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Stronger Toll-like receptor 1/2, 4, and 7/8 but less 9 responses in peripheral blood mononuclear cells in non-infectious exacerbated asthmatic children

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ABSTRACT

Toll-like receptors (TLR) initiate innate and often affect adaptive immune response. This study aimed to determine if TLR response and T regulatory cell (Treg) function in peripheral blood mononuclear cells (PBMC) correlate with clinical severity in non-infectious asthma. TLR1–9 expression and representative response cytokine TNF- α , IL-6, and IFN- β secretions were analyzed after stimulation by TLR1–9 ligands from 17 non-infectious asthmatic children. TNF- α production was higher in TLR1/2 (median 385.4 vs. 250.3 pg/ml in 1 μ g/ml Pam3CSK4, $p=0.0078$), TLR4 (2392.4 vs. 1355.9 in 1 μ g/ml LPS; $p=0.0005$), and TLR7/8 (10,776.2 vs. 4237.0 pg/ml in 1 μ g/ml R848, $p=0.0079$) of patients in exacerbation than those in convalescence and healthy controls despite equal TLR expression. TNF- α production stimulated by TLR9 agonist was significantly lower in exacerbation (17.7 vs. 34.9 pg/ml in 1 μ g/ml ODN2216, $p=0.0175$), while IL-6 production had similar patterns but was significantly lower in TLR3 signaling (119.7 vs. 245.0 pg/ml in 0.1 μ g/ml poly(I:C), $p=0.0033$). IFN- β production by TLR3 agonist also decreased in exacerbation but not statistically significant. Six older children showed decreased FOXP3 percentage in CD4+CD25^{high} and decreased suppression capability in exacerbation but restored in stabilization (82.8% vs. 90.0%, $p=0.0061$ and 60.9% vs. 81.7%, $p=0.0071$; respectively). In conclusion, normalizing imbalanced TLR signaling and enhancing Treg cell capability may guide possible therapeutic strategies for non-infectious asthma in exacerbation.

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Introduction

Innate responses to microbes are mediated largely by Toll-like receptors (TLR), which recognize a diverse family of ligands produced by viruses, bacteria, and fungi (Eder et al. 2004; Fagerås Böttcher et al. 2004; Koppelman et al. 2001; Medzhitov and Janeway 2000). Genetic polymorphisms in nucleotide substitutes, including TLR2 (–16,934), CD14 (–159 promoter) and TLR4 (Gly299Asp and Ile399Thr) alter structural dimensions and binding affinity, and have a significant trend to develop allergy (Eder et al. 2004; Fagerås Böttcher et al. 2004; Koppelman et al. 2001). Functionally, elevated supernatant TLR2 and TLR3 expressions in airway epithelium and infiltrated neutrophils contacting common

pathogens serve as TLR ligands that enhance TLR response for the up-regulation of IFN- γ -induced protein 10 (IP-10) and IL-10. These reflect the degree of acute airflow obstruction and inhibition of T regulatory (Treg) cell differentiation (Kulkarni et al. 2011; Wark et al. 2007; Wood et al. 2011).

In the process of chronic inflammation in asthma, circulating lymphocytes predominantly infiltrate the airway sub-mucosa, cross-talk antigen-presenting cells, and secrete chemokines and inflammatory cytokines to attract more eosinophils influx into the airway, thereby leading to airway remodeling and fibrosis (Hamid and Tulic 2009). The study investigated TLR response and Treg function from such potentially peripheral blood mononuclear cells (PBMC) destined to be located in the airway sub-mucosa to determine if these contribute to the exacerbation of non-infectious chronic inflammation.

To prevent infectious confounders from enhancing selective TLR signaling, we compared TLR1–9 signaling from PBMC in asthmatic children without detectable infectious pathogens during the exacerbation and convalescence stages, and with non-allergic healthy controls by evaluating representative TLR response inflammatory cytokines of TNF- α and IL-6, both as chemoattractants and degranulation factors for neutrophils and eosinophils that viciously

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Table 1
Severity scoring of asthma in exacerbation.

Variable	Clinical asthma score		
	1 point	2 point	3 point
Cerebral function	Normal	Decreased or agitated	Coma or unconsciousness
Heart rate (min)	121–140	141–160	>160
Respiratory rate			
<2 years	≤40		≥46
2–3 years	≤34		≥40
4–5 years	≤30		≥36
6–12 years	≤26		≥31
>12 years	≤23		≥28
Sat O ₂ (room air)	>95		<90
Auscultation (wheezing)	End expiratory	End expiratory + inspiratory with stethoscope only	Loud audible without stethoscope
Retraction	Intercostal	Intercostal + subcostal	Intercostal + subcostal + supraclavicular
Dyspnea (speaks)	In sentences	In partial sentences	In single word
Inspiratory/expiratory ratio	5/4–5/3	Short cries 1/1	Grunts <1/1
Severity of asthma			
	Mild	Moderate	Severe
PEFR (% predicated)	>80		<60
Asthma score	8–11	12–17	18–24

enhance airway inflammation (Abbas and Lichman 2005; Hamid and Tulic 2009; Lukacs et al. 1995; Rock and Kono 2008), and IFN- β which confers the degree of Th1 deviation to anti-viral capability (Kawai and Akira 2005; Schaub et al. 2006; Takeuchi and Akira 2010).

Methods

Patients

Patients experiencing acute exacerbation of asthma and admitted to Chang Gung Children's Hospital (Taoyuan, Taiwan) between June 2009 and February 2010 were recruited after the hospital's Human Investigation Committee approved this study and the patients' parents or guardians provided informed consent. An acute exacerbation was defined as loss of lung function by FEV1 (Bousquet et al. 2007) requiring oral corticosteroids, as determined by a pediatric allergist within 24 h after being brought to the emergency or outpatient department. The severity of symptoms was scored using the system in Table 1 (Ben-Zvi et al. 1982). Asthma was confirmed 4–6 weeks later by bronchial provocation testing. Atopy was confirmed by serum CAP/MAST specific IgE and family history, and/or allergic skin prick testing. Age-matched non-allergic healthy controls without a family history and detectable CAP/MAST IgE were also studied.

