$CD56^{bright}CD16^{-}$ to $CD57^{+}CD56^{dim}CD16^{+}$ NK cell ratio discriminates disease activity and renal involvement in patients with systemic lupus erythematosus

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Abstract

Objective

We aimed to discriminate subpopulations of peripheral natural killer (NK) cells of patients with systemic lupus erythematosus (SLE) and evaluate their usability in monitoring disease activity.

Methods

The total number of NK cells and their subpopulations were determined by flow cytometry in 68 patients with SLE and 35 healthy controls. Clinical data were extracted from medical records, including serum anti-double-stranded-DNA (anti-dsDNA), complement C3 and C4, and urine protein. Disease activity in patients with SLE was assessed using the SLE Disease Activity Index-2000 (SLEDAI-2K).

Results

The percentages and absolute numbers of NK cells decreased, and the proportions of three major NK cell subsets defined by cell maturation status altered in SLE patients. The frequency of $CD56^{bright}CD16^{-}$ NK (immature, Im NK) cells increased, while that of the $CD57^{+}CD56^{dim}CD16^{-}$ subset (mature, more differentiated, MD NK) decreased in patients with high-activity SLE, resulting in a significant increase in the Im NK-to-MD NK ratio as compared with that in patients with low-activity SLE. The area under the receiver operating characteristic curve indicated that the ratio was 0.722 in severe SLE and 0.773 in lupus nephritis, with optimal cut-off levels of 0.075 and 0.108, respectively. The ratio correlated positively with the SLEDAI-2K score, proteinuria, and serum anti-dsDNA antibody levels but negatively with C3 and C4 levels.

Conclusion

Our data indicate that the imbalance in Im NK and MD NK cells may play a role in lupus development and serve as a predictive biomarker to assess disease activity and renal involvement in patients with SLE.

Key words

systemic lupus erythematosus, natural killer cell subsets, disease activity, renal involvement

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Introduction

Systemic lupus erythematosus (SLE) is a chronic, heterogeneous, multisystem autoimmune disease that mainly affects the kidneys, characterised by the deposition of immune complexes and autoantibodies produced by abnormally activated and hyperproliferating autoreactive B and T cells (1, 2). Evidence suggests that natural killer (NK) cells act as a bridge between innate and adaptive immunity. They can directly recognise and kill target cells and regulate other immune cells and may play crucial roles in lupus development (3-5).

NK cells ($CD3^{-}CD56^{+}$) are relatively abundant; they constitute 5–20% of lymphocytes in peripheral blood and other tissues (6, 7). The NK cell differentiation spectrum ranges from immature (Im NK, $CD56^{bright}CD16^{-}$) to mature subsets (Ma NK, $CD56^{dim}CD16^{+}$) (8-10). Im NK cells, representing a pro-inflammatory NK cell subset, contribute to autoimmune inflammation. In contrast, Ma NK cells exhibit cytotoxicity and have a regulatory role in suppressing or preventing autoimmune disease. Ma NK cells can be further divided into less differentiated (LD NK, $CD57^{-}CD56^{dim}CD16^{+}$) and more differentiated (MD NK, $CD57^{+}CD56^{dim}CD16^{+}$) subsets based on their expression of NKG2A, killer-cell immunoglobulin-like receptors, and CD57 (9, 11). LD NK cells are highly responsive to cytokines such as IL-12 and IL-18. In contrast, MD NK cells progressively lose their ability to respond to cytokines while their cytotoxic function is maintained or improved (8, 12).

Reducing the total NK cell number and imbalances in NK cell subpopulations play crucial roles in the development of autoimmune diseases such as rheumatoid arthritis, primary Sjögren's syndrome, systemic sclerosis, psoriasis and Primary sclerosing cholangitis (13-17). In patients with active SLE, NK cells overexpress the surface marker CD94/NKG2A, whereas CD16, KIR, and CD57 are under-expressed, suggesting impaired differentiation (18-20). Furthermore, circulating NK cells are decreased in number, with altered subpopulations and impaired function (5, 18-27). In previous studies in patients with SLE, Im NK cells were elevated in some studies (24, 27) but normal in others (5, 21, 22), whereas MD NK cells were reduced (21, 22). Single-cell RNA sequencing of kidney biopsies from lupus nephritis (LN) patients has revealed an increase of Im NK cells and Ma NK cells in the kidney. Im NK cells showed high expression of genes including *KIT, IL7R, TCF7, RUNX2*, and the chemokine receptor-encoding gene *CXCR4*, while Ma NK cells, which exhibited increased expression of cytotoxic genes such as *PRF1, GZMB, GNLY,* and *CXCR4* and *CX3CR1* (28). However, it remains obscure whether and how NK cells and/or their subsets are involved in SLE pathogenesis, lupus activity, clinical characteristics, and organ involvement.

