	-
IVH	30 <sup>b</sup>	39 ± 8.1	24	6	37.5	58.3	54.2	83.3	61.1

IVX = HIV-1 seronegative IVDUs; IVH = HIV-1 seropositive IVDUs

<sup>a</sup> unknown history of antiretroviral drug treatment; <sup>b</sup> all subjects received tenofovir as antiretroviral drugs.

received tenofovir as antiretroviral drugs. All IVDUs subjects had history for multiple intravenous and inhalation drug behaviors. Their drugs used within the 3-month period prior to blood collection were mainly heroin, methamphetamine, domicum and cannabis for both IVX and IVH groups. Within the IVX group, the percentage of participants that used injecting drugs was 66% heroin, 52.4% methamphetamine and 28.6% domicum, and inhalation drugs was 68.8% methamphetamine and 56.3% cannabis (Table 1). For the IVH group, the percentage was 37.5% heroin, 58.3% methamphetamine and 54.2% domicum, and inhalation drugs was 83.3% methamphetamine and 61.1% cannabis (Table 1).

**Percentages and absolute counts of NK cells, helper CD4<sup>+</sup> T cells and cytotoxic CD8<sup>+</sup> T cells in the study populations**

Mean percentages and absolute counts of each lymphocyte subset among the study populations were compared and shown in Table 2. The mean percentages of helper CD4<sup>+</sup> T cells of IVH group and HIV-1+ve group were significantly lower than those of IVX group and healthy group. No statistically significant difference among the two HIV-1 infected groups (IVH group vs. HIV-1+ve group) and among the two HIV-1 seronegative groups (IVX group vs. healthy group) were found. The mean absolute counts of helper CD4<sup>+</sup> T cells from IVH group were significant decreased ( $p \leq 0.001$ ) when compared with those from HIV-1+ve, IVX, and the healthy groups. Interestingly, the

absolute counts of helper CD4<sup>+</sup> T cells from IVX group were significantly higher than those of healthy and HIV-1+ve groups ( $p$  value  $\leq 0.05$ ) while those of healthy and HIV-1+ve groups were comparable.

For cytotoxic CD8<sup>+</sup> T cells, the mean percentages in HIV-1 +ve group and IVH group were significantly higher ( $p \leq 0.05$ ) than those of healthy group and IVX group. No statistically significant differences among the HIV-1+ve and IVH groups and among the IVX and healthy groups were seen (Table 2). Comparison of the absolute counts of cytotoxic CD8<sup>+</sup> T cells among the study groups showed that HIV-1+ve group had a significant increase ( $p \leq 0.05$ ) in the absolute counts when compared with those of IVH, IVX and the healthy groups. Moreover, no significant difference among the healthy, IVX and IVH groups were observed.

Among the study groups, the mean percentage of NK cells was significantly decreased ( $p \leq 0.05$ ) in IVH group when compared to those from healthy group but did not show any significant difference to those of HIV-1+ve group and IVX group. No significant differences in mean percentages of NK cells were found among the HIV+ve, IVX and the healthy groups (Table 2). For the absolute counts of NK cells, the IVH group showed significant decrease ( $p \leq 0.05$ ) in the absolute counts when compared with those of HIV+ve, IVX and the healthy groups. No significant differences among the healthy, IVX and HIV-1+ve groups were found.

**Table 2. Comparison of percentages and absolute counts of helper T cells (CD3<sup>+</sup>CD4<sup>+</sup>), cytotoxic T cells (CD3<sup>+</sup>CD8<sup>+</sup>) and NK cells (CD3<sup>+</sup>CD16<sup>+</sup>CD56<sup>+</sup>) in the 4 study groups.**

Parameter	Mean ± SD				p-value
	Healthy gr.	IVX gr.	HIV-1 +ve gr.	IVH gr.	
<b>Helper T cells</b>					
%	33.25 ± 5.58	36.39 ± 8.78	22.39 ± 9.71*	19.99 ± 6.36*	$\leq 0.05$
cells/ $\mu$ l	784 ± 261	1104 ± 526	732 ± 581	358 ± 206**	$\leq 0.001$
<b>Cytotoxic T cells</b>					
%	30.61 ± 6.07	26.07 ± 8.16	49.38 ± 13.21*	51.09 ± 9.52*	$\leq 0.05$
cells/ $\mu$ l	657 ± 268	757 ± 390	1380 ± 874*	948 ± 406	$\leq 0.05$
<b>NK cells</b>					
%	18.99 ± 5.30	15.62 ± 7.74	14.55 ± 10.87	11.10 ± 6.47 <sup>§</sup>	$\leq 0.05$
cells/ $\mu$ l	458 ± 189	451 ± 303	380 ± 320	188 ± 115**	$\leq 0.05$

\* Statistical difference when compared to the Healthy and IVX groups.

\*\* Statistical difference when compared to the Healthy, IVX and HIV+ve groups.

§ Statistical difference when compared to the Healthy group.