To prevent confounding TLR response enhancement from infectious pathogens, patients with clinical manifestations of fever, thick rhinorrhea, cough with sputum, and/or identified pathogens from common virus polymerase chain reactions (PCRs) for rhinovirus, enterovirus, respiratory syncytial virus, metapneumovirus, influenza, mycoplasma pneumonia, culture from nasopharyngeal aspiration and/or serology were excluded.

Study design

Participants without manifestations of virus infections provided nasal and/or throat swabs to further exclude subtle and occult infection. Subjects with acute asthma received assessment for TLR response and Treg function before taking quick-relief medication. These were repeated 4–6 weeks post-exacerbation when their asthma had been stabilized according to clinical symptoms or/and

spirometry. Subjects also underwent atopy evaluation. Medical management was based on the updated Global Initiative for Asthma (GINA) guidelines (Bousquet et al. 2007).

Expression of TLR1–9 on PBMC

From each patient, 10–20 ml venous blood was collected into heparin-containing syringes and delivered to our laboratory within 24–72 h. The PBMC were isolated by Ficoll-Hypaque (Pharmacia Biotech, Piscataway, NJ) centrifugation. RPMI 1640 supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT), 2 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin (complete medium) was used as culture complete media for further analysis.

Antibodies for flow cytometry of human TLR and isotype controls were purchased from Imgenex Corp. (phycoerythrin-conjugated [PE]-conjugated TLR1–8) and eBioscience (PE-conjugated TLR9, San Diego, CA). TLR1, TLR2, TLR4, TLR5, and TLR6 were detected by cell surface staining and TLR3, TLR7, TLR8 and TLR9 by intracellular staining. For intracellular staining, PBMC were fixed in 4% formaldehyde for 15 min and permeabilized with 0.1% saponin for 30 min on ice. For both protocols, the cells were incubated with the corresponding TLR antibodies. A total of 10,000 viable lymphocytes were collected and analyzed by flow cytometry (FACSCalibur, BD Biosciences Corp., San Jose, CA).

TLR ligands stimulation

To determine TLR responses, PBMC (2×10^5) in 100 μ l complete media were added to each of the duplicate ligand- or medium-containing wells and incubated at 37 °C for 24 h with 5% CO₂. Two stimulant dosages of TLR1–9 agonists at a log range were chosen to test the dose-dependent effect: 1.0, 10 μ g/ml Pam3CSK4 (TLR1/2 ligand, Invivogen, San Diego, CA); 0.1, 1.0 μ g/ml poly(I:C) (TLR3 ligand, Sigma–Aldrich Co., St. Louis, MO); 0.1, 1.0 μ g/ml LPS (TLR4 ligand, Invivogen, San Diego, CA); 1.0, 10 μ g/ml flagellin (TLR5 ligand, Calbiochem Corp., San Diego, CA); 1.0, 10 μ g/ml zymosan (TLR2/6 ligand, Invivogen, San Diego, CA); 1.0, 10 μ g/ml R848 (TLR7/8 ligand, Invivogen, San Diego, CA); and 1.0, 10 μ g/ml ODN 2216 (TLR9 ligand, Coley Pharmaceuticals, Wellesley, MA) for TNF- α and IL-6 production and additional INF- β production for

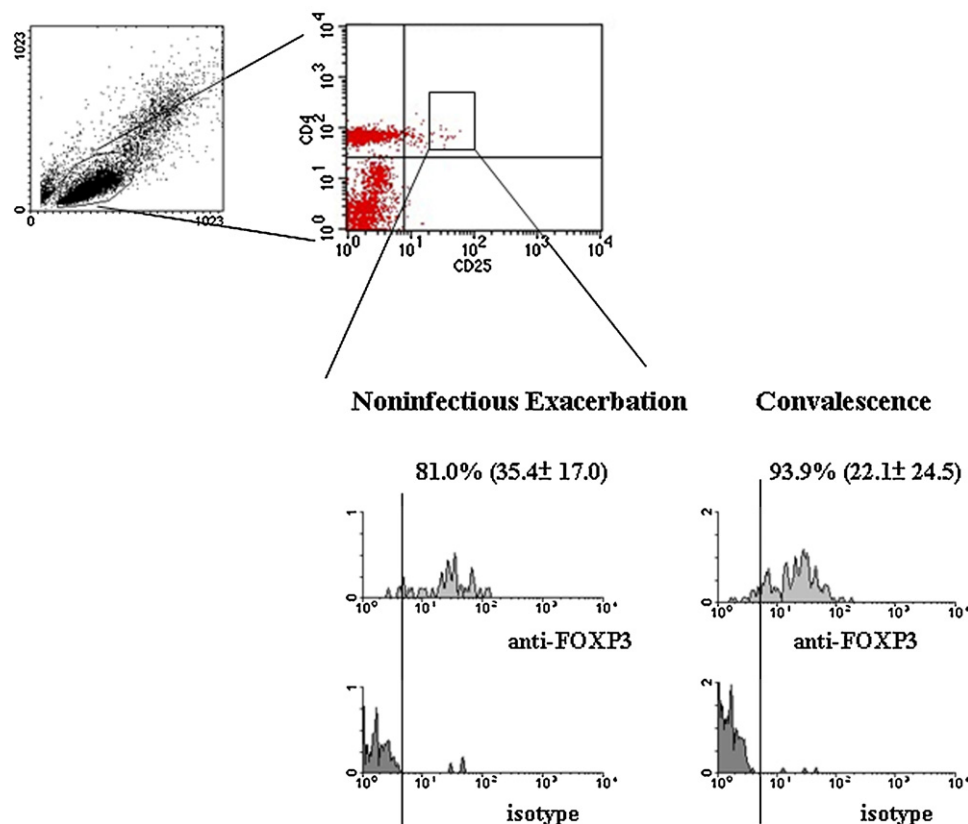
Asthmatic patient P1

Fig. 1. The lymphocyte population was first gated by the ellipse shape in FSC and SSC dot plot, second by PerCP conjugated anti-CD4 and FITC conjugated anti-CD25. In this second dot plot, CD4⁺CD25^{high} cells were third gated by the rectangle shape for intracellular PE-conjugated anti-FOXP3 staining. The representative histograms in patient P1 showed 81.0% of FOXP3⁺/CD4⁺CD25^{high} cells in exacerbation and restored to 93.9% in the convalescence stage.

