Low adenosine triphosphate activity in CD4+ cells predicts infection in patients with lupus nephritis

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Abstract

Objective

The ImmuKnow (Cylex) assay has been reported to predict the risk of infection in some diseases; however, it is uncertain whether ImmuKnow can predict the risk of infection in lupus nephritis (LN) patients receiving immunosuppressive therapy.

Methods

The ImmuKnow Immune Cell Function Assay (Cylex, Inc., Columbia, MD, USA) was applied to measure the activity of CD4+ T cells, as a marker of global immune-competence. The correlation between changes in T cell activation and the relative risk of over-immunosuppression as well as infection was studied. The amount of adenosine triphosphate (ATP) produced by CD4+ T cells in response to phytohemagglutinin (PHA) was measured for 74 LN patients without infection, 22 LN patients with severe infection (i.e. required hospitalisation), and 28 healthy controls.

Results

No correlation was found between the ATP level and systemic lupus erythematosus (SLE) activity. The mean ATP level was significantly lower in LN patients with infection than that in healthy controls ($p<0.01$) and non-infected LN patients ($p<0.01$). The mean ATP level in non-infected LN patients was not significantly different compared to healthy controls. A cut-off ATP value of 300 ng/mL predicted infection in LN patients with a specificity of 77% and a sensitivity of 77%. Multi-variable partial correlation coefficient between the ATP assay and severe infection was $r = -0.040$, $p<0.001$; CRP was $r=0.962$, $p<0.001$.

Conclusion

The ImmuKnow assay may be effective in identifying an increased risk of infection in LN patients but is not correlated with SLE activity. Combined CRP value will increase the diagnostic rate of severe infection in SLE. Larger studies are required to establish clinical advantages of this assay in SLE treatment.

Key words

ImmuKnow, infection, systemic lupus erythematosus, lupus nephritis

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Introduction

Systemic lupus erythematosus (SLE) is a multi-system autoimmune disease; clinical manifestations vary from relatively minor skin and joint symptoms to severe life-threatening major organ involvement. Infections are among the most important causes of morbidity and mortality in SLE patients due to the use of immunosuppressive therapy (1, 2). Certain factors increase the susceptibility of infection in patients with SLE, including $CD24^+$ $CD25^+$ T regulator cells, complement deficiency (3), and mannose-binding lectin (MBL) deficiency (4). Several studies have identified active lupus as a risk factor for infection; higher SLE disease activity index (SLEDAI) scores increased the risk of infection and were associated with increased rates of hospitalisation (3). Glucocorticoids and immunosuppression therapy are usually administered to control SLE activity in SLE patients. However, inadequate immunosuppression leads to disorders of the immune system, and increases mortality and hospitalisation (5, 6). In a retrospective study, it was observed that an increase in the corticosteroid dose was associated with increased mortality in patients admitted to the ICU (5).

Therefore, balancing immunosuppression therapy to prevent SLE activity, while minimising the risk of infection or drug toxicity, is a central issue. In 2002, the Food and Drug Administration (FDA) approved the ImmuKnow (Cylex) Immune Cell Function Assay for clinical use to detect cell-mediated immunity in adults undergoing immunosuppressive therapy after organ transplantation. The ImmuKnow assay is used to measure the concentration of ATP released from CD4 cells after stimulation (7). Specifically, the ATP value in phytohemagglutinin (PHA)-stimulated $CD4^+$ T cells is measured to predict postoperative immune stability and adverse events such as rejection or infection. Several studies have described the application of the ImmuKnow assay to monitor patient immune status during immunosuppressive treatment after kidney (8-10), liver (11, 12), lung (13, 14), small bowel (15), and pancreas transplantation (16, 17). This assay can also be used to predict the risk of infection in rheumatoid arthritis (RA) patients (18). Results of these studies indicated that the mean ATP level was significantly lower in patients with infection compared to healthy controls and patients without infection, but there was no correlation with RA activity. However, it is not yet known whether the ImmuKnow assay is useful for identifying SLE or lupus nephritis (LN) patients at increased risk of infection or whether the assay can be correlated with SLE activity.