In this study, we hypothesised that dysdifferentiation among NK cell subpopulations might reflect immune homeostasis dysregulation in SLE. We compared peripheral blood NK cell subpopulations between patients with SLE and healthy controls (HCs) and among patients with different disease statuses and assessed the correlations between the Im NK-to-MD NK ratio and SLE disease activity and clinical features.

Materials and methods

Study participants

Patients with SLE were recruited from the Department of Rheumatology and Immunology of the Affiliated Drum Tower Hospital of Nanjing University Medical School (n=34) or the Department of Rheumatology and Immunology, Huaian No. 1 People's Hospital (n=34) and sex- and age-matched HCs (n=35) were recruited from both hospitals between April and June 2021. All patients and HCs were Han Chinese. All patients with SLE satisfied the 2019 European League Against Rheumatism/American College of Rheumatology classification criteria for SLE (29). None of the participants had a medical history of other autoimmune diseases, such as inflammatory bowel disease or rheumatoid arthritis, human immunodeficiency virus infection, hepatitis virus infection, or malignancies. None of the female patients with SLE were lactating or pregnant. The demographic

and clinical characteristics of the study participants are presented in Tables I and II. No significant differences were observed among patients with SLE with low or high disease activity and HCs regarding whether they were recruited from Nanjing Drum Tower Hospital or Huaian No. 1 People's Hospital. There was a significant difference in age between the low- and high-activity SLE groups but not between the SLE and HC groups. No disparities in sex were observed among the three groups.

Ethical considerations

This study was approved by the ethics committees of participating hospitals (nos. 2021-662-01 and KY-2022-010-01). Written informed consent for venous blood specimen collection was obtained from all participants.

Disease assessment

Based on the SLE Disease Activity Index 2000 (SLEDAI-2K) system (30), we classified patients into a low-activity SLE group (SLEDAI-2K score ≤10; patients with low to intermediate disease activity) and a high-activity SLE group (SLEDAI-2K >10; patients with high disease activity) (31). Patients with SLE were classified into three groups according to the anti-dsDNA antibody titres (negative (Neg; ≤20 IU/mL), lowly positive (Low; 20–200 IU/mL), and highly positive (High; >200 IU/mL)), which were determined based on the upper level of the normal range (20 IU/mL) and maximum of the detection range (200 IU/mL) of the ELISA kit we used. Meanwhile, patients with SLE were divided into two groups according to the complement C4 expression level (Low, ≤0.1 g/L and High, >0.1 g/L), which corresponded to the upper level of the normal range (0.1 g/L) detected by turbidimetry.

Isolation of peripheral blood mononuclear cells (PBMCs) and flow cytometry

PBMCs were isolated from whole heparinised blood collected from all patients with SLE and HCs through density gradient centrifugation using Ficoll-Paque (STEMCELL Technologies, Vancouver, Canada). NK cell populations were quantified using flow cytometry based on staining with fluorochrome-conjugated antibodies targeting the following cell surface markers: live/dead-FVS780 (Cat. no.: 565388, Becton Dickinson (BD) Biosciences, San Jose, CA, USA; 1:1000), anti-human-CD3 (HIT3a)-FITC (Cat. no.: 300306, Bio-

Table I. Clinical and laboratory test results of patients with SLE and healthy controls.