TLR3 stimulation (Deering and Orange 2006). TNF- α , IL-6 (R & D Systems, Minneapolis, MN) and INF- β (TFB, Fujirebio, Inc., Tokyo) production was assessed by ELISA.

T regulatory cell assessment

To correlate TLR response with Treg cell function, we modified a commercially available human Treg staining kit (eBiosciences, San Diego, CA, USA) following manufacturer's instructions (Wark et al. 2007; Wood et al. 2011). Briefly, PBMC were surface-labeled with PerCP (peridinin chlorophyll protein)-conjugated anti-CD4 (SK3, BD Biosciences, San Jose, CA) and FITC (fluorescein isothiocyanate)-conjugated anti-CD25 (2A3, BD Biosciences). The Treg cells were then sorted from the CD4⁺CD25^{high} cells and quantified by intra-cellularly labeled PE-conjugated anti-FOXP3 (PCH101; eBiosciences, San Diego, CA). Gating strategies were representatively shown in Fig. 1. The suppressive capacity was determined by culturing 1×10^4 (CD4⁺CD25[−]) T cells in a 1:1 mixture of CD4⁺CD25^{high} cells in the presence of 2.5 μ g/ml of soluble anti-human CD28 (CD28.2; eBioscience) and 2.5 μ g/ml anti-CD3 mAb (OKT3; eBioscience) coated 96-well plates (Bio-Whittaker, Walkersville, MD, USA) for two days. At the end of the culture periods, cell proliferation of (CD4⁺CD25[−]) T cells was assessed by incubation ³H-thymidine for 16 h (Lin and Chiang 1997).

Statistical analysis

The Wilcoxon signed rank and/or paired *t* tests were used to compare the cytokines after TLR ligand stimulation in the same asthmatic children in the exacerbation and convalescence stages,

while the Mann–Whitney *U* test was used to compare asthmatic children and healthy controls. A *p* < 0.05 was considered statistically significant.

Results

Patients' characteristics

Despite excluding virus infection by clinical symptoms, three patients were positive for respiratory syncytial virus antigen, one for influenza by PCR, one for influenza by culture, one for parainfluenza by culture, and two for serologic mycoplasma IgM. These eight patients were not recruited into this study. Total 17 patients (four females) met the enrollment criteria (Table 2). All had comorbid allergic rhinitis and 14 were sensitive to mites. The age of asthma exacerbation ranged from 6 to 17 years (median, 8.0 years), while the score severity during exacerbation ranged from 12 to 19 (mean, 15.6), and <5 during the convalescence stage after a short course of treatment (5–7 days of prednisolone and bronchodilators) (Ben-Zvi et al. 1982). Mild to moderate doses of inhaled steroids were given to seven patients as maintenance therapy as recommended by the GINA treatment guidelines (Bousquet et al. 2007).

TLR expressions in PBMC

In the 17 non-infectious asthmatic children in exacerbation, all TLR expression of PBMC were evaluated and summarized (Table 3; representatively shown in Fig. 2). The mean surface TLR1, TLR2, TLR4, TLR5, and TLR6 expressions in PBMC gating were 54.2%,