Materials and methods

Blood samples

Peripheral blood samples were obtained from 96 LN patients who received immunosuppressive therapy in Shanghai First People's Hospital between January 1, 2010, and January 1, 2013. Control blood samples were obtained from 28 healthy volunteers. The approval of the Medical Ethics Committees of the respective institutions and informed consent from all patients were obtained for the collection of additional venous blood during routine clinical management of the patients.

Testing of immune cell function

Immune cell function was measured using the ImmuKnow® assay (Cylex). Briefly, sodium heparin anti-coagulated blood (250 μL per reaction) was incubated with and without PHA for 15–18 h at 37°C. $CD4^+$ T cells were then positively selected using magnetic beads coated with an anti-CD4 monoclonal antibody (Dynal, Oslo, Norway). CD4 beads were provided by Cylex as part of the ImmuKnow kit. Bead-isolated cells were lysed, and the amount of ATP released was measured by luminescence by using a luciferin/luciferase detection system (Cylex). The results are expressed in nanograms of ATP per milliliter of blood.

Immunosuppressive therapy

Prednisolone was administered to 96 patients at 5–60 mg per day. Cyclophosphamide was administered to 79 patients. FK506 and MMF were administered to 3 patients and 9 patients, respectively.

Definition of infection

The diagnosis of infection was made according to clinical, microbiological, and imaging criteria. Severe infections were defined as those requiring intravenous antimicrobial therapy or an infection caused by opportunistic pathogens such as *Mycobacterium* species, herpes zoster, cytomegalovirus, and *Pneumocystis jiroveci*. Systemic inflammatory response syndrome was defined as a condition with body temperature less than 36°C or greater than 38°C; heart rate greater than 90 beats per minute; tachypnea (high respiratory rate, with greater than 20 breaths per minute) or an arterial partial pressure of carbon dioxide less than 4.3 kPa (32 mmHg); leukocytes less than 4×10^9 cells/L or greater than 12×10^9 cells/L; or the presence of more than 10% immature neutrophils. The infectious patient sample was collected when his diagnosis of infection was clearly. "After infection sample" was collected at one week after infection be cured.

SLE activity score

The systemic lupus erythematosus disease activity index (SLEDAI) 2 K was used to measure lupus activity (19).

Statistical analysis

The unpaired Student's *t*-test or Mann-Whitney *U*-test was used to evaluate continuous data by performing a comparative analysis between patients with and without severe infection, whereas the χ^2 test or Fisher's exact test was used for categorical data. Receiver operating characteristic (ROC) analysis was performed to describe the diagnostic accuracy available to date. Multivariable analysis was performed to determine the independent value of the ATP assay in diagnosis of severe infection. Multi-variable correlation analysis was applied to evaluate the ATP level in relation to infection after adjusting for potential confounders: prednisolone dosage and cumulative CTX dosage. All statistical analyses were performed with SPSS 17.0 (SPSS, Chicago, IL, USA). For all statistical evaluations, $p<0.05$ was considered statistically significant.

Table I. Characteristics of study subjects.

	Health n=28	Non-infected SLE n=74	Infected SLE n=22	*p*-value
At diagnosis				
Age	42.68 ± 12.14	42.32 ± 14.84	41.87 ± 12.66	NS [a]
Sex (F/M)	25/3	68/6	20/2	$p<0.01$ [b]
Proteinuria		4.24 ± 2.42	4.42 ± 2.78	NS [a]
Plasma albumin		32.44 ± 7.55	32.31 ± 6.85	NS [a]
		99.20 (31,652)	109.59 (33,467)	NS [b]
SLEDAI at diagnosis		19.25 ± 5.5	19.55 ± 9.45	NS [a]
ESR	10.45 ± 10.34	60.27 ± 27.83	113.52 ± 22.86	$p<0.01$ [a]
Hs-CRP	2.30 ± 1.12	8.12 ± 3.43	75.11 ± 15.34	$p<0.01$ [a]
ANA		Nucleolus 2 (2.70%) Peripheral 1 (1.35%) Mixed 3 (4%) Homogeneity 30 (40.5%) Particles 35 (47%) Centromere 3 (4%)	Mixed 1 (4.55%) Homogeneity 12 (54.55%) Particles 9 (40.91%)	
Pathological classification		III n=9 (12.2%) IV n=35 (47.3%) V n=5 (6.75%) IV + V n=7 (9.46%) -typeable n=18 (24.32%)	III n=2 (9.09%) IV n=11 (50%) V n=1 (4.55%) IV+Vn=5 (22.73%) Non-typeable n=3 (13.66%)	
At Study				
Immunosuppressant		PSL 1 PSL + CTX 65 PSL + FK 1 PSL + MMF 8	PSL + CTX 14 PSL + FK 1 PSL + MMF 5 PSL + MMF + FK 2	
Average steroid (mg/d)		20.33 ± 17.15	27.73 ± 14.86	$p<0.01$ [a]
Cumulative CTX (mg)		8.35 ± 4.51	4.81 ± 2.38	$p<0.01$ [a]
Average MMF (mg)		1.24 ± 0.36	1.17 ± 0.41	NS [a]
Proteinuria (g/d)		1.8 ± 2.77	1.73 ± 1.37	NS [a]
Creatinine (μmol/L)		115.12 (28,629)	118.9 (19,769)	NS [b]
Albumin (mg/L)		32.69 ± 7.91	36.2 ± 8.26	$p<0.05$ [a]
ESR (mm/h)		34.68 ± 22.48	34.64 ± 26.55	NS [a]
Haemoglobin (g/L)		116.4 ± 1.87	113.52 ± 23.59	NS [a]
WBC ($\times 10^9$/L)		6.37 ± 2.24	7.49 ± 3.4	$p<0.05$ [a]
Neutrophil %		60.17 ± 21.17	85.9 ± 29.14	$p<0.05$ [a]
lymphocyte %		22.53 ± 12.39	16.46 ± 17.78	NS [a]
IgG (mg/L)		13.51 ± 5.0	14.79 ± 6.73	NS [a]
IgM (mg/L)		0.93 ± 0.53	1.00 ± 0.58	NS [a]
IgA (mg/L)		2.73 ± 1.12	2.32 ± 0.79	NS [a]
C3 (mg/L)		0.79 ± 0.28	0.79 ± 0.33	NS [a]
SLEDAI score		7.03 ± 6.02	8.33 ± 6.92	$p<0.05$ [a]

Values are given as means ± standard deviation. NS: not significant; WBC: white blood cells; Ig: immunoglobulin; PSL: prednisolone. [a] Mann-Whitney U-test; [b] χ^2 test.

Results

Relationship between immune cell function and infection

The ImmuKnow assay was used to study immune cell function in 124 blood samples obtained from 96 LN patients (non-infected and infected SLE) and 28 healthy volunteers. Demographic characteristics of the study population are presented in Table I.

According to the manufacturer's instructions, immune-cell reactivity levels measured with ImmuKnow in healthy individuals can be stratified as follows: low (ATP <225 ng/mL), moderate (ATP 226–524 ng/mL), and strong (ATP >525 ng/mL) (6). However, this stratification may not be directly applicable to Chinese subjects because it was based on a clinical trial in the United States. Therefore, in the present study, ATP levels were measured in healthy volunteers as controls.

The number of LN patients who developed infections and required hospitalisation and their clinical characteristics are presented in Tables I and II. There were 2 cases of herpes zoster; 9 cases of bacterial pneumonia; 1 case each of acute upper respiratory infection,

Table II. Characteristics of infected LN subjects.

No.	Age	Sex	Diagnosis	WBC ($\times 10^9$/L)	Neutro-phil(%)	CRP (mg/L)	SIRS	Immunosuppressant	ATP level (ng/mL) Infection	Recover
1	45	F	bacterial pneumonia	6.23	63.8	62.2	(-)	Pred (30mg/d)+CTX (3g)	450	426
2	63	F	bacterial pneumonia	3.2	85	70.3	Yes	Pred (20mg/d)+CTX (7.6g)	147	291
3	57	F	bacterial pneumonia	7.8	79.4	66.1	(-)	Pred (5mg/d)+CTX (9.8g)	216	359
4	40	F	catheter-related staphylococcus aureus	6.83	71.7	73.4	(-)	Pred (30mg/d)+CTX (4.2g)	358	361
5	50	F	bacterial pneumonia	7.4	84.5	71.6	(-)	Pred (30mg/d)+CTX (7.4g)	267	259
6	42	F	bacterial pneumonia	10.94	68.4	68	(-)	Pred (30mg/d)+CTX (4g)	268	353
7	28	F	cellulitis	12.89	92.6	74.8	(-)	Pred (40mg/d)+CTX (4g)	221	289
8	50	F	pulmonary aspergillosis	5	77	68.4	(-)	Pred (50mg/d)+CTX (3.2g)	232	249
9	30	F	pneumocystis pneumonia	4.2	52.3	74.5	(-)	Pred (10mg/d)+CTX (7.8g)	78	196
10	43	F	pulmonary aspergillosis	15.3	66.5	112	Yes	Pred (30mg/d)+CTX (4.6g)	224	496
11	41	F	herpes zoster	9.32	68.3	80	(-)	Pred (50mg)+CTX (2g)	223	301
12	59	F	pulmonary aspergillosis	5.7	49.5	73.9	(-)	Pred (20mg/d)+CTX (5.8g)	489	413
13	31	F	Klebsiella pneumoniae	5.28	64.1	74.1	(-)	Pred (30mg/d)+CTX (4g)	298	601
14	25	M	central nervous system (streptococcus pneumonia)	5.3	54	50	(-)	Pred (40mg/d)+CTX (3.2g)	149	187
15	42	F	pulmonary aspergillosis	6.4	52.7	70.5	(-)	Pred (10mg/d)+FK506(0.07mg/kg/d)	281	412
16	42	F	acute upper respiratory infection	6.7	69	57.1	(-)	Pred (15mg/d)+MMF (1g/d)	159	333
17	21	F	bacterial pneumonia	3.9	69.9	71.6	(-)	Pred (60mg/d)+MMF (2g/d)	264	512
18	22	F	cytomegalovirus pneumonia	6.34	54.8	105.2	(-)	Pred (10mg/d0+MMF (1.5g/d)	433	401
19	38	F	bacterial pneumonia	10.6	67	55.4	(-)	Pred (20mg/d)+MMF (1g/d)	297	385
20	43	F	herpes zoster	2.43	4.9	94.2	Yes	Pred (10mg/d)+MMF (1g/d)	197	299
21	20	F	bacterial pneumonia (E. coli)	12.65	84.8	96.8	(-)	Pred (40mg/d)+MMF (1g/d)+FK506(0.07mg/kg/d)	112	293
22	22	M	viral pneumonia	10.38	80.1	82.4	(-)	Pred (30mg/d)+MMF (1g/d)+FK506(0.07mg/kg/d)	317	368

SIRS: systemic inflammatory response syndrome.

pneumocystis pneumonia, cellulitis, catheter-related *Staphylococcus aureus* infection, and a central nervous system infection; and 4 cases of pulmonary aspergillosis.

In our study, patients with infection were classifies as ''infected'' and those without infection were classified as "non-infected" LN patients. No differences were observed between the two groups with respect to the modified systemic lupus erythematosus disease activity index 2000 (M-SLEDAI-2K) scores, proteinuria, plasma albumin, or serum creatinine (Scr) at diagnosis. Furthermore, no differences were observed in the mean age (Kruskal-Wallis test), but a significant difference was seen in the sex ratio ($p<0.01$, χ^2 test) between the healthy volunteers and LN patients. Erythrocyte sedimentation rate (ESR) and hs-CRP levels ($p<0.01$, Kruskal-Wallis test) were significantly increased in infected LN patients compared to non-infected patients (Table I). The average dose of steroid and cumulative CTX dose administered differed between the non-infected and infected LN patients; however, the dose

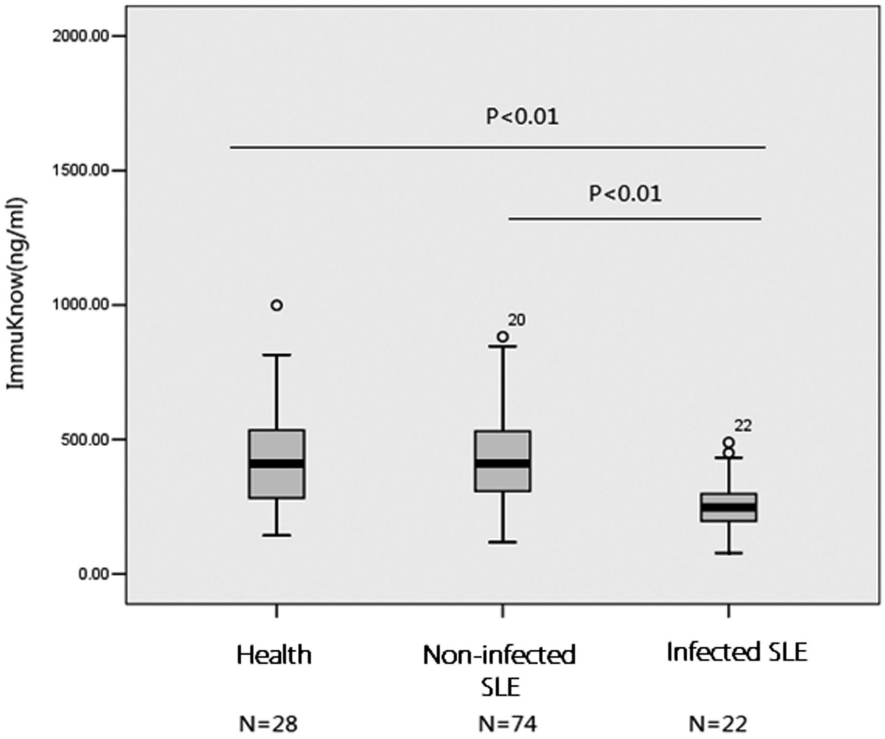

Fig. 1. Box-and-whisker diagram of adenosine triphosphate (ATP) levels measured in healthy individuals, non-infected SLE patients, and SLE patients with infection. Lower and upper box borders indicate 25th and 75th percentiles, respectively. Lines within boxes correspond to median ATP levels. Whiskers below and above the boxes define the minimum and maximum values, respectively. ATP levels in healthy individuals, non-infected SLE patients, and SLE patients with infection were 410 (281, 550), 412 (306, 533), 248 (187, 302), respectively (median [25th, 75th], ng/mL).

of MMF did not vary. Plasma albumin and Scr also differed between the non-infected and infected LN patients. The WBC count and neutrophils were significantly higher in infected LN patients than in non-infected patients, but serum levels of immunoglobulin A, M, G, and complement C3 levels showed no differences. Moreover, the SLEDAI scores showed an increasing trend in infected LN patients.

In the present study, the mean ATP level measured in healthy controls was in the moderate range (410 [281, 550] ng/mL, median [25th, 75th]), although wide differences in ATP levels were observed in the healthy controls, as reported previously. The mean ATP level measured in infected LN patients (248 [187, 302] ng/mL, median [25th, 75th]) was significantly lower than those observed in either healthy controls (p<0.01) or non-infected LN patients (412 [306, 533] ng/mL, median [25th, 75th], p<0.01). The mean ATP level in non-infected LN patients was not significantly different from that in healthy controls (Fig. 1).

However, the ATP level increased significantly after infection in LN patients [(248 (187, 302) ng/mL *vs*. 355 (290, 412) ng/mL, p<0.01] (Fig. 2).

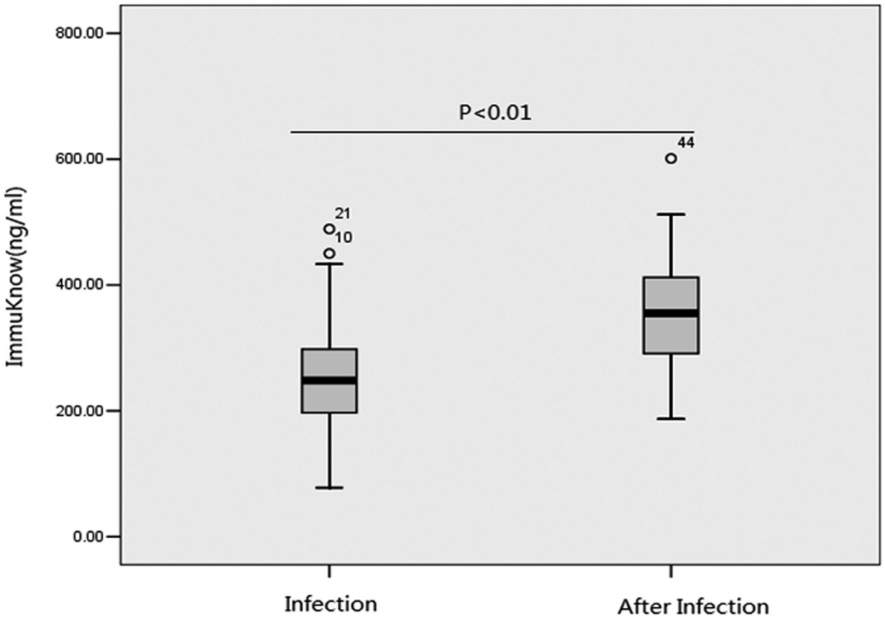

Fig. 2. Box-and-whisker diagram of adenosine triphosphate (ATP) levels measured in SLE patients with and after infection. ATP level in SLE patients with and after infection were 248 (187, 302) and 355 (290, 412), respectively (median [25th, 75th], ng/mL).

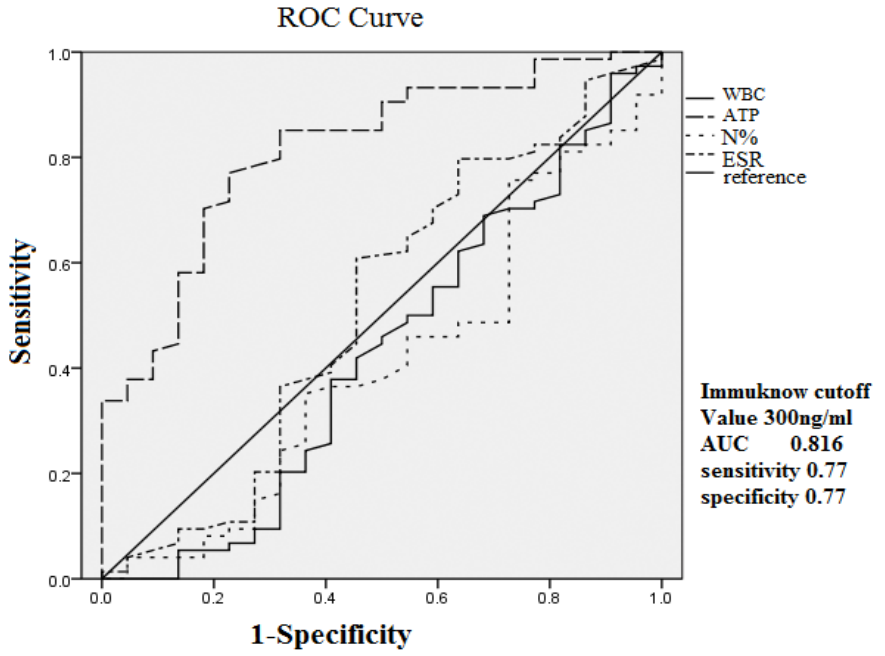

Fig. 3. Receiver Operating Characteristics (ROC) curve for ImmuKnow and other markers of infection of infected and non-infected patients in predicting subsequent infections in SLE. The estimated areas under the ROC curve were 0.816 for infection, and the best cut-off ATP value was 300 ng/mL for infection.

ROC curve of ATP for identifying infection

The estimated area under the receiver operating characteristic (ROC) curve of ATP was 0.816 for identifying infection, and AUC of CRP , ESR, WBC count and neutrophil percentage were 0.637, 0.518,0.428 and 0.410, respectively. ATP had the highest area under the curve for predicting infection. The best cut-off of the ATP value was 300 ng/mL for infected LN patients. The cut-off ATP value of 300 ng/mL for identifying infected patients showed 77% specificity and 77% sensitivity (Fig. 3).

Relationships between immune cell function, SLE activity, and renal function

No correlations were found between ATP activity and proteinuria (r=0.40, p=0.696); serum creatinine (r=0.109, p=0.291); plasma albumin (r=0.008, p=0.939); ESR (r=0.155, p=0.132); HGB (r=0.006, p=0.957); IgG, IgM, IgA, C3, or SLEDAI (Table III). However, a negative correlation was found between ATP and WBC count (r=–0.328, p=0.01) and neutrophil percentage (r=–0.231, p=0.024).

Multi-variable analysis to determine the validity of ATP assays in the diagnosis of severe infection

Multi-variable correlation analysis was used to determine the independent value of the ATP assay in the diagnosis of severe infection. After adjusting for

Table III. Pearson correction analysis between $CD4^+$ T cell activity detected by ImmuKnow assay (ng/ml) and immunoglobulin levels, complement C3, and SLEDAI score.

ImmuKnow (ng/ml)	IgG	IgA	IgM	C3	SLEDAI score
Pearson correlation	0.03	0.07	-0.07	-0.05	0.143
Sig. (2-tailed)	0.78	0.51	0.48	0.63	0.167

the confounding effects, the average steroid dose and cumulative CTX dose, the partial correlation coefficients between the ATP assay and severe infection were r=–0.040, $p<0.001$; CRP was r=0.962, $p<0.001$; however, the WBC was r=0.138, $p=0.22$ and neutrophil count was r=0.102, $p=0.365$, respectively. These results indicated that the CRP value and ATP assays might be potentially useful for the diagnosis of severe infection in SLE.

Discussion

In this study, we investigated the immune function in LN patients treated with steroids, and CTX, FK506, or MMF. The results showed that ATP activity, as determined by the ImmuKnow assay, correlated with infection, but not with SLE activity.

An international multicentre clinical trial with the ImmuKnow assay examined the ATP levels in healthy volunteers and transplant recipients. These results showed that ATP levels of 226–524 ng/mL accurately reflected a moderate immune response. Most of the ATP levels measured in the peripheral blood of Chinese healthy controls were within the ''moderate'' range; therefore, the manufacturer's stratification system was applicable to Chinese patients. Our result indicated that ATP levels ranged from 281 to 550 ng/mL in healthy controls.

A meta-analysis indicated that ATP levels were strongly predictive of infection among transplant patients (20). Among 504 patients, including 243 kidney, 150 liver, 86 heart, and 25 small intestine transplant recipients who provided 1833 specimens, the analysis showed that ATP levels of 25 ng/mL were associated with a 12-fold increased probability of infection among patients with strong immune responses. However, we did not observe an adequate cut-off value for infection. Longitudinal monitoring is necessary to assess immunity and infection in individual patients as a guide to direct therapy. Our study indicated that immune cell function in infected LN patients was significantly lower than that in healthy controls or non-infected LN patients, suggesting that a low ImmuKnow result predicts an infectious syndrome. The ImmuKnow assay is useful for identifying patients at an increased risk of infection, not only among transplant recipients and RA patients but also among LN patients.

Zhou *et al*. reported that a cut-off ATP value of 238 ng/mL identified patients with infection after renal transplantation with 100% specificity and a positive predictive value (PPV) and 92.9% sensitivity (21). Hwang *et al*. reported that a cut-off ATP value of 225 ng/mL identified patients with infection after liver transplantation with 81.4% specificity and a PPV and 59.7% sensitivity (22). Kobashigawa *et al*. reported that a cut-off ATP value of 200 ng/mL identified patients with infection after heart transplantation with 71.7% specificity and a PPV and 71.7% sensitivity (23). Our study showed that the best cut-off ATP value for infection was 300 ng/mL. The cut-off ATP value of 300 ng/mL to identify patients with infection displayed 77% specificity and a PPV with 77% sensitivity.

A broad spectrum of infections have been reported in SLE, including bacterial, mycobacterial, viral, fungal, and parasitic infections, with respiratory and urinary tracts being the most commonly involved sites (4, 24). Our study showed that one-third of LN patients developed serious infections during follow-up. Common bacteria such as *Escherichia coli*, *Staphylococcus* spp., and *Streptococcus pneumoniae* were the most frequent isolates. In a previous study, ATP activity in $CD4^+$ cells did not show a correlation with CD4 cell counts (7). Because the ImmuKnow test measures the amount of ATP after stimulation with PHA, thereby providing a gauge of patient global T-cell responsiveness, the results are not correlated solely with the $CD4^+$ cell count before stimulation. It is not clear why ATP activity in $CD4^+$ T cells was positively associated with neutrophil counts in the present study. The present study showed a negative correlation between ATP and WBC count (r=–0.328, $p=0.01$) and neutrophil percentage (r=–0.231, $p=0.024$).

As observed in RA, our data also indicated that the results of the ImmuKnow assay were not correlated with SLE activity. No correlation was observed between ATP activity and SLEDAI scores, complement C3 level, and ESR measured in SLE patients without infection. This lack of correlation may be due to the presence of other immune cells. The discovery of a new lineage of $CD4^+$ effector or T-helper type 17 (Th17) cells that selectively produce interleukin-17 (IL-17) has provided new insights into the immune regulation, host defense, and pathogenesis of autoimmune and other chronic inflammatory disorders, including SLE (25). IL-17 is a proinflammatory cytokine that induces other cytokines such as tumor necrosis factor-alpha (TNF-α), IL-1β, IL-6, and IL-23, some of which can activate other lineages of $CD4^+$ T cells. SLE progresses by regulating Th1 and Th17 cell function via B-T cell interactions and cytokine network regulation. (26). The Cylex ImmuKnow Immune Cell Function Assay measures the concentration of ATP in circulating CD4 cells following in vitro stimulation with phytohemagglutinin (PHA) as an indicator of immune cell function (6). However, it is difficult to count the Th1 or Th2 or Th17 CD4 cells and distinguished Th1 or Th2 cells from total CD4 cells.

In conclusion, in LN patients treated with immunosuppression therapy, the monitoring of immune cell function using the ImmuKnow assay may permit the identification of patients at increased risk of infection. However, immune cell function, as measured with the ImmuKnow assay, showed no correlation with SLE activity in the present study. Furthermore, a higher steroid dose was administered in the infected group than

in the non-infected group, which may potentially affect ATP levels. In present studies, we observe infected patients had higher SLEDAI scores than no infected patients. So we consider that a higher steroid dose still cannot control lupus activity, which indicates it may be associated with difference in severity of diseases. No obvious differences were observed between the healthy control and non-infected LN patients in the present study. Larger studies are required to establish the clinical advantages of the ImmuKnow assay as a test for the optimal monitoring of the immune function in LN patients treated with immunosuppression therapy.

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