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ORIGINAL ARTICLE

Activation status of CD56^{dim} natural killer cells is associated with disease activity of patients with systemic lupus erythematosus

Manman Liu[1,2] · Junli Liu[3] · Xiaoyan Zhang[1] · Yichuan Xiao[3] · Gengru Jiang[1] · Xinfang Huang[1,4]

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Abstract

Objectives Decreased natural killer (NK) cells have been reported in systemic lupus erythematosus (SLE) patients. However, the role of NK cells in the pathogenesis of SLE is not well understood. In this study, we aimed to characterize NK cell subsets, phenotypes, and cytokine-secreting functions and investigate the clinical relevance of NK cells in SLE patients.

Methods Peripheral blood samples from 81 SLE patients and 59 healthy donors (HDs) were collected. The frequency and phenotype of NK cells were measured by flow cytometry. Intracellular interferon-γ (IFN-γ) production by NK cells was evaluated by flow cytometry after stimulation with interleukin-12 (IL-12) and IL-18.

Results The percentages of NK cells in the peripheral blood of SLE patients were significantly lower than those in HDs, and the percentages of CD56^{dim} NK cells among total NK cells showed a trend toward decrease. The CD56^{dim} NK cells in SLE patients showed increased production of IFN-γ and displayed relatively activated phenotypic characteristics, including significant increases in NKp44, NKp46, and CD69 and decreased expression of CD16 and CD158a/h/g. Furthermore, CD56^{dim} NK cells in active SLE patients had higher percentages of NKp44^{+} cells and lower percentages of CD158a/h/g^{+} cells than those in inactive SLE patients. The percentages of CD158a/h/g^{+} cells among CD56^{dim} NK cells were negatively correlated with the systemic lupus erythematosus disease activity index (SLEDAI) and positively correlated with C3 and C4 levels.

Conclusion CD56^{dim} NK cells in SLE patients show a reduced proportion tendency among total NK cells and are activated, which partially reflects the disease activity. CD158a/h/g expression on CD56^{dim} NK cells may be considered an index of disease activity.

Key Points
- *In patients with SLE, the proportion of CD56^{dim} NK cells showed a decreased trend and CD56^{dim} NK cells were phenotypically activated which partially reflects the disease activity.*
- *CD158a/h/g expression on CD56^{dim} NK cells were decreased which may be used as an indicator for evaluating disease activity in SLE patients.*

Keywords CD158a/h/g · CD56^{dim} natural killer cell · Disease activity · Interferon-γ · Phenotype · Systemic lupus erythematosus

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✉ Xinfang Huang
huangxinfang@xinhuamed.com.cn; hxf343@126.com

[1] Department of Renal and Rheumatology, Xin Hua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China

[2] Department of Rheumatology and Immunology, Nanjing Jiangning Hospital, The Affiliated Jiangning Hospital of Nanjing Medical University, Nanjing 211100, China

[3] The Key Laboratory of Stem Cell Biology, Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, University of Chinese Academy of Sciences, Shanghai 200031, China

[4] Department of Rheumatology, Shanghai East Hospital, Tongji University, School of Medicine, Shanghai 200120, China

Introduction

Natural killer (NK) cells constitute approximately 5–15% of the lymphocytes in human peripheral blood. NK cells were first named for their capacity to mediate spontaneous "natural" cytotoxicity toward certain tumor- and virus-infected cells. NK cells are also rapid producers of interferon-γ (IFN-γ), as well as TNF-α, GM-CSF, and other cytokines and chemokines [1]. NK cells traditionally belong to the innate immunity system, but it is now accepted that these cells shape and influence adaptive immunity and participate in autoimmune diseases [2, 3]. Two major subsets of NK cells are well established in human subjects: $CD56^{dim}$ and $CD56^{bright}$ cells. $CD56^{dim}$ NK cells are fully mature, predominant in the peripheral blood, and primarily mediate cytotoxic responses. In contrast, $CD56^{bright}$ NK cells are more immature, predominant in the tissues, and secondary lymphoid organs and have been considered primarily as cytokine producers that play a limited role in cytolytic responses [1]. Although $CD56^{bright}$ NK cells are more efficient at producing cytokines overall, $CD56^{dim}$ NK cells can also contribute significantly to early cytokine production because these cells are a significantly greater fraction of the total NK cell pool and can more rapidly secrete cytokines [4]. NK cells can produce a variety of inflammatory cytokines in response to activating receptor stimulation as well as inflammatory cytokine-induced activation signaling [5]. The combination of interleukin-12 (IL-12) and IL-18 is the best combination for inducing strong IFN-γ production [6].