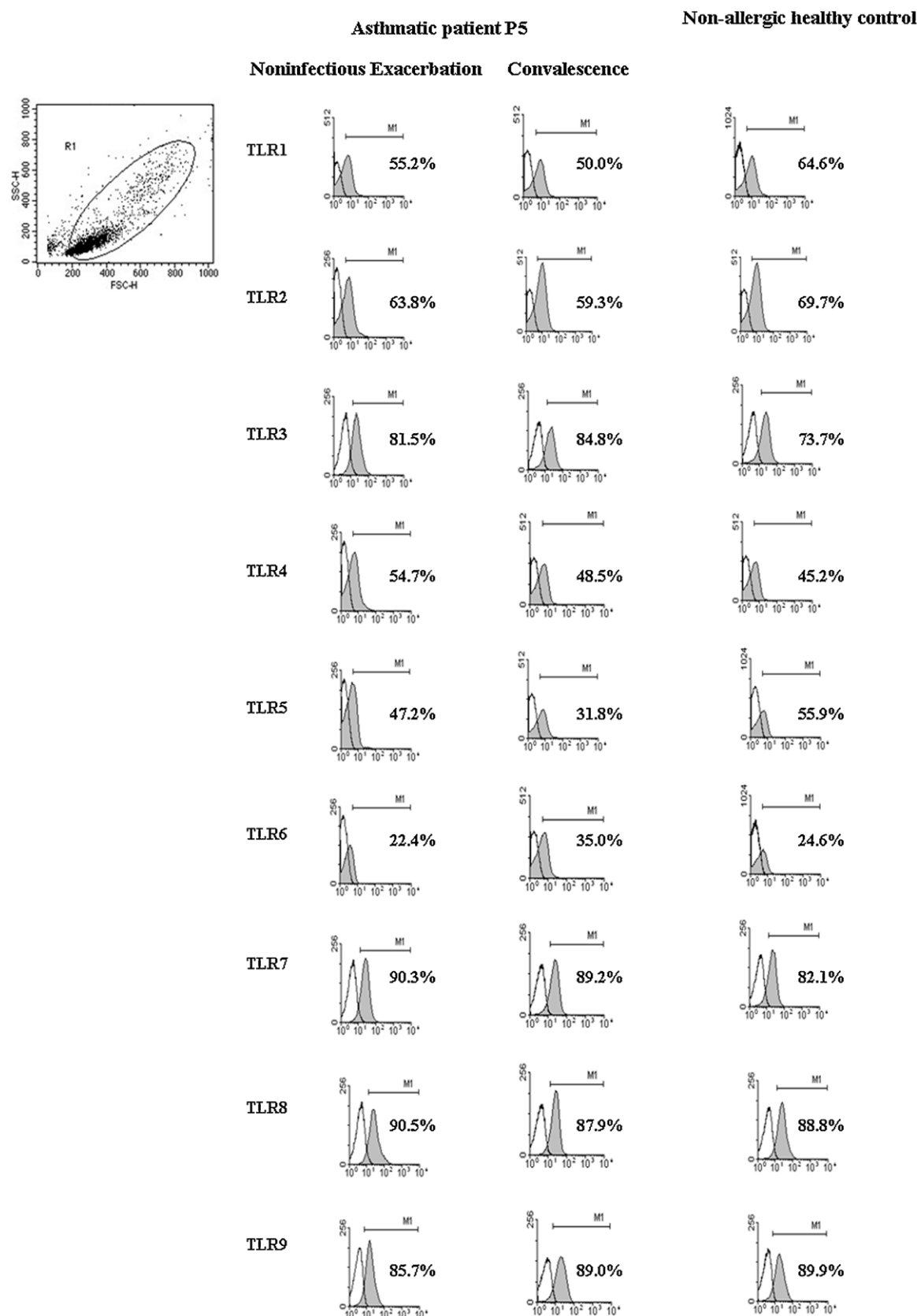


Fig. 2. PBMC gating in FSC and SSC dot plot, representative histograms showing mean surface TLR1, TLR2, TLR4, TLR5, and TLR6 expression of 55.2%, 63.8%, 54.7%, 47.2% and 22.4%, respectively. Mean intracellular TLR3, TLR7, TLR8, and TLR9 expression were prominent at 81.5%, 90.3%, 90.5%, and 85.7%, respectively, in a noninfectious exacerbated asthmatic child P5. There were no significantly different TLR expressions on PBMC in asthmatic patients (in the exacerbation and convalescence stages) and healthy controls. Blank lines represented isotype controls and gray areas represented the respective anti-TLR antibodies. Duplicate experiments were performed.

Table 2Clinical features of exacerbated asthma patients without detectable pathogens ($n = 17$).

Patient/sex	Age (years)	Severity	IgE	FEV1 (bronchodilator)		Detectable allergen	Associated allergy
				Pre-%	Post-%		
P1/F	13	16	407	57	76	Mite	Rhinitis
P2/M	5	14	112	32	87	Mite, egg white	Rhinitis
P3/F	17	17	49	46	74	Not-detectable	Rhinitis
P4/M	6	10	135	69	97	Mite	Rhinitis
P5/M	5	13	1669	56	84	Mite, cockroach, dog dander	Rhinitis
P6/M	9	18	705	43	79	Mite, cockroach	Rhinitis
P7/M	6	19	84	34	75	Not-detectable	Rhinitis
P8/M	16	18	308	55	84	Mite, cockroach	Rhinitis
P9/M	6	14	4159	62	81	Mite, dog dander	Conjunctivitis, rhinitis
P10/F	5	17	645	28	75	Mite	Conjunctivitis, rhinitis
P11/M	6	10	621	49	77	Mite, crab, shrimp	Rhinitis
P12/F	10	16	938	61	89	Mite	Conjunctivitis, rhinitis
P13/M	11	14	245	53	79	Mite	Conjunctivitis, rhinitis
P14/M	17	17	67	44	73	Non-detectable	Rhinitis
P15/M	13	13	2721	38	85	Mite, dog dander, cockroach	Rhinitis
P16/M	11	18	1189	51	94	Mite, dog dander, cat dander	Rhinitis
P17/M	8	17	110	38	81	Mite	Conjunctivitis, rhinitis

57.7%, 45.6%, 29.7%, and 24.8%, respectively. The mean intracellular TLR3, TLR7, TLR8, and TLR9 expression were prominent, at 84.2%, 89.1%, 88.4%, and 89.4%, respectively. Similar and increasing percentage patterns were observed in respective lymphocytes CD3+ and monocytes CD14+ gating (data not shown). There were no significantly different TLR expressions in PBMC in non-infectious asthmatic patients (during the exacerbation or convalescence stage in the same patient) and in the healthy controls.

Functional TLR responses by mutual ligands stimulation

Compared to non-infectious asthmatic children in the convalescence stage, TNF- α production from PBMC stimulated by TLR1/2 (Pam3CSK4; 1, 10 μ g/ml), TLR4 (LPS; 1 μ g/ml), and TLR7/8 (R848; 1, 10 μ g/ml) agonists in the exacerbation stage was significantly higher and presented with a dose-dependent trend (Table 4; Fig. 3A). In contrast, TNF- α production from TLR9 agonist (ODN 2216) significantly decreased in the exacerbation stage.

Exacerbated asthmatic PBMC had less TNF- α production under stimulation by TLR3 agonist, but not statistically significant.

In line with TNF- α secretion, IL-6 production in the exacerbation stage was significantly higher after TLR1/2 and TLR7/8 stimulation, but lower after TLR9 ligand stimulation. Furthermore, IL-6 production after TLR3 agonist stimulation was also significantly lower (Fig. 3B).

There were no significant differences in TNF- α and IL-6 production through TLR agonists between stable asthmatic children and healthy controls using the Mann–Whitney test (representatively demonstrated in Fig. 3).

IFN- β production by PBMC through TLR3 ligand stimulation

Along with TNF- α and IL-6 production by MyD88-dependent downstream TLR signaling, IFN- β secretion by MyD88-independent TLR signaling was also evaluated by TLR3 agonist poly I:C stimulation (Alexopoulou et al. 2001). Poly I:C could

Table 3

Percentage of TLR expression in lymphocytes isolated from asthmatic patients with non-infectious exacerbation and stabilization.