**Table 3. Comparison of NK cytotoxicity function in the 4 study groups.**

E/T ratio	Mean $\pm$ SD of % lysis of NK cytotoxicity in				p-value
	Healthy gr.	IVX gr.	HIV-1 +ve gr.	IVH gr.	
80:1	41.78 $\pm$ 7.99	52.02 $\pm$ 13.68	41.06 $\pm$ 11.23	33.77 $\pm$ 10.02*	$\leq$ 0.05
40:1	30.91 $\pm$ 8.77	35.58 $\pm$ 15.40	29.64 $\pm$ 8.29	23.20 $\pm$ 9.71*	$\leq$ 0.05

\* Statistical difference when compared to the Healthy, IVX, and HIV+ve groups.

### NK cell cytotoxicity in the study populations

Comparison of the mean percentages of EGFP-K562 target cell lysis at the E:T ratios of 80:1 and 40:1 by NK cells from the 4 study groups showed that the IVH group showed significantly decreased of NK cytotoxicity ( $p \leq 0.05$ ) at the E:T ratio of 80:1 and 40:1 when compared to the other groups (Table 3). Interestingly, the mean lysis percentages of NK cytotoxicity in IVX group at both E/T ratios were significantly higher ( $p \leq 0.05$ ) than those in HIV-1+ve, IVH and the healthy groups, particularly at E:T ratio of 80:1. Moreover, no difference of the mean percentages lysis activity between the healthy and the HIV-1+ve groups were shown.

### Discussion

It is estimated that worldwide there are almost 13 million people who are IVDUs. Out of these nearly 2 million, or 15%, are also living with HIV which is typically far greater than it is among the rest of the adult population, with IVDUs bearing a 28 times higher prevalence.<sup>22</sup> In Thailand, there were an estimated 440,000 people living with HIV in Thailand in 2015 with an estimation of adult HIV prevalence of 1.1% out of Thailand's population of more than 60 million. Among 6,900 new HIV infections in 2015, unsafe injecting drug use is the second biggest transmission route which account for 12%.<sup>23</sup> During HIV-1 infection several immunological abnormalities have been found in IVDUs.<sup>5,8</sup> It has been known that the increased risk for the disease progression in HIV-infected individuals is associated with the decline of helper CD4<sup>+</sup> T cells and the accumulation of cytotoxic CD8<sup>+</sup> T cells with a more rapid helper CD4<sup>+</sup> T cell decline among HIV-1 infected IVDUs.<sup>24</sup> Biological factors such as effect of opioids, co-infection with other diseases such as TB or hepatitis C, even a difference in tropism with virulent strain of HIV transmitted among the IVDUs are all responsible for the faster decline in helper CD4<sup>+</sup> T cells.<sup>25,26</sup> A decline in both percentages and absolute counts of helper CD4<sup>+</sup> T cells with marked increases in percentages and absolute counts of cytotoxic CD8<sup>+</sup> T cells in our IVH group support the above findings. Although there was no history of how fast the decrease of helper CD4<sup>+</sup> T cells in our IVH group as only one blood sample at one time point was obtained, our IVH group showed more helper CD4<sup>+</sup> T cell decline than those in HIV+ve group. This may indicate the problems with an irregular adherence to antiretroviral drugs, loss of follow-up or lower access to health services in our IVH group. Unlike the decline helper T cells found in IVH group, our HIV+ve group showed higher helper CD4<sup>+</sup> T cell counts than the IVH group implying that there was a good suppressing HIV replication in these asymptomatic HIV-1 infected blood donors. Unfortunately, the history of

antiretroviral drug treatment was not available for this group.

NK cells have been described for the innate mechanism as the first line defense against HIV-1 infection and may help mediating adaptive immune responses.<sup>10-12</sup> It has been shown that NK cell effect or function that mediated NK cell cytotoxicity are impaired during the course of HIV-1 disease<sup>26</sup> but enhanced during HIV-1 viremia.<sup>27</sup> Previous studies on the NK profiles from the cohort of HIV-1 exposed, uninfected IVDUs demonstrated the enhancement of NK activity, high NK activation as represented by CD38<sup>+</sup> cells, and the CD4 and CD8 cell-mediated resistance to HIV-1 infection.<sup>18,28,29</sup> However, there is limited information available regarding the effect of HIV-1 infection on the NK profile in Thai IVDUs. Our NK cytotoxicity study in IVDUs showed significantly decreased of lysis activity in IVH group when compared to those of healthy, HIV-1+ve and IVX groups. When defining the percentage and absolute count of NK cells, our study showed significant decrease of both the percentage and the absolute count of NK cells in IVH group when compared to those of healthy, HIV-1+ve and IVX groups. This data suggested the inverse effect to the NK cell numbers due to HIV-1 infection along with the use of injecting drugs. Our findings also support that the drug abuse has immune-modulating effects which could alter the progression of HIV infection.<sup>30,31</sup>