NK cell activation and function are controlled by several germline-encoded membrane receptors which are generally divided into inhibitory and activating receptors. Activating receptors include FcγRIIIA (CD16), activating forms of killer cell immunoglobulin-like receptors (KIRs, KIR2DS and KIR3DS), 2B4, NKG2D, and the natural cytotoxicity receptors (NCRs) NKp30, NKp44, and NKp46. Inhibitory receptors comprise NKG2A and KIRs, such as KIR2DL1 (CD158a) [7]. $CD56^{dim}$ NK cells bear the low affinity Fc-γ-receptor CD16, which plays a prominent role in activating $CD56^{dim}$ NK cells [8]. Following stimulation, CD16 is downregulated by shedding and intracellular uptake [9]. Under certain activation conditions, NK cells are activated and express TRAIL, which interacts with TNF-related apoptosis-inducing ligand-receptor (TRAIL-R) to induce apoptosis of target cells [5].

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of autoantibodies. Increasing evidence indicates that NK cells are involved in the pathogenesis of SLE. Several studies showed that SLE patients had decreased NK cell numbers and proportions in the peripheral blood, impaired NK cell cytotoxic functions, impaired NK cell differentiation, altered phenotypes, and altered NK cell cytokine production [10–17]. In addition, studies conducted in a model of lupus also revealed the contribution of NK cells to lupus pathogenesis [18, 19]. Although it is not known whether these abnormalities are a cause or consequence of the pathological process underlying SLE, NK cells clearly contribute to the pathology. However, the relationship of NK cells with the clinical parameters of SLE has not been extensively studied. In addition, we recently discovered that blood IFNG expression levels correlate positively with anti-double-stranded DNA (ds-DNA) autoantibody levels and SLE activity [20]. Additionally, a study by Huang et al. also reported that NK cells participate in the pathogenesis and development of SLE by activation and production of IFN-γ in an amyloid-induced experimental lupus-like syndrome model [19]. Therefore, in this study, we investigated the phenotypes and IFN-γ-secreting function of NK cells and their relationship with the clinical parameters of the disease to provide comprehensive insights into the changes in NK cells in SLE patients.

Materials and methods

Patients and healthy donors

A total of 81 patients with SLE (93.8% female, median age: 35 (10–70) years) and 59 healthy donors (HDs) (79.7% female, median age: 33 (23–67) years) were included in this study. All SLE patients were recruited from the inpatient department of Xin Hua Hospital affiliated to Shanghai Jiao Tong University School of Medicine and met the 1982 American College of Rheumatology revised criteria for the classification of SLE [21]. No other exclusion criteria were applied. Clinical and laboratory data were documented at the time of inclusion. Disease activity was assessed using the SLEDAI 2000, and a score ≥ 5 was considered active SLE [22, 23]. Of the 81 patients with SLE, six were sampled twice during the course of induction therapy. HDs were selected from the Department of Medical Examination Center and did not have a history of autoimmune diseases, infectious diseases, malignancies, or immunosuppressive therapy. The study was approved by the ethics committee of Xin Hua Hospital and was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants. The clinical and laboratory characteristics of the patients and HDs are summarized in Table 1.

Isolation of peripheral blood mononuclear cells and flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation using the lymphoprep premium kit (StemCell Technologies). The cells were stained with combinations of the appropriate antibodies (Abs) against different cell surface markers for 30 min on ice. Stained cells were analyzed on a four-laser flow cytometer (Gallios, Beckman Coulter), and the data were processed using FlowJo version 10.1 software (TreeStar). The Abs used in this study included anti-CD3 FITC (Clone: OKT3), anti-CD56 PE-Cyanine5.5 (CMSSB), anti-CD16 APC (eBioCB16, CB16), anti-NKp30 PE-Cyanine7

Table 1 Baseline demographics of SLE patients and healthy donors

Characteristics	HDs ($n = 59$)	SLE patients ($n = 81$)	
		Inactive SLE ($n = 32$)	Active SLE ($n = 49$)
Demographic data			
Female sex, n (%)	47 (79.7%)	31 (96.9%)	45 (91.8%)
Age, years, median (range)	30 (23–67)	26 (11–70)	39 (10–69)
Disease duration, years, median (range)		2.5 (0–20)	2 (0.04–23)
Naïve SLE [a], n (%)		0	9 (18.4%)
SLEDAI, median (range)		2 (0–4)	8 (5–18)
Features of SLE, present, n (%)			
Neuropsychiatric		0	0
Arthritis		0	5 (10.2%)
Nephritis		17 (53.1%)	33 (67.3%)
Proteinuria (> 0.5 g/24 h)		2 (6.3%)	29 (59.2%)
Pericarditis		1 (3.1%)	15 (30.6%)
Pleuritis		2 (6.3%)	17 (24.7%)
Rash		0	16 (32.7%)
Oral ulceration		1 (3.1%)	8 (16.3%)
Positive anti-dsDNA		9 (28.1%)	45 (91.8%)
Hypocomplementemia		16 (50%)	43 (87.8)
Leukopenia		0	12 (24.5%)
Thrombocytopenia		1 (3.1%)	7 (14.3%)
Positive ANA		26 (81.3%)	41 (83.7%)
Positive APL		2 (6.3%)	1 (2.0%)
Medical therapy, n		32	40
Prednisone [b]		32	40
≤ 30 mg/day,		27	15
> 30 mg/day,		5	25
HCQ		25	20
Immunosuppressants (IS) [c]		19	14
MMF or CsA		7	8
CYC		4	2