	TLR1		TLR2		TLR3		TLR4		TLR5		TLR6		TLR7		TLR8		TLR9	
	E	S	E	S	E	S	E	S	E	S	E	S	E	S	E	S	E	S
P1	62.4	67.5	63.8	59.3	76.8	79.5	61.8	78.2	33.7	45.6	21.8	33.5	92.4	85.7	84.5	91.4	76.1	89.6
P2	54.8	52.4	75.2	78.9	84.2	88.7	55.9	69.1	31.9	42.7	41.5	48.7	83.7	91.5	92.4	79.6	74.5	91.2
P3	72.1	68.4	82.4	85.1	74.6	78.9	39.6	47.9	45.2	38.1	32.5	45.1	85.4	92.4	87.1	94.7	85.6	94.2
P4	75.2	67.9	57.6	65.2	82.5	84.9	48.7	41.9	35.9	45.7	25.4	33.2	75.6	88.7	82.5	89.4	77.3	89.3
P5	55.2	50.0	63.8	59.3	81.5	84.8	54.7	48.5	47.2	31.8	22.4	35.0	90.3	89.2	90.5	87.9	85.7	89.0
P6	54.5	62.5	58.7	64.5	79.1	75.6	44.9	52.7	38.9	47.6	42.5	31.8	84.5	79.6	77.6	91.4	79.2	90.4
P7	71.2	77.5	54.3	67.2	67.9	84.3	62.9	54.8	51.9	66.4	21.9	30.4	95.2	87.6	83.2	95.7	93.3	81.7
P8	65.4	72.8	74.3	78.2	69.4	71.3	57.3	64.9	58.7	42.5	25.4	34.5	85.4	92.8	80.5	93.4	95.3	87.0
P9	70.2	68.4	59.6	61.4	82.4	79.6	41.9	48.7	57.6	43.6	41.2	31.4	89.2	78.5	79.6	89.6	78.3	94.9
P10	56.2	72.2	57.9	67.6	64.5	71.8	54.2	59.7	60.7	53.2	27.9	44.2	84.6	95.4	77.5	91.6	79.6	91.7
P11	81.4	78.2	81.2	59.8	72.6	78.9	65.4	74.9	52.4	41.9	35.7	24.3	89.5	95.2	85.4	79.9	84.5	69.3
P12	66.2	68.4	77.5	74.3	69.7	74.5	51.4	47.6	59.5	42.3	35.4	44.1	79.7	84.6	85.1	96.6	82.7	79.5
P13	55.2	58.7	58.9	64.3	81.4	73.9	72.6	64.9	39.5	48.2	39.5	27.8	89.4	79.6	93.2	81.6	84.3	69.4
P14	67.7	82.5	59.3	66.8	74.9	81.5	61.6	72.9	34.9	52.4	35.2	21.9	87.6	96.8	92.5	84.3	74.5	89.1
P15	75.2	74.2	77.5	81.6	81.9	73.2	54.3	46.4	47.9	32.8	31.5	47.2	85.6	97.3	82.3	94.0	87.4	93.8
P16	82.1	75.1	78.5	74.9	79.5	73.1	59.6	71.9	57.1	39.5	33.5	47.2	84.6	95.7	91.8	80.3	69.5	83.8
P17	81.5	77.4	69.8	74.3	75.9	84.7	61.9	54.4	42.9	55.7	45.2	28.9	79.5	86.1	82.2	94.3	90.2	79.7
Comparison*																		
Wilcoxon signed rank	0.5001		0.1051		0.2143		0.1213		0.5307		0.4418		0.1213		0.1002		0.1956	
Paired t	0.3613		0.3288		0.1493		0.1862		0.6467		0.3169		0.1241		0.1244		0.1841	
Controls ($n = 17$)	48.8–85.6		52.4–91.7		63.7–85.9		38.9–74.9		29.4–69.7		19.4–48.1		78.2–98.5		76.1–96.7		65.7–98.7	

Abbreviations: E, exacerbation; S, stabilization.

* $p < 0.05$ was significant in the same asthmatic patients when comparing exacerbation and stabilization stages.

Table 4

TNF- α , IL-6 and IFN- β production from PBMC after TLR stimulation in non-infectious asthmatic children in the exacerbation and convalescence stages.