Of interest is that the NK functional activity in our IVX group was significantly increased when compared to the other 3 groups. The increased NK cell lysis activity in the IVX who are a high-risk group, could due to different mechanisms related either to immune responses and/or to genetic background contribute to the resistance to infection in the highly exposed but uninfected (EU) IVDUs. Our results support the contentions of enhanced innate immune cell function in EU individuals especially when compare with IVDUs who became HIV-1 infected.<sup>28</sup> There was no difference of the mean percentages NK cell lysis activity between the healthy and the HIV-1+ve groups. This may be due to the asymptomatic stage of our HIV+ve individuals whose NK cell functions were still normal. When defining the percentage and absolute count of NK cells, our study demonstrated that not only there was no significant difference of the mean percentages of NK lysis activity among the HIV+ve, IVX and the healthy groups, but also there was no difference in percentages and the absolute counts of NK cells. Our findings are in line with de Souza et al. who reported that NK cell cytotoxicity and NK cell counts are not different between Thai HIV-1 seronegative and Thai HIV-1 seropositive subjects.<sup>32</sup>

In conclusion, our study shows that IVHs have alterations in the lymphocyte subsets evidenced by a decrease in helper



CD4<sup>+</sup> T cells and an increase in cytotoxic CD8<sup>+</sup> T cells along with a lower number of NK cells and their impaired cytotoxic function. The data suggest that the failure of innate immune NK cell function and their number with possible link to decrease in helper CD4<sup>+</sup> T cells and increase in cytotoxic CD8<sup>+</sup> T cells, may contribute to the ineffective immune surveillance and potentially predisposing them to progressive disease.

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Author's contributions: SJ and SA were contributors for performing experiments including collection of data, analysis; PB and RC were responsible on recruitment of IVDUs and blood collection. RS oversaw the collection of data, analysis and interpretation; WK and KP were the main contributors to the design and direction of the study including writing the manuscript. KP is the corresponding author.

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# Instructions for authors

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Example: 250 abstract; 3500 text; 35 references; 2 tables; 4 figures

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Abstract must be written in a structured format with the following headings: background; objective; methods; results; and conclusion. The major points of the article should be summarized in 150 (case reports) to 250 words (original research and review articles), in the order of their appearance in the manuscript. Abbreviations should be kept to an absolute minimum. References are not allowed in the abstract.

Keywords (at least 5 words or key phrases)

A minimum of 5 key words or brief phrases should be listed below the abstract for indexing purposes. The Medical Subject Headings (MeSH) used by the US National Library of Medicine’s Index Medicus (MEDLINE) are preferred.

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This section must have the following headings: Introduction, Methods, Results, Discussion, and Conclusion. In the text, cite references sequentially in superscript arabic numerals, e.g., <sup>1,2,3</sup>. Tables must be numbered sequentially in the text with Arabic numerals (1, 2, 3, 4, etc). Figures must be numbered sequentially in the text with Arabic numerals (1, 2, 3, 4, etc).

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This section should state the specific purpose, research objective, or hypothesis of the study and should provide a context or background information for the study. The aims of the manuscript should be clearly stated. Papers most closely related to the issue of the study may be mentioned. The introduction should not contain either findings or conclusions.

#### Methods

This section should be concise but provide sufficient detail to allow the work to be repeated by others. The source of material should be given in detail, where possible. Describe the design, subjects, setting, interventions, and main outcome measures. The explanation of the experimental methods provides technical information, apparatus details, and procedures. Describe statistical methods with sufficient detail to enable a reader with access to the original data to verify the reported results. For all research studies including human subjects (excluding Case Reports) the specific IRB that has approved the research must be indicated. Additionally a statement that informed consent was obtained from all research participants must be included. The clinical trial registration number and place of registry should be informed for clinical trial studies.

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Provide and quantify the main outcomes of the study. The data should be interpreted concisely, without repeating data already presented in the results section. Identify limitations of the presented data including plausible explanations for discrepancies between the data and the literature, any differences not expected from the initial hypothesis presented in the introduction and a measured description of the conclusions of the study with implications for future research, biological understanding and/or clinical applications.

### 6.4 Acknowledgements

Conflict of interest (in the past 3 years)

Source of funding with grant numbers (if applicable)

Author contributions

### 6.5 References

not more than total of 35 for original research papers  
not more than 70 for review papers

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#### Examples

- 1 Rose ME, Huerbin MB, Melick J, Marion DW, Palmer AM, Schiding JK, et al. Regulation of interstitial excitatory amino acid concentrations after cortical contusion injury. *Brain Res.* 2002;935:40-6.
- 2 Corporate Author Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension.* 2002;40:679-86.

#### Books and other monographs

- 1 Personal Author(s) Murray PR, Rosenthal KS, Kobayashi GS, Pfaller MA. *Medical microbiology.* 4th ed. St. Louis: Mosby; 2002.
- 2 Chapter in a Book Meltzer PS, Kallioniemi A, Trent JM. Chromosome alterations in human solid tumors. In: Vogelstein B, Kinzler KW, editors. *The genetic basis of human cancer.* New York: McGraw-Hill; 2002. p. 93-113.

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Tables should be numbered in the order in which they are first cited in the text with Arabic numerals (1, 2, 3, 4, etc). They should be on separate pages, one table per page. Each table should have a concise heading that makes it comprehensible without reference to the text of the article. Use horizontal lines only at the top and bottom of the table and between column headings and the body of the table. Use no vertical lines. Explain any nonstandard abbreviations in the footnote of the table, e.g., Abbreviations: CT, computed tomography; MRI,



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