Values are expressed as the median (range) or number (percentage)*SLEDAI* systemic lupus erythematosus disease activity index, *dsDNA* double-stranded DNA, *ANA* antinuclear antibodies, *APL* antiphospholipid antibodies, *HCQ* hydroxychloroquine, *MMF* mycophenolate mofetil, *CsA* cyclosporin, *CYC* cyclophosphamide

[a] Naïve SLE patients are newly diagnosed SLE patients who were not taking glucocorticoids, HCQ or IS

[b] Dose of glucocorticoid per day at the time of blood drawn (calculated as prednisone equivalents)

[c] Patient receiving immunosuppressants represents the one who received CYC, MMF, CsA, methotrexate (MTX), azathioprine (AZA) or leflunomide (LEF) within 1month

(AF29-4D12), anti-NKp44 APC (44.189), anti-NKp46 eFluor 450 (9E2), anti-CD158a/h/g eFluor 450 (HP-MA4), anti-TRAIL PE (RIK-2), anti-CD69 eFluor 450 (FN50), and anti-IFN-γ PE (4S.B3) (eBioscience).

Intracellular IFN-γ detection

Freshly isolated PBMCs were cultured in RPMI 1640 containing 10% FBS in the presence of IL-12 (20 ng/ml) and IL-18 (25 ng/ml) (R&D Systems) with GolgiPlug (Becton Dickinson) for 4 h. The cells were harvested and stained with anti-CD3 FITC, anti-CD16 APC, and anti-CD56 PE-Cyanine5.5 for 30 min on ice. After fixation and permeabilization with the cytofix/cytoperm kit (Becton Dickinson), the cells were stained with anti-IFN-γ PE for 30 min on ice, washed with permeabilization buffer, and analyzed by flow cytometry.

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 6 software. All group results are expressed as the mean ± SEM. Differences between groups were evaluated by an

unpaired *t* test for continuous parametric variables and by a non-parametric Mann-Whitney U test for skewed-distribution variables. The Wilcoxon signed rank test for matched pairs was used to evaluate SLE patients who had completed two visits. A spearman's test was used to test for correlation. Two-tailed *p*-values below 0.05 were considered statistically significant.

Results

SLE patients showed a trend toward reduced proportion of circulating CD56^{dim} NK cells

The flow cytometry gating strategy for the analysis of NK cells and their subsets are shown in Fig. S1A. The percentages of NK cells in lymphocytes were markedly reduced in SLE patients (mean ± SEM; 6.679 ± 0.6661) compared with those in HDs (mean ± SEM; 14.36 ± 1.206; $p < 0.0001$) (Fig. 1a, left). In addition, though they were not statistically significant, SLE patients showed a tendency of having lower proportion of CD56^{dim} NK cells (mean ± SEM; HD: 95.17 ± 0.6845, SLE: 89.94 ± 1.650; $p = 0.0721$) and higher proportion of CD56^{bright} NK cells (mean ± SEM; HD: 4.876 ± 0.6831, SLE: 10.14 ± 1.660; $p = 0.0702$) among total NK cells compared with HDs (Fig. 1a, middle and right). Therefore, a decrease in the CD56^{dim} subset probably was responsible for the decrease in total NK cell numbers in SLE patients. The percentages of NK cells were lower in both inactive (mean ± SEM; 5.782 ± 1.063; $p < 0.0001$) and active SLE patients (mean ± SEM; 7.315 ± 0.8481; $p < 0.0001$) compared with those in HDs (mean ± SEM; 14.36 ± 1.206) but were similar between inactive and active SLE patients. No difference was observed in CD56^{dim} NK cell proportions and CD56^{bright} NK cell proportions among HDs and inactive and active SLE patients (Fig. 1b). There was a trend toward a decrease in CD56^{bright} NK cell proportions and a trend toward an increase in CD56^{dim} NK cell proportions after treatment or follow-up in six SLE patients, but no significant differences were found (Fig. S1B).