	Agonist (low dose)	Median TNF-alpha (95% confidence interval) (pg/ml)		<i>p</i>	Agonist (high dose)	Median TNF-alpha (95% confidence interval) (pg/ml)		<i>p</i>
		Exacerbation	Stable			Exacerbation	Stable	
TLR1/2	Pam3CSK4 1 μg/ml	385.4 (286.4–497.7)	250.3 (184.4–293.3)	0.0078	Pam3CSK4 10 μg/ml	644.9 (460.6–742.4)	356.8 (265.1–438.6)	0.0090
TLR3	Poly(I:C) 0.1 μ g/ml	12.1 (6.6–91.1)	19.1 (17.4–74.6)	0.6616	Poly(I:C) 1 μ g/ml	61.9 (50.7–161.1)	88.0 (71.2–143.9)	0.5948
TLR4	LPS 0.1 μ g/ml	507.6 (452.5–1454.1)	517.5 (488.2–1048.3)	0.5001	LPS 1 μ g/ml	2392.4 (1860.0–4494.5)	1355.9 (1065.8–2319.3)	0.0005
TLR5	Flagellin 1 μ g/ml	142.1 (26.6–401.7)	96.2 (70.1–171.6)	0.2513	Flagellin 10 μ g/ml	186.0 (123.3–1036.6)	245.2 (159.1–846.1)	0.7271
TLR2/6	Zymosan 1 μ g/ml	22.1 (15.2–204.4)	30.3 (29.6–96.7)	0.7315	Zymosan 10 μ g/ml	127.7 (64.4–301.6)	199.2 (130.6–296.0)	0.1053
TLR7/8	R848 1 μg/ml	10,776.2 (7146.5–15,482.8)	4237.0 (3234.1–7748.5)	0.0079	R848 10 μg/ml	18,189.2 (13,157.5–22,244.5)	10,134.0 (7514.8–12,164.1)	0.0051
TLR9	ODN 2216 1 μg/ml	17.7 (14.2–54.4)	34.9 (23.8–84.2)	0.0175	ODN2216 10 μg/ml	57.0 (44.8–109.7)	141.2 (95.8–240.4)	0.0038
	Agonist (low dose)	Median IL-6 (95% confidence interval) (pg/ml)		<i>p</i>	Agonist (high dose)	Median IL-6 (95% confidence interval) (pg/ml)		<i>p</i>
		Exacerbation	Stable			Exacerbation	Stable	
TLR1/2	Pam3CSK4 1 μg/ml	9348.6 (7305.3–12,408.5)	6974.0 (5875.1–9039.2)	0.0288	Pam3CSK4 10 μg/ml	13,495.7 (10,907.5–17,577.8)	10,544.6 (8419.7–13,196.5)	0.0028
TLR3	Poly(I:C) 0.1 μ g/ml	119.7 (85.7–243.3)	245.0 (185.0–953.5)	0.0033	Poly(I:C) 1 μ g/ml	464.1 (390.7–1135.2)	957.0 (917.3–1765.6)	0.0004
TLR4	LPS 0.1 μ g/ml	12,587.5 (9467.8–14,396.5)	11,247.0 (9595.7–13,241.1)	0.1665	LPS 1 μ g/ml	14,579.0 (12,951.2–18,493.2)	12,984.2 (12,211.8–14,972.7)	0.0570
TLR5	Flagellin 1 μ g/ml	2973.0 (1951.2–5627.2)	1754.4 (1750.9–4976.2)	0.5001	Flagellin 10 μ g/ml	8702.9 (5972.6–10,846.2)	5341.0 (4535.9–8563.1)	0.0865
TLR2/6	Zymosan 1 μ g/ml	100.7 (53.8–632.2)	180.4 (139.9–841.1)	0.2143	Zymosan 10 μ g/ml	983.0 (683.2–2944.2)	1538.6 (1126.3–2546.5)	0.5621
TLR7/8	R848 1 μg/ml	16,717.2 (13,889.7–18,843.5)	11,970.8 (10,084.3–15,840.5)	0.0176	R848 10 μg/ml	17,792.8 (13,966.1–18,870.4)	14,729.0 (11,798.5–17,172.4)	0.2413
TLR9	ODN 2216 1 μg/ml	78.0 (36.5–238.9)	234.0 (157.6–849.4)	0.0011	ODN 2216 10 μg/ml	187.4 (140.0–1321.8)	698.5 (603.8–2123.6)	0.0038
	Agonist (low dose)	Median IFN-beta (95% confidence interval) (pg/ml)		<i>p</i>	Agonist (high dose)	Median IFN-beta (95% confidence interval) (pg/ml)		<i>p</i>
		Exacerbation	Stable			Exacerbation	Stable	
TLR3	Poly(I:C) 0.1 μ g/ml	27.2 (20.8–33.8)	32.4 (26.5–40.5)	0.2053	Poly(I:C) 1 μ g/ml	37.1 (28.0–74.7)	59.0 (45.3–84.1)	0.5961

Cytokine production from PBMC stimulated by TLR agonists was presented by subtracting the duplicated values of stimulant supernatants with TLR agonists from those with only blank media. There were no significant differences between asthmatic children in the convalescent stage and healthy controls (data not all shown) (some in Fig. 2). Bold numbers represented significant difference, by Wilcoxon signed rank test and consistent with paired *t*-tests (data not shown).

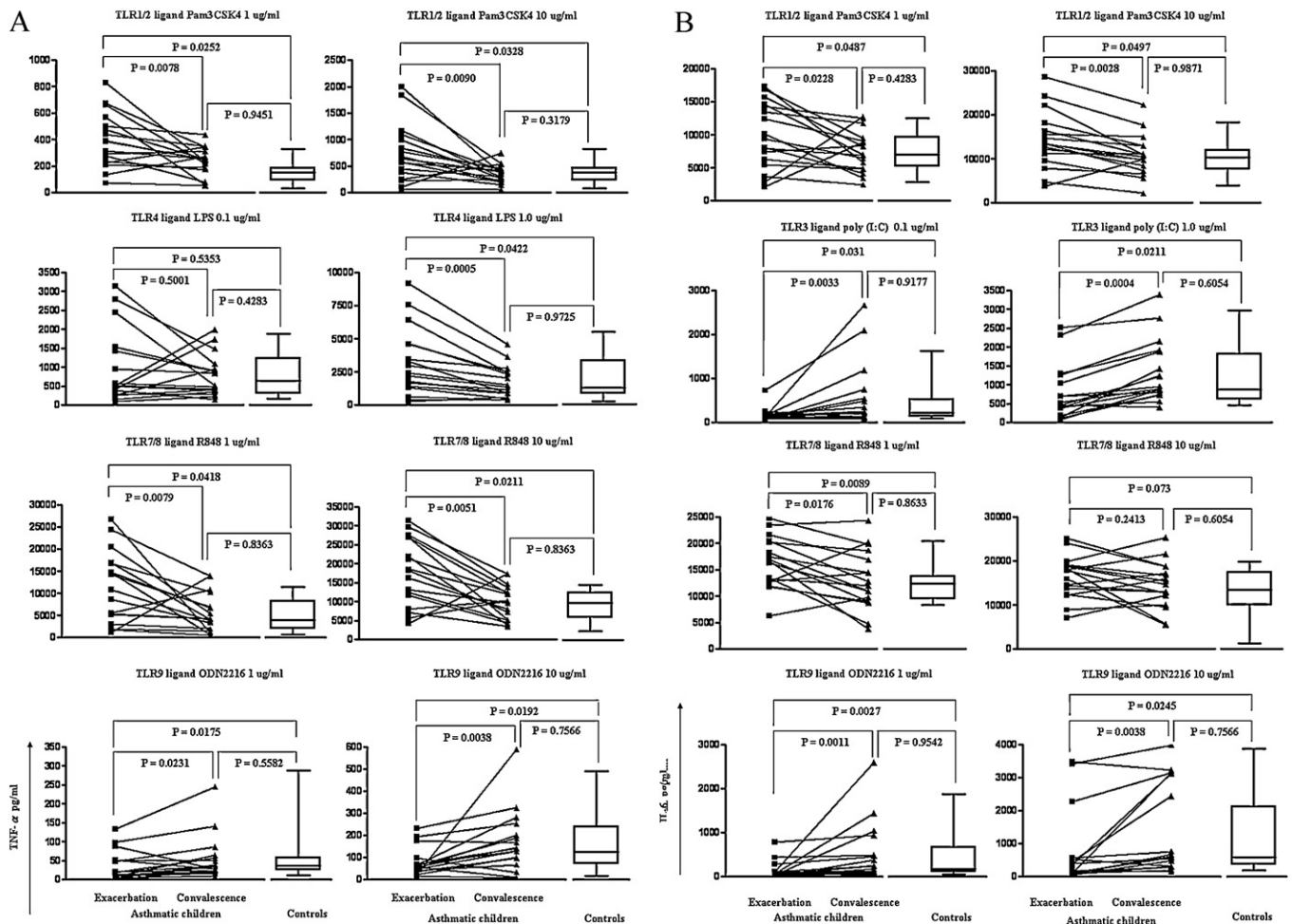


Fig. 3. Comparing non-infectious asthmatic children in the convalescent stage, TNF- α production from PBMC stimulated by TLR1/2 (Pam3CSK4; 1, 10 μ g/ml), TLR4 (LPS; 1 μ g/ml), and TLR7/8 (R848; 1, 10 μ g/ml) agonists were significantly higher in the exacerbation stage. (A) TNF- α production from TLR9 agonist (ODN 2216) significantly decreased during exacerbation. (B) Consistent with TNF- α secretion, IL-6 production during exacerbation was significantly higher after TLR1/2, TLR7/8, and TLR9 agonist stimulation, but significantly lower after TLR3 agonist stimulation. TNF- α and IL-6 production was not significantly different between stable asthmatic children and healthy controls, by the Mann–Whitney test.

penetrate the cytoplasmic membrane to trigger TLR3 signaling effects, although most TLR3 receptors are located within the intra-cytoplasm (Deering and Orange 2006; Lun et al. 2009). Under poly I:C stimulation, IFN- β production had a trend of being mildly diminished in the exacerbation stage, but not significantly different compared to the convalescence stage in the same patients.

Decreased Treg cell suppression in non-infectious asthmatic patients

Even though the percentage of CD4+CD25^{high} in six older patients was similar to the healthy controls, the percentage of CD4+CD25^{high} FOXP3+ and of FOXP3+/CD4+CD25^{high}, and the suppression capability were significantly lower in the exacerbation stage but all restored in the convalescence stage (Table 5).

Discussion

Noninfectious exacerbated asthmatic PBMC enhanced TLR1/2, TLR4, and TLR7/8 responses by robust TNF- α and IL-6 production after stimulation by their respective TLR agonists. Such in vitro observations provide possible explanations for the increased comorbidity or/and susceptibility to bronchiolitis and pneumonia in asthmatic children, even when there are no detectable infectious

triggers (Miller et al. 2008; Sheikh et al. 2002; Thorburn et al. 2009; Weinberger 2004). The common pathogens in bronchiolitis and pneumonia are *respiratory syncytial virus*, *influenza virus*, *Streptococcus pneumoniae*, *Hemophilus influenza* and *Mycoplasma pneumoniae*. All of these are able to serve as critical components of TLR ligands: *Streptococcus pneumoniae* as TLR1/2 (Alexander and Rietschel 2001; Takeuchi et al. 2002; Yamamoto et al. 2002), *respiratory syncytial virus* and *Hemophilus influenza* as TLR4 (Haynes et al. 2001; Heumann et al. 2003; Kurt-Jones et al. 2000; Poltorak et al. 1998), and *influenza virus* as TLR7/8 (Diebold et al. 2004; Heil et al. 2004) to augment inflammatory cytokine cascades in exacerbated asthmatic PBMC. This implies that non-infectious exacerbated asthmatic PBMC may amplify corresponding TLR response to these superimposed common pathogens, and in unfortunate cases, cause bronchiectasis and severe parenchymal damage. Thus, vaccination strategies for these common pathogens are suggested to prevent asthmatic children from pathogen-triggering colonization (Miller et al. 2008; Sheikh et al. 2002; Thorburn et al. 2009; Weinberger 2004). Such colonization orchestrates stronger TLR response for more inflammation cytokines production, including TNF- α , which inhibits Treg cell development, especially during the exacerbation period (Lin et al. 2008). In a mice model, pneumococcal conjugate vaccine can decrease colonization, accelerate Treg cell development, and subsequently inhibit airway inflammation (Thorburn et al. 2010).

Table 5
Treg cell percentage and suppression function in six selected older patients.

Patient	Lymphocyte (PBMC) %				FOXP3+/ CD4+CD25 ^{high}		Proliferation incubated thymidine (c.p.m.)					
	CD4+CD25 ^{high}		CD4+ CD25 ^{high} FOXP3+				CD4+CD25–		CD4+CD25–plus CD4+CD25 ^{high}		Suppression percentage (%) ^a	
	E	S	E	S	E	S	E	S	E	S	E	S
P1	2.3	3.1	1.5	2.3	81.0	93.9	2145	3256	1029	752	52.0	77.0
P8	2.8	2.5	1.9	2.0	84.7	92.2	4698	3318	1550	607	67.0	81.7
P13	4.2	3.8	2.4	3.2	82.7	85.4	4178	3698	1596	547	61.8	85.2
P14	1.5	1.2	1.0	1.1	79.7	88.2	2798	3265	1259	397	55.0	87.8
P15	2.8	3.2	1.7	2.2	85.7	88.6	3846	3179	1454	579	62.2	81.8
P16	3.3	3.4	2.4	2.5	82.7	91.5	2976	2249	1148	486	67.4	76.4
Comparison [*]												
Wilcoxon signed rank	0.8074		0.0313		0.0313		0.4375		0.0313		0.0313	
Paired <i>t</i>	0.8438		0.0367		0.0061		0.4836		0.0010		0.0017	
Non-atopic healthy controls (<i>n</i> = 7)	1.4–5.8		1.0–3.7		82.8–95.4		1878–4215		215–764		62.7–89.4	

Abbreviations: E, exacerbation; S, stabilization.

^a Suppression percentage = c.p.m. in CD4+CD25– minus [CD4+CD25– plus CD4+ CD25^{high}]/CD4+CD25– × 100.^{*} *p* < 0.05 was significant in the same asthmatic patients when comparing exacerbation and stabilization stages. Bold numbers represented significance.

Aero-allergenecity has been proposed via functional mimicry of TLR complex proteins. In addition to infection as triggering factors (Wark et al. 2002; Wood et al. 2011), the non-infectious *Dermatophagoides pteronyssinus* (Der p) is the most common exposure allergen in Taiwan (Huang et al. 2003; Niu et al. 2006) and may be the possible triggering factor in this study because most of the patients (14 out of 17) are mite-sensitive. Der p has a structural homology with MD-2 (also known as LY96) as the lipopolysaccharide (LPS)-binding component for the TLR4 signaling (Trompette et al. 2009). Cultured Der p-stimulated asthmatic PBMC have been shown to secrete more supernatant TNF- α , possibly through TLR4 receptor, which can down-regulate FOXP3 expression and abrogate Treg cell suppression (Lin et al. 2008).

Moreover, the number and function of Treg cells have been shown to decrease in asthmatic PBMC, lung tissue, and bronchoalveolar lavage (Akdis et al. 2004; Grindebacke et al. 2004; Hartl et al. 2007), which is consistent with the findings here of decreased percentages of FOXP3 expression in CD4+CD25^{high} and suppression capability in selected non-infectious exacerbated asthmatic PBMC that are restored in the convalescence stage. Anti-TNF treatment (etanercept) can counterbalance selective TLR activation and restore such impaired Treg function (Lin et al. 2008). Thus, Der p priming mite-sensitive exacerbated asthmatic PBMC increase inflammatory cytokines (i.e., TNF- α and IL-6) through, at least, waxing TLR4 signaling and more TNF- α production, thereby diminishing Treg function. Further investigations are needed to clarify the hypothesis that other unknown components within the Der p allergen augment TLR1/2 and TLR7/8 pathways.

In contrast, TLR3 and TLR9 response are diminished in non-infectious exacerbated asthmatic PBMC. These two TLR recognize double-strand RNA and CpG DNA virus pathogens (e.g. herpes simplex virus), and mainly drive interferon type I cytokine profile to kill and eradicate virus-related pathogens. This favorably shifts to a Th1 response in infected healthy subjects. Thus, noninfectious exacerbated asthmatic PBMC responsible for the Th2 shift have relatively less ability to behave as Th1 response for anti-viral cytokine secretions like IFN- β .

To examine the mechanism of selective TLR response during exacerbation, the present study simultaneously evaluated whether the level of respective TLR1–9 expression in PBMC affected the selective TLR response. The expression on the surface of PBMC was grossly equal in asthmatic children in the non-infectious exacerbation and convalescence stages, and healthy controls (Table 2; representative demonstration in Fig. 2). In contrast, TLR expression

on subsets of PBMC in exacerbated asthmatic patients had dynamic variations (Liu et al. 2010; Lun et al. 2009; Simpson et al. 2003; Wark et al. 2002) because approximately 80% of exacerbated asthma were induced by various virus infections that were able to directly enhance correspondent TLR expression (Liu et al. 2010; Simpson et al. 2003; Wark et al. 2002; Wood et al. 2011). However, this group of virus-induced exacerbated asthma was not enrolled. Aside from infection to increase TLR expression, the effect of inhaled steroids (as maintenance therapy for moderate and severe asthma) only enhanced TLR2 expression in human respiratory epithelial cells in an in vitro study (Homma et al. 2004; Wood et al. 2011). It is possible that the inconsistency of TLR2 expression in the patients here were due to a relatively lower dose of inhaled steroids or/and the different TLR2 dynamic expression between respiratory epithelium and PBMC.

Similar patterns of cytokine profiles were also observed in whole blood stimulated by higher doses of TLR agonists (data not shown). Therefore, cognate downstream TLR1/2, TLR7/8 and/or TLR4 signaling to evaluate NF- κ B, JNK and MAP signaling including translation and transcription factors of MyD88, IRAK-4, IRAK-1, IRF-5 and IKK- β expression, are candidates in further study to clarify their potential in augmenting selective higher TLR responses in exacerbated non-infectious asthmatic PBMC (Kawai and Akira 2005; Takeuchi and Akira 2010).

This study utilized PBMC to investigate TLR signaling and Treg function. However, it cannot conclude that immune cells in situ from the airways will present the same profile. These observations should, therefore, be performed on cells obtained from bronchoalveolar lavage and airway biopsy in non-infectious mite-sensitive asthmatic children and controls. A comprehensive evaluation must be demonstrated by animal studies and clinical application, if ethical standards permit.

In conclusion, there are potentially higher TLR1/2, TLR7/8, and TLR4 response in non-infectious exacerbated asthmatic PBMC through significantly increased TNF- α accompanying IL-6 production, but lower response in TLR9 or TLR3 stimulation. The orchestration of inflammation by TNF- α in asthma is partly ascribed to stronger selective TLR response, possibly inhibiting the Treg function of expanding anti-TNF- α treatment as a potential therapy beyond the scope restricted to severe refractory asthma (Berry et al. 2006; Howarth et al. 2005). In clinical trials, a TLR9 agonist vaccine for the effective prevention of allergic rhinitis (Creticos et al. 2006) may be applied to some asthma sub-groups. Fluctuating TLR response and Treg function in non-infectious exacerbated

asthmatic PBMC may provide possible therapeutic strategies to prevent asthma attacks and attenuate its severity.

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