Variable	Low-activity SLE (n=27)	High-activity SLE (n=41)	*p*
Demographic data			
Hospital, n (%)			0.083*
Nanjing Drum Tower Hospital	10 (37.0)	24 (58.5)	
Huaian No. 1 People's Hospital	17 (63.0)	17 (41.5)	
Age (years) (mean ± SEM)	45.8 ± 2.7	35.4 ± 2.1	0.003$^{\Phi}$
Sex (female), n (%)	22 (81.5)	39 (95.1)	>0.05*
Disease duration (months) (IQR)	96.0 (34.5–176.0)	63.0 (6.3–118.5)	0.072#
SLEDAI-2K score (range)	5.8 (8)	15.7 (19)	<0.001#
SDI median (range)	0.76 (6)	0.58 (7)	0.424#
Organ damage, n (%)			
Leukocytopenia	11 (40.7)	26 (63.4)	0.066*
Thrombocytopenia	5 (18.5)	14 (34.1)	0.160*
Autoimmune haemolysis	4 (14.8)	4 (9.8)	0.703*
Neuropsychiatric	2 (7.4)	4 (9.8)	1.000*
Alopecia	9 (33.3)	29 (70.7)	0.002*
Oral ulcers	8 (29.6)	16 (39.0)	0.428*
SCLE/DLE	2 (7.4)	0 (0)	0.154*
ACLE	10 (37.0)	25 (61.0)	0.053*
Pleural or pericardial effusion	5 (18.5)	27 (65.9)	<0.001*
Arthralgia/arthritis	7 (25.9)	23 (56.1)	0.014*
Proteinuria (>0.5 g/24 h)	6 (8.8)	24 (35.3)	0.003*
Cardiopulmonary PAH	4 (14.8)	6 (14.6)	1.000*
Gastrointestinal vasculitis	0 (0)	3 (4.4)	0.271*
Fever	6 (8.8)	20 (29.4)	0.027*
Raynaud's phenomenon	9 (33.3)	15 (36.6)	0.784*
Laboratory tests, n (%)			
Anti-dsDNA[a]			0.002*
Neg	13 (54.2)	6 (14.6)	
Low	8 (33.3)	19 (46.3)	
High	3 (12.5)	16 (39.0)	
C3 (g/L) (IQR)	0.76 (0.6–1.0)	0.6 (0.4–0.8)	0.007*
C4 (g/L)[b]			0.032*
Low	8 (29.6)	23 (56.1)	
High	19 (70.4)	18 (43.9)	
IgG (g/L) (IQR)	13.4 (8.9–14.5)	13.6 (8.4–21.1)	0.692*
IgA (g/L)	2.7 (1.7–3.2)	2.0 (1.3–2.9)	0.149*
ESR (mm/h) (IQR)	36.0 (22.0–74.0)	30.0 (22.8–59.5)	0.650*
Hb (g/L) (IQR)	111.5 (84.0–123.8)	100.0 (77.0– 114.0)	0.144*
ALB (g/L) (IQR)	36.7 (34.3–39.5)	32.6 (26.3–24.8)	<0.001*
Positive ANA	27 (100)	41 (100)	1.000*
Anti-SSA	15 (55.6)	26 (63.4)	0.517*
Anti-SSB	2 (7.4)	11 (26.8)	0.061*
Anti-Ro 52	16 (59.3)	20 (58.8)	0.397*
Anti-Sm	9 (33.3)	17 (41.5)	0.500*
Positive APL	4 (14.8)	10 (24.4)	0.379*
Infection, n (%)	10 (37.0)	16 (39.0)	0.869*
Medications, n (%)			
Prednisone dose (mg/day) (IQR)	20.0 (10.0–50.0)	37.5 (25.0–50.0)	0.026*
Hydroxychloroquine, n (%)	26 (96.3)	40 (97.6)	0.763*
Immunosuppressive therapy[c]	21 (77.8)	36 (87.8)	0.272*

[a]Neg, 20 IU/mL; low, 20–200 IU/mL; high, >200 IU/mL

[b]Low C4, ≤0.1 g/L, high C4, >0.1 g/L

[c]Patients with SLE received one or more of the following drugs: cyclophosphamide, mycophenolate mofetil, cyclosporin A, tacrolimus, azathioprine, leflunomide, or methotrexate within 1 month.

#Mann-Whitney test, *χ^2, $^{\Phi}$one-way ANOVA followed by Bonferroni *post-hoc* tests.

IQR: interquartile range; C3: complement 3; C4: complement 4; IgG: immunoglobulin G; IgM: immunoglobulin M; ESR: erythrocyte sedimentation rate; Hb: haemoglobin; ALB: albumin; SCLE: subacute cutaneous lupus erythematosus; DLE: discoid lupus erythematosus; ACLE: acute cutaneous lupus erythematosus.

Table II. Bivariate logistic regression analysis of patients with SLE with factors independently related to clinical features.

Variable	Low-activity SLE (n=27)	High-activity SLE (n=41)	Crude OR (95% CI)	Adjusted OR (95% CI)
Im NK-to-MD NK ratio, n (%)	2 3 (33.8)	45 (66.2)	5.156 (1.746, 15.229)	6.443 (1.571, 26.430)
Sex (female), n (%)	22 (81.5)	39 (95.1)	4.432 (0.793, 24.776)	8.571 (1.145, 64.16)
ALB (g/L) (IQR)	36.7 (34.3–39.5)	32.6 (26.3–24.8)	0.766 (0.658, 0.893)	0.704 (0.574, 0.863)
Hospital, n (%)			0.417 (0.154, 1.130)	-
Nanjing Drum Tower Hospital	1 0 (37.0)	24 (58.5)		
Huaian No. 1 People's Hospital	17 (63.0)	17 (41.5)		
Prednisone dose (mg/day) (IQR)	20.0 (10.0–50.0)	37.5 (25.0–50.0)	1.023 (1.002, 1.046)	-
Immunosuppressive therapy, n (%)	21 (77.8)	36 (87.8)	2.057 (0.559, 7.572)	-

*$p<0.05$.

Legend, San Diego, CA, USA; 1:200), anti-human-CD14 (HCD14)-FITC (Cat. no.: 325604, BioLegend; 1:200), anti-human-CD19 (HIB19)-FITC (Cat. no.: 302206, BioLegend; 1:200), anti-human-CD56 (NCAM16.2)-BB700 (Cat. no.: 566573, BD Biosciences; 1:200), anti-human-CD16 (3G8)-BV605 (Cat. no.: 302040, BioLegend; 1:200), and anti-human-CD57 (NK-1)-PE (Cat. no.: 560844, BD Biosciences; 1:200). Freshly isolated PBMCs were incubated with the antibodies in PBS containing 1% FBS at 4°C for 20 min. NK cells and their subpopulations were quantified using flow cytometry on an LSR Fortessa instrument (BD Biosciences). The data were analysed using FlowJo software v. 10.4 for Windows (BD Biosciences).

Statistical methods

The normality of data distribution was tested using the Shapiro-Wilk test. We used parametric or non-parametric tests based on a normal or non-normal distribution. Parametric continuous data are reported as standard error mean (SEM), whereas non-parametric continuous data are expressed as median (interquartile range). For normally distributed data, mean values were compared using Student's *t*-test (two groups) or one-way analysis of variance (ANOVA) (multiple groups). For non-normally distributed data, medians were compared using the Mann-Whitney U-test (two groups) or Kruskal-Wallis one-way ANOVA (multiple groups). Categorical data are expressed as a percentage and were evaluated using the chi-square test. We generated a receiver operating characteristic curve (ROC) curve to assess the sensitivity and specificity of the Im NK-to-MD NK ratio in predicting SLE disease activity. We used Spearman's rank correlation test to investigate correlations between two continuous non-parametric variables. All statistical analyses were conducted using SPSS 23 (IBM, Segrate, Italy) and GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). $p<0.05$ was considered significant.

Results

The proportion of NK cell subsets is altered in patients with SLE

To investigate the role of NK cell ($CD3^-CD14^-CD19^-CD56^+$ lymphocytes) subsets in the pathogenesis of SLE, we measured and compared the frequency and numbers of NK cell subpopulations in the peripheral blood of patients with SLE and HCs. Ma NK cells were further divided into LD and MD subsets according to CD57 expression (Fig. 1A). Both the percentage and absolute numbers of total NK cells were significantly decreased in the two SLE groups (low- and high-activity disease) compared to the HC group; the relative and absolute numbers of total NK cells tended to be lower in the high-activity SLE group than in the low-activity SLE group, although the difference was not significant (Fig. 1B (a, b)). Among total NK cells, the percentage of Ma NK cells was decreased, whereas that of Im NK cells was increased in both patient groups compared to the HC group. However, the percentage of Ma NK cells tended to be reduced, whereas that of Im NK cells tended to be increased in high-activity SLE compared to low-activity SLE, although significance was not reached. Furthermore, the population of MD NK cells gradually decreased among the HC and low- and high-activity SLE groups. In contrast, no significant difference in the LD NK cell population was observed among the groups (Fig. 1C (a)). The absolute numbers of total, Im, and Ma (LD and MD) NK cells were lower in both patient groups than in HCs (Fig. 1C (b)).

To assess the association between NK cell subpopulations and SLE disease activity, we calculated the Im NK-to-Ma NK ratio and found that the ratio was higher in patients with SLE than in HCs; however, significance was not reached, despite a trend for increased ratios in the low- and high-activity SLE groups (Fig. 1D), indicating that the reduced Ma NK cell levels may be attributed to decreased MD NK cell levels rather than decreased LD NK cell levels in active SLE. These data suggested that altered frequency or numbers of circulating NK cell subpopulations may play a role in the development of SLE.

The Im NK-to-MD NK ratio correlates with SLE disease activity

To further explore whether the development and differentiation of NK cell subsets are correlated with SLE disease activity, we compared the correlations between the numbers of Im NK, Ma NK, LD NK, and MD NK cells and SLE disease activity (Supplementary Fig. S1). A higher frequency of Im NK and LD NK cells was positively correlated with a higher SLEDAI-2K score and anti-double-stranded-DNA (anti-dsDNA) antibody expression level but