CD56^{dim} NK cells but not CD56^{bright} NK cells were phenotypically activated in active SLE patients

We further analyzed the expression of activating and inhibitory NK cell receptors including NKp30, NKp44, NKp46, and CD158a/h/g on both CD56^{dim} and CD56^{bright} NK cells. The percentages of NKp44^{+} cells among CD56^{dim} NK cells were increased in active SLE patients (mean ± SEM; 1.173 ± 0.2700) compared with those in inactive SLE patients (mean ± SEM; 0.7319 ± 0.3844; $p = 0.0424$) and HDs (mean ± SEM; 0.2917 ± 0.04276; $p = 0.0005$) (Fig. 2a). Additionally, CD56^{dim} NK cells from inactive SLE patients (mean ± SEM; 59.38 ± 4.414) had a significantly higher proportion of NKp46^{+} cells than those from HDs (mean ± SEM; 43.28 ± 4.140; $p = 0.0190$) (Fig. 2a). The expression of NKp30 on CD56^{dim} NK cells was not different between SLE patients and HDs (data not shown). A lower frequency of CD158a/h/g^{+} cells among CD56^{dim} NK cells was found in active SLE patients (mean ± SEM; 10.99 ± 1.489) compared with those in inactive SLE patients (mean ± SEM; 20.27 ± 3.852; $p = 0.0413$) and HDs (mean ± SEM; 20.45 ± 2.915; $p = 0.0082$)

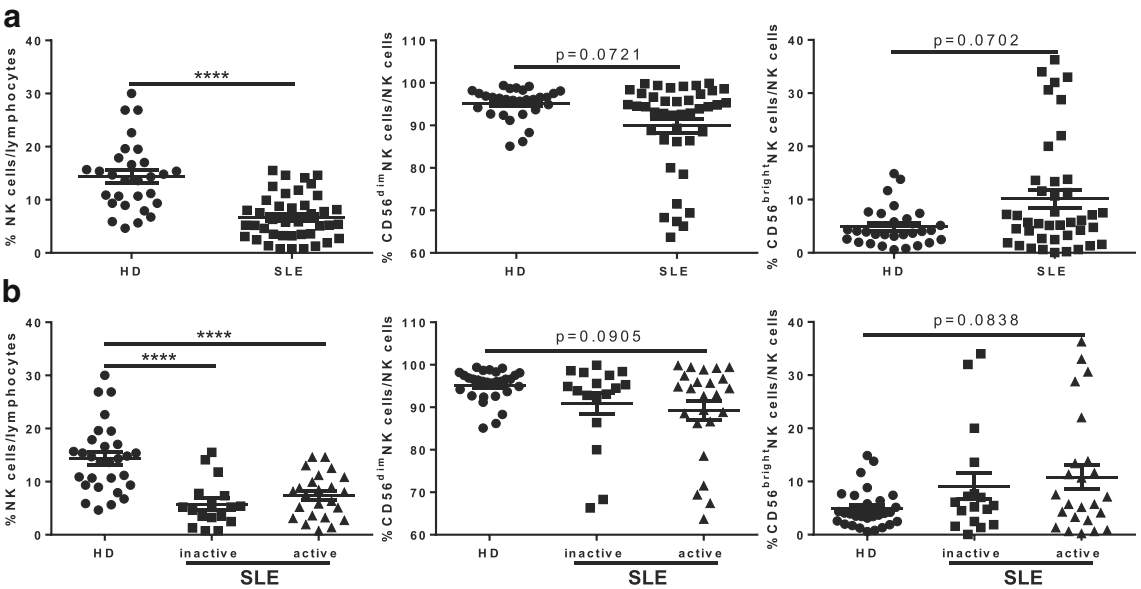

Fig. 1 Frequencies of NK cells and NK cell subsets in the peripheral blood samples of SLE patients. Freshly isolated peripheral blood mononuclear cells (PBMCs) were stained with antibodies against CD3, CD56 and CD16 and analyzed by flow cytometry. (**a**) Percentages of total NK cells (left) or the proportions of CD56^{dim} (middle) and CD56^{bright} NK cells (right) in healthy donors (HDs) ($n = 29$) and SLE patients ($n = 41$). (**b**) SLE patients were categorized into two groups, inactive and active patients, according to disease activity. ****$p < 0.0001$, by Mann–Whitney U-test.

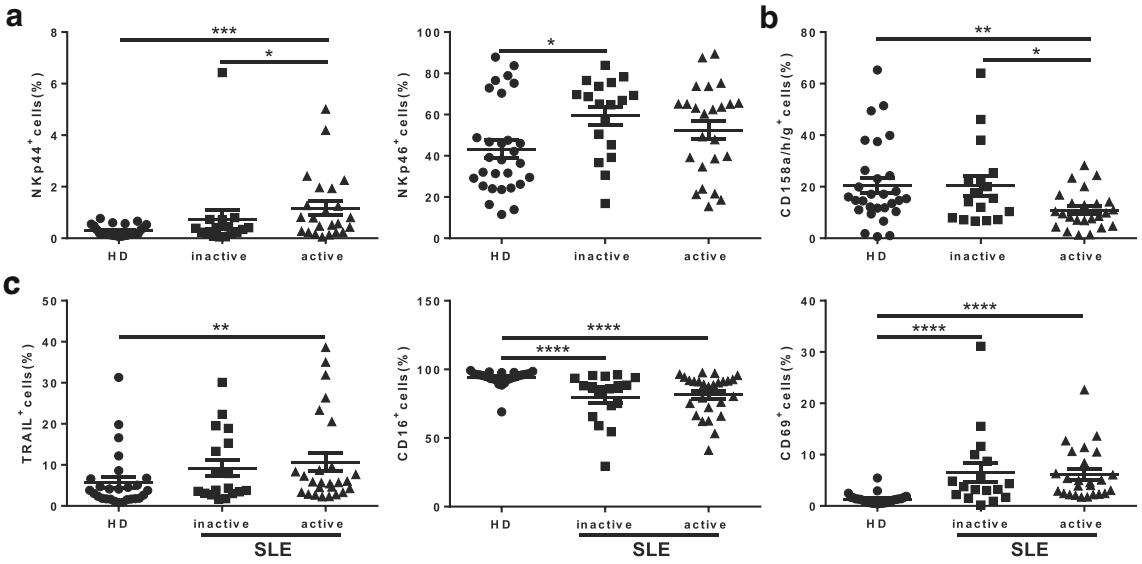

Fig. 2 Relatively activated phenotype of CD56^{dim} NK cells in active SLE patients. Freshly isolated PBMCs were stained with antibodies against CD3, CD56 and the natural killer receptors and analyzed by flow cytometry. (**a**–**b**) Frequencies of CD56^{dim} NK cells expressing NKp44, NKp46, CD158a/h/g, TRAIL, CD16, and CD69 in SLE patients ($n = 40 \sim 45$) grouped by disease activity and HDs ($n = 26 \sim 30$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, by Mann–Whitney U test

(Fig. 2b). We also analyzed the expression of TRAIL, CD16, and CD69, which are all signs of NK cell activation. CD56^{dim} NK cells from active SLE patients had higher percentages of TRAIL^{+} cells (mean ± SEM; 10.70 ± 2.161) compared with those from HDs (mean ± SEM; 5.742 ± 1.333; $p = 0.0059$) (Fig. 2c left). The activation of NK cells via CD16 leads to the downregulation of surface CD16. The proportions of CD16^{+} cells among CD56^{dim} NK cells were significantly lower in inactive (mean ± SEM; 79.86 ± 4.186; $p < 0.0001$) and active SLE patients (mean ± SEM; 81.50 ± 2.966; $p < 0.0001$) compared with those in HDs (mean ± SEM; 93.92 ± 1.090) (Fig. 2c middle). Additionally, CD69 is an early cell activation marker, and we found that CD56^{dim} NK cells from inactive (mean ± SEM; 6.551 ± 1.835; $p < 0.0001$) and active SLE patients (mean ± SEM; 6.108 ± 1.006; p < 0.0001) more frequently expressed CD69 compared with those from HDs (mean ± SEM; 1.324 ± 0.1989) (Fig. 2c right). Intriguingly, the expression of NKp30, NKp44, NKp46, CD158a/h/g, TRAIL, and CD16 on CD56^{bright} NK cells did not differ between SLE patients and HDs, except CD69, which was increased in SLE patients (data not shown). Together, CD56^{dim} NK cells but not CD56^{bright} NK cells were phenotypically activated in SLE patients, especially in active patients.

Increased IFN-γ production by CD56^{dim} but not CD56^{bright} NK cells in SLE patients

IFN-γ secretion was one of the main functions of activated NK cells. We examined the IFN-γ production of spontaneous and IL-12/18-stimulated activated NK cells. The median fluorescence intensity (MFI) of IFN-γ in both spontaneous and stimulated CD56^{dim} NK cells was higher in inactive (mean ± SEM; 664.9 ± 38.08, 939.1 ± 107.2; $p = 0.0023$, $p = 0.0029$, respectively) and active SLE patients (mean ± SEM; 657.8 ± 38.60, 822.3 ± 54.62; $p = 0.0058$, $p = 0.0047$, respectively) compared with those in HDs (mean ± SEM; 534.3 ± 16.95, 646.3 ± 26.17, respectively) (Fig. 3a). As shown in Fig. 3b, SLE patients with hypocomplementemia displayed significantly higher IFN-γ expression in both spontaneous (mean ± SEM; 675.5 ± 33.38; $p = 0.0004$) and stimulated CD56^{dim} NK cells (mean ± SEM; 925.0 ± 65.84; $p < 0.0001$) compared with those from HDs (mean ± SEM; 534.3 ± 16.95, 646.3 ± 26.17, respectively) and slightly increased IFN-γ expression in stimulated CD56^{dim} NK cells compared with those from SLE patients without hypocomplementemia (mean ± SEM; 699.2 ± 66.77, $p = 0.0507$, not statistically significant). Anti-dsDNA did not affect CD56^{dim} NK cell secretion of IFN-γ protein (data not shown). For CD56^{bright} NK cells, there were no significant differences in both spontaneous and stimulated IFN-γ production between cells from SLE patients and HDs (data not shown). Overall, these data demonstrated that the numerically decreased CD56^{dim} NK cells but not CD56^{bright} NK cells in SLE patients were activated and secreted IFN-γ. Thus, the subsequent analysis focused on CD56^{dim} NK cells.

Decreased expression of CD158a/h/g on CD56^{dim} NK cells was associated with disease activity and low complement levels

We observed a negative correlation between the SLEDAI and the percentages of CD158a/h/g^{+} cells among CD56^{dim} NK

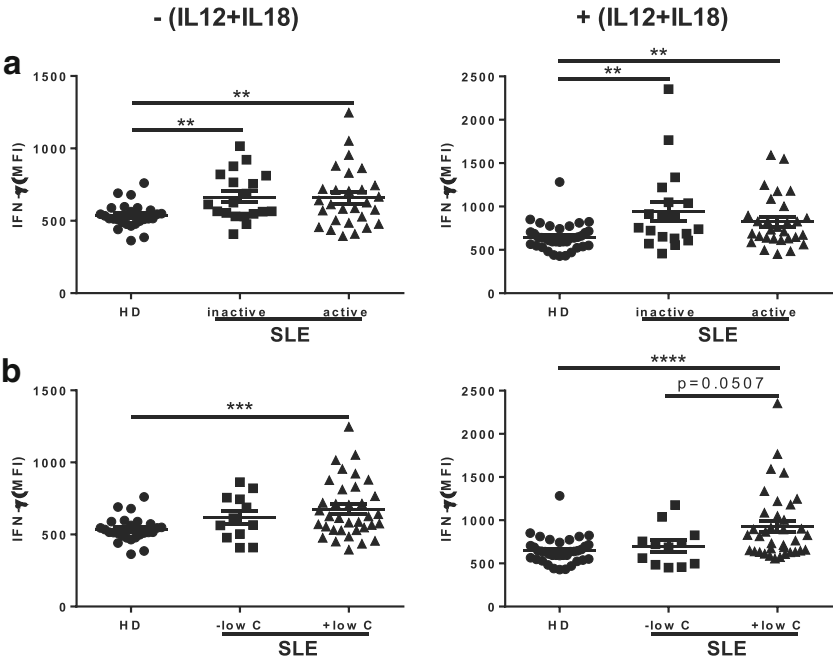

Fig. 3 Increased IFN-γ production by CD56^{dim} NK cells in SLE patients. PBMCs were cultured with or without IL-12 (20 ng/ml) and IL-18 (25 ng/ml) in the presence of GolgiPlug for 4 h, stained with antibodies against CD3, CD56, CD16 and IFN-γ, and analyzed by flow cytometry. Median fluorescence intensity (MFI) of IFN-γ in CD56^{dim} NK cells without (left) and with (right) IL-12/IL-18 stimulation from healthy donors (n = 26 ~ 36) and SLE patients (n = 47 ~ 48) grouped by disease activity **(a)** and complement levels **(b)**, respectively. ** $p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, by Mann–Whitney U test.

cells (r = −0.4779, $p = 0.0016$) (Fig. 4a). The percentages of CD158a/h/g^{+} cells among CD56^{dim} NK cells were lower in SLE patients with hypocomplementemia (mean ± SEM; 12.64 ± 1.604; $p = 0.0269$) compared with those in HDs (mean ± SEM; 20.45 ± 2.915), but no such difference was observed compared with those in SLE patients without hypocomplementemia (mean ± SEM; 20.83 ± 5.511) (Fig. 4b). Serum C3 levels (r = 0.4533, $p = 0.0038$), as well as C4 levels (r = 0.4110, $p = 0.0115$), were positively correlated with the percentages of CD158a/h/g^{+} cells among CD56^{dim} NK cells (Fig. 4c). Anti-dsDNA antibody levels did not impact the CD158a/h/g expression of CD56^{dim}NK cells (data not shown). There was no relationship between the lupus activation marker, SLEDAI, anti-dsDNA antibody, or complements

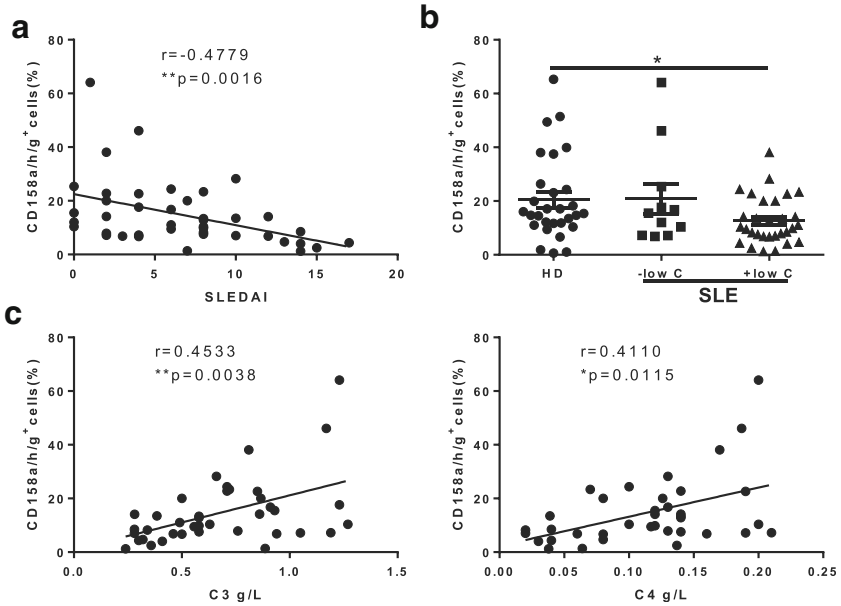

Fig. 4 The expression of CD158a/h/g on CD56^{dim} NK cells was negatively correlated with disease activity and positively correlated with complement levels. (**a**) Correlations between systemic lupus erythematosus disease activity index (SLEDAI) scores and percentages of CD158a/h/g^{+} cells among CD56^{dim} NK cells (n = 41). (**b**) Percentages of CD158a/h/g^{+} cells among CD56^{dim} NK cells from healthy donors (n = 29) and SLE patients without (n = 11) or with (n = 30) decreased complement levels (Mann–Whitney U-test). (**c**) Correlations between complement 3 (left, n = 39) or complements 4 (right, n = 37) levels and the percentages of CD158a/h/g^{+} cells among CD56^{dim} NK cells. Symbols represent individual subjects. *$p < 0.05$, ** $p < 0.01$, correlations were analyzed using Spearman's correlation analysis

and other receptors or activation markers of CD56^{dim} NK cells, including NKp44, NKp46, TRAIL, CD16, and CD69 (data not shown).

CD56^{dim} NK cell phenotype and IFN-γ secretion in relation to immunosuppressive treatment

The changes in CD56^{dim} NK cells may be affected by medication, and so we compared the phenotype and IFN-γ secretion of CD56^{dim} NK cells after subgrouping according to different treatments, including glucocorticoids (GCs), hydroxychloroquine (HCQ), and immunosuppressants (IS). Lower frequencies of CD16^{+} CD56^{dim} NK cells were found in the high-dose group (GCs > 30 mg/d) (mean ± SEM; 73.38 ± 4.620) compared with those in the naive group (mean ± SEM; 86.98 ± 6.828; $p = 0.0167$) and the low to moderate dose group (GCs ≤ 30 mg/d) (mean ± SEM; 83.22 ± 2.970; $p = 0.0344$), as well as in patients taking HCQ (mean ± SEM; 76.68 ± 3.569) compared with those in patients that were not taking HCQ (mean ± SEM; 85.81 ± 2.888; $p = 0.0314$) (Fig. 5a). In addition, patients taking IS (mean ± SEM; 21.41 ± 4.048) had a higher frequency of CD158a/h/g^{+} cells among CD56^{dim} NK cells compared with those in patients that were not taking IS (mean ± SEM; 11.43 ± 1.777; $p = 0.0040$) (Fig. 5b). Patients taking HCQ had slightly decreased IFN-γ MFI in stimulated CD56^{dim} NK cells compared with those from patients that were not taking HCQ (Fig. S2, $p = 0.0668$, not statistically significant). NKp44, NKp46, TRAIL, and CD69 expression on CD56^{dim} NK cells did not change among the treatments (data not shown).

Discussion

Consistent with almost all previous studies, we confirmed that NK cell percentages were lower in SLE patients than in HDs, which might be a common phenomenon in SLE. Furthermore, we showed a downward trend in the proportion of CD56^{dim} NK cells among total NK cells in SLE patients, but it was not statistically significant, which may be related to small sample size. Interestingly, the proportion of CD56^{dim} NK cells in SLE patients was significantly decreased when direct comparison using the unpaired *t* test was performed ($p = 0.0129$). There are several possible mechanisms that might explain the reduction in peripheral blood total NK cells and CD56^{dim} NK cells, including activation-induced cell death, impaired differentiation, cell apoptosis induced by reactive oxygen species (ROS), and the use of medication [9]. The follow-up during induction therapy suggested a trend toward an intraindividual increase in the proportion of CD56^{dim} NK cells over time. Our data are partially consistent with a previous observation that showed higher proportions of CD56^{bright} NK cells in SLE patients compared with those in HDs [24], but our results were slightly different from two previous reports that showed that the distribution of NK cells into the CD56^{bright} and CD56^{dim} NK subsets was not significantly different between SLE patients and HDs [13, 25]. Together, our results support the possibly inverse correlation between NK cells, specifically CD56^{dim} NK cells, and the disease course. High expression of the early cell activation marker CD69 on CD56^{dim} NK cells from SLE patients revealed that those cells were strongly activated, which was consistent with most previous studies. It has been shown that activation of CD56^{dim} NK cells results in the downmodulation of CD16 by shedding or internalization [9, 26]. We showed that expression of CD16 on CD56^{dim} NK cells was strongly reduced in SLE patients, which was also found in Huang et al. [12], indicating the activation state of CD56^{dim} NK cells. NKp44 is expressed by IL-2-activated NK cells but not by resting NK cells. We found increased proportions of NKp44^{+} CD56^{dim} NK cells in active SLE patients compared with those in inactive SLE patients and HDs, which indicated that CD56^{dim} NK cells are activated in active SLE patients. TRAIL is another marker of NK cell activation, and the increased expression of TRAIL on CD56^{dim} NK cells indicated the activation of these cells in SLE patients. The increase in activating receptors and the decrease in inhibitory

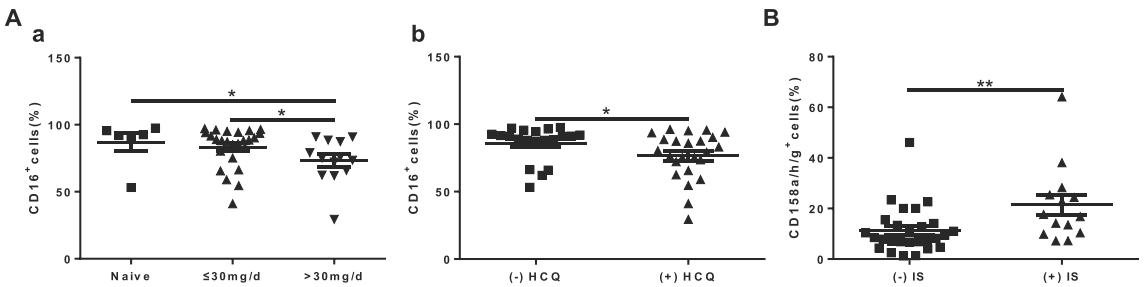

Fig. 5 CD16 and CD158a/h/g expression on CD56^{dim} NK cells in SLE patients in relation to immunosuppressive treatment. (**a**) We divided the SLE patients receiving glucocorticoids into two groups according to their prednisone dose: a low to moderate dose (≤ 30 mg/day) group and a high dose (> 30 mg/day) group. Percentages of CD16^{+} cells among CD56^{dim} NK cells from naive patients ($n = 5$), the low to moderate dose ($n = 25$) and high-dose groups ($n = 13$). (**b**) Percentages of CD16^{+} cells among CD56^{dim} NK cells from SLE patients who were not taking ($n = 20$) and who were taking hydroxychloroquine (HCQ) ($n = 24$). (**c**) Percentages of CD158a/h/g^{+} cells among CD56^{dim} NK cells from SLE patients who were not taking ($n = 27$) and who were taking immunosuppressants (IS) ($n = 14$). *$p < 0.05$, ** $p < 0.01$, by Mann-Whitney U test

receptors might affect the immune response in patients with SLE. However, the phenotypic analyses of NK cells in SLE patients in our study have yielded some different results with others. For instance, we reported increased NKp46 expression, which was found in Lin et al. [16], but not in Hervier et al. [11]. Above differences might be reasonably caused by the different techniques used, patients' selection, eventual pretreatments, and size of the population analyzed. But overall, results of previous research and our work indicate that $CD56^{dim}$ NK cells in SLE patients showed relatively activated phenotypic characteristics. Taken together, our data suggest that $CD56^{dim}$ NK cells are mainly responsible for the decrease in total NK cells, and $CD56^{dim}$ NK cells from SLE patients, especially those from active SLE patients, are significantly activated. What is noteworthy is that almost all previous studies confirmed impaired cytotoxicity of circulating $CD56^{dim}$ NK cells in SLE patients. A possible explanation for the discrepancy between activated phenotype and reduced cytotoxic ability of $CD56^{dim}$ NK cells in SLE patients was that NK cells were activated to promote autoimmune response following induction of autoimmunity before they were suppressed and degenerated [27, 28]. In addition, dysfunctional KIR expression and NK education might also partially explain the impaired cytotoxicity of NK cells [29].

Another interesting finding was that the expression of CD158a/g/h on $CD56^{