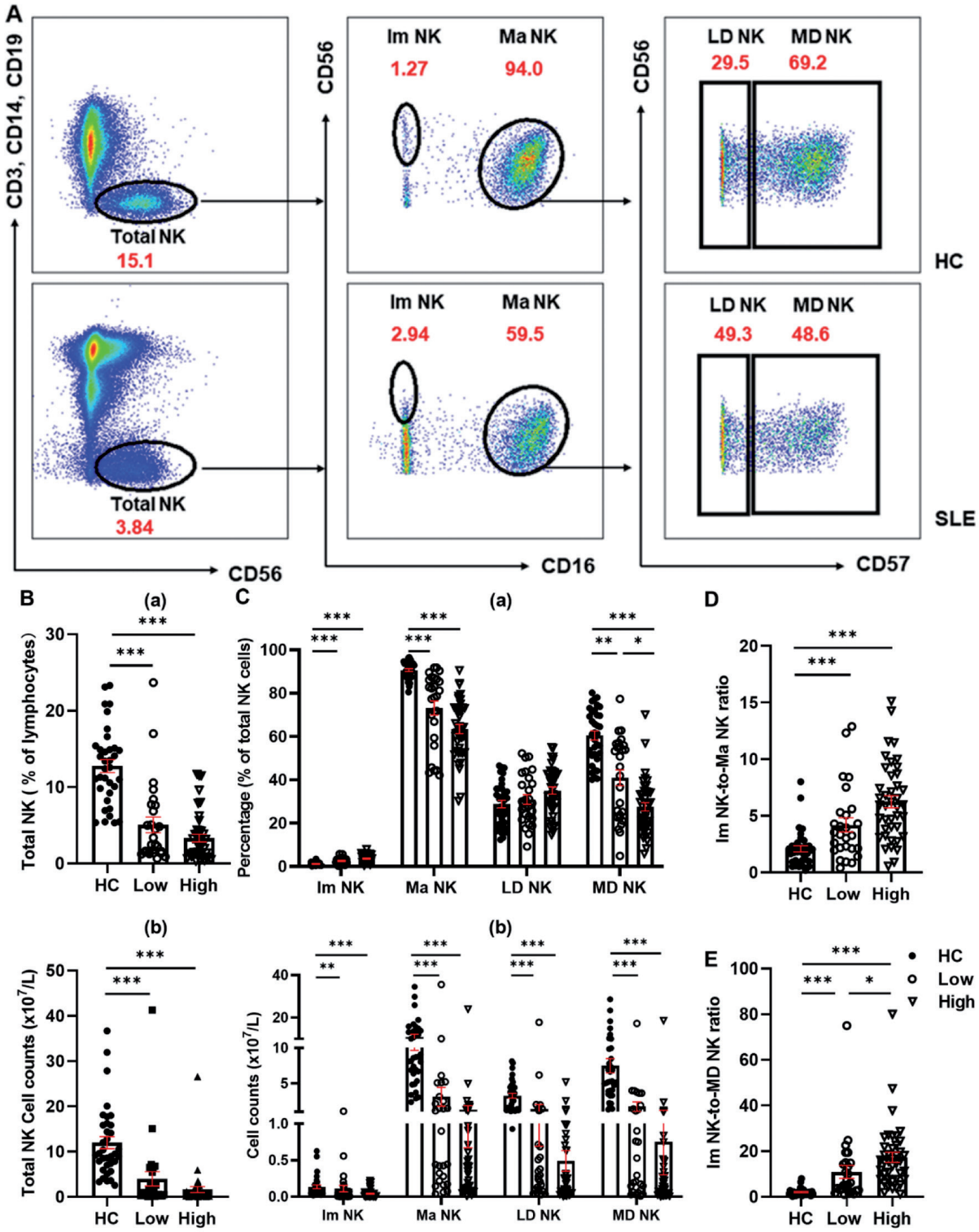

Fig. 1. Determination of NK cell subpopulations in peripheral blood of patients with SLE and HCs.
A: Flow cytometry gating strategy for NK cell subpopulations.
B: Relative numbers of circulating NK cells and their subpopulations (HCs, n=35; patients with low-activity SLE, n=27; patients with high-activity SLE, n=41; Kruskal-Wallis H-test).
C: Absolute numbers of NK cells and their populations (HCs, n=35; patients with low-activity SLE, n=27; patients with high-activity SLE, n 41; Kruskal-Wallis H-test).
D: Im NK-to-Ma NK ratio (HCs, n=35; patients with low-activity SLE, n=27; patients with high-activity SLE, n=41; Kruskal-Wallis H test) and (E) Im NK-to-MD NK ratio in patients with SLE with different disease activity and HCs (HCs, n=35; patients with low-activity SLE, n=27; patients with high-activity SLE, n=41; Kruskal-Wallis H-test).
The horizontal line indicates the mean. Im: immature; Ma: mature; LD: less differentiated; MD, more differentiated; HCs: healthy controls.
Note: demographic data of HCs: age: 39.4 ±1.6 years (mean ± SEM); sex: female: 30 (85.7%). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

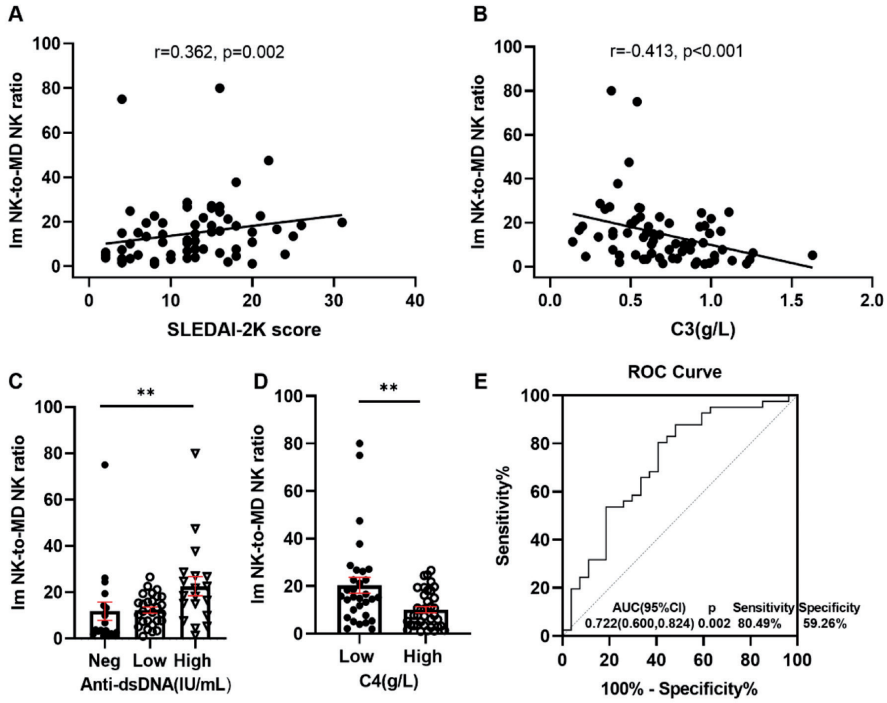

Fig. 2. Correlation between the Im NK-to-MD NK ratio and SLE disease activity. Correlation between the Im NK-to-MD NK ratio and (**A**) SLEDAI-2K score (n=68; Spearman's rank correlation test) or (**B**) serum C3 level (n=68; Spearman's rank correlation test).
C: Comparison of the Im NK-to-MD NK ratio among different serum anti-dsDNA titres. Based on the serum anti-dsDNA antibody level, patients with SLE were grouped into negative (Neg; ≤20 IU/mL; n=18), lowly positive (Low; 20–200 IU/mL; n=27), and highly positive (High; >200 IU/mL; n=22) (Kruskal-Wallis H-test) groups.
D: Comparison of the Im NK-to-MD NK ratio among patients with different serum C4 levels. Based on the serum C4 level, patients with SLE were grouped into low-activity (≤0.1 g/L; n=31) and high-activity (>0.1 g/L; n=37) (Mann-Whitney U-test) groups.
E: ROC curve analysis for an elevated Im NK-to-MD NK ratio in measuring SLE disease activity. The optimal predicted cut-off point was 0.075, and the highest Youden index value was 0.397 for patients with high-activity SLE (n=68). C3: complement C3; C4: complement C4.
$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

negatively with lower serum levels of complement C3 and C4 in patients with SLE, suggesting that Im NK cells play a role in lupus development. As the differentiation of Im NK to MD NK cells indicates the ability of NK cells to respond to cytokines (32), we calculated the Im NK-to-MD NK ratio in patients with SLE. The Im NK-to-MD NK ratio was significantly increased in patients with SLE (both with low- and high-activity disease) compared to HCs (Fig. 1E). The ratio was further elevated in patients with high disease activity compared to those with low disease activity (Fig. 1E). Next, we analysed the correlation between the Im NK-to-MD NK ratio and SLE disease activity. The ratio was correlated significantly positively with the SLEDAI-2K score (Fig. 2A) and significantly negatively with the C3 level (Fig. 2B). Next, patients with SLE were classified into three groups according to the anti-dsDNA antibody titres (negative (Neg; ≤20 IU/mL), lowly positive (Low; 20–200 IU/mL) and highly positive (High; >200 IU/mL)), which were determined based on the upper level of the normal range (20 IU/mL) and maximum of the detection range (200 IU/mL) of the ELISA kit we used (Fig. 2C). We found that the Im NK-to-MD NK ratio was increased in patients with SLE with high anti-dsDNA antibody titres, but not in those with low or negative anti-dsDNA antibody titres. Meanwhile, patients with SLE were divided into two groups according to the complement C4 expression level (Low, ≤0.1 g/L and High, >0.1 g/L), which corresponded to the upper level of the normal range (0.1 g/L) detected by turbidimetry (Fig. 2D). The results demonstrated that the Im NK-to-MD NK ratio was significantly increased in the low C4 SLE group.

In ROC curve analysis, the AUC was 0.722, and the best cut-off point for the Im NK-to-MD NK ratio to discriminate patients with SLE with high disease activity was 0.074. Applying a threshold of 0.075, sensitivity was 80.49%, specificity was 59.26%, and the Youden index was 0.397 (Fig. 2E). These data indicated that the Im NK-to-MD NK ratio reflects the disease status of patients with SLE.

The Im NK-to-MD NK ratio may serve as a predictive biomarker for LN activity

LN is one of the essential clinical complications of SLE and is often correlated with increased mortality (2). To assess whether the dysregulation of NK cell subpopulations is involved in LN, we analysed the correlation between the Im NK-to-MD NK ratio and LN. The Im NK-to-MD NK ratio was significantly positively correlated with proteinuria (Fig. 3A) but negatively with hemoglobin (Hb) (Fig. 3B) and albumin (ALB) (Fig. 3C) levels. After correcting for confounders using a forward binary logistic regression model, the Im NK-to-MD NK ratio and female sex were found to be positively associated with high disease activity compared to low disease activity, with adjusted odds ratios (ORs) (95% confidence interval (CI)) of 6.443 (1.571, 26.430) and 8.571 (1.145, 64.16), respectively. In contrast, the ALB level was negatively correlated with high disease activity *versus* low disease activity, with an adjusted OR (95% CI) of 0.704 (0.574, 0.863) (Table II). Potential confounding factors, including recruitment from two different hospitals and different drugs used for treatment (glucocorticoids and immunosuppressive agents), did not show correlations in the low- and high-activity SLE groups (Table II). We further compared the correlations between the Im NK, Ma NK, LD NK, and MD NK cell populations and clinical features of SLE (Suppl. Fig. S2). The frequency of Im NK cells, but not that of other NK cell subsets, was positively correlated with proteinuria. In contrast, it was nega-

tively associated with serum ALB levels, suggesting the role of Im NK cells in the development of LN.

In ROC curve analysis, after excluding three participants with extreme Hb values, the AUC was 0.773, and the best cut-off point for the Im NK-to-MD NK ratio to discriminate LN was 0.108. Applying a threshold of 0.108, the sensitivity was 84.62%, the specificity was 76.19%, and the Youden index was 0.608 for patients with SLE with LN (Fig. 3D). These data suggested that the Im NK-to-MD NK ratio is related to clinical features of SLE and could be applied as a predictive biomarker for LN.

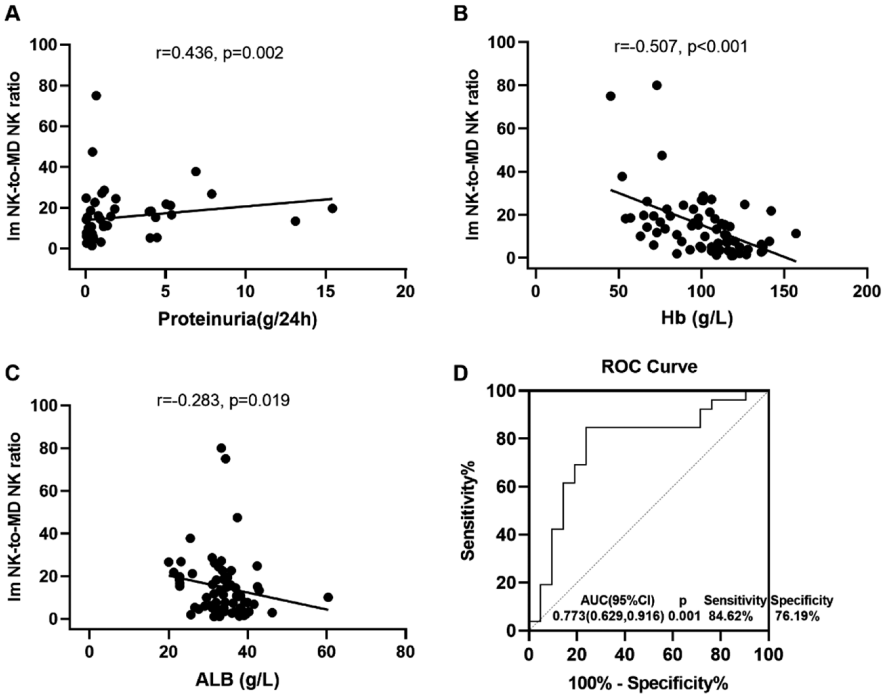

Fig. 3. Correlations between the Im NK-to-MD NK ratio and clinical features of SLE. Correlations between the Im NK-to-MD NK ratio and (**A**) proteinuria (n=50), (**B**) Hb level (n=67), and (**C**) ALB level (n=68) (all: Spearman's rank correlation test).

D: The ROC curve for an elevated Im NK-to-MD NK ratio in the prediction of LN. The optimal predicted cut-off point was 0.108, and the highest Youden index value was 0.608 for patients with SLE with LN (n=47).

Hb: haemoglobin; ALB: albumin.

Discussion

Our study showed that the absolute numbers of total NK cells and their subpopulations were reduced in patients with SLE. Among NK cells, the percentage of Im NK cells was elevated, whereas that of Ma NK cells (comprising both LD and MD NK cells) was reduced. Our results showed that the peripheral Im NK-to-Ma NK ratio was higher in patients with SLE than in HCs, which is in line with previous findings (14). However, the Im NK-to-Ma NK ratio was not significantly increased in high-activity SLE compared to low-activity SLE. Furthermore, we found that the frequency of MD NK cells gradually decreased from the HC group to the low-activity SLE group to the high-activity SLE group. In contrast, LD NK cell numbers showed no significant difference among the three groups. These results suggest that the reduction in Ma NK cells mainly resulted from the loss of MD NK cells; the higher the SLE disease activity, the higher the loss of MD NK cells. Based on these findings, we evaluated the correlations between the Im NK-to-MD NK ratio, disease activity, and clinical features of SLE.

According to previous studies, Im NK cells and MD NK cells play different roles in the pathogenesis of SLE (5, 18, 20-27, 33). In the present study, we found that the Im NK-to-MD NK ratio in circulation was increased in SLE and correlated with disease activity and LN, suggesting that the loss of MD NK may be responsible for aggravating the disease. Type I IFN signalling through STAT1 induces early NK cell activation (34). Huang *et al*. showed that plasmacytoid dendritic cell-secreted IFN-α stimulated circulating NK cells to undergo activation-induced apoptosis, reducing circulating NK cells in patients with active SLE (35). Ma NK cells, but not Im NK cells, are likely to suffer from apoptosis when undergoing oxidative stress (36). Furthermore, MD NK cells exhibit higher oxidative stress levels. They are more susceptible to apoptosis than LD NK cells, which may explain the more robust reduction in MD NK cells in patients with SLE (22). However, the underlying mechanism remains largely unknown. Therefore, identifying the factors that cause the accelerated loss of MD NK cells will be helpful for the development of treatments to control the progression of the disease. Together, our data showed that the Im NK-to-MD NK ratio is a valuable marker for monitoring SLE disease activity and the development of LN.

Various factors, including increased expression of NKG2D ligands, chemoattractant chemokines (*e.g.* CX3CL1), and pro-inflammatory cytokines (*e.g.* TNF-α) in kidney tissues, potentially promote Ma NK cell migration (37, 38). Furthermore, circulating Im NK cells may have the capacity to home to tissues, increasing the number of tissue-resident NK cells (39). Single-cell RNA sequencing identified two clusters of tissue-resident infiltrating NK cells, Im and Ma NK cells, which showed the potential to promote inflammatory activities and exacerbate kidney damage in patients with SLE (28, 40). We found that the Im NK-to-MD NK ratio in the circulation was correlated with SLE clinical features; it was associated positively with proteinuria but negatively with Hb and ALB. At a cut-off value of 0.108, the Im NK-to-MD NK ratio showed a sensitivity of 84.62% and a specificity of 76.19% in distinguishing patients with LN. Considering that drug treatment may significantly influence our result, we used the forward bivariate logistic regression model and excluded the confounding factors, such as patients with

SLE recruited from different hospitals and taking various drugs. Thus, our result showed that the changes in NK cell subpopulations were mainly related to lupus itself rather than the drugs. Consistent with our point of view, a recent study showed that both percentages and absolute numbers of circulating total NK cells, especially those of MD NK cells, of treatment-naïve SLE patients significantly decreased (22). These findings may suggest that SLE triggers the migration of peripheral blood NK cells, particularly MD NK cells, to inflamed locations, further promoting immune injury. Our data indicate that the Im NK-to-MD NK ratio can serve as a helpful marker for monitoring the development of LN.

This study had some limitations. The data were obtained from two centres. The study had a cross-sectional design and a small sample size, limiting our ability to judge the causal relationship between the Im NK-to-MD NK ratio and SLE disease activity. In addition, we measured NK cells and their subsets only at a single time point; thus, the results may not reflect relationships over time. Therefore, further study is required to survey the relationships between the Im NK-to-MD NK ratio and clinical features of other autoimmune diseases.

Our study indicated that the Im NK-to-MD NK ratio might serve as a predictive biomarker of disease activity in patients with SLE. We found that an elevated Im NK-to-MD NK ratio was correlated with increased SLE disease activity, SLEDAI-2K score, anti-dsDNA level, and serum C3 and C4 consumption; moreover, an elevated ratio was associated with SLE clinical features, including increased proteinuria and decreased circulating Hb and ALB levels, and may serve as a predictive biomarker to assess disease activity and renal involvement in patients with SLE. Further work will be needed for an in-depth understanding of the changes in the NK cell frequency and subpopulations and their pathogenic roles in SLE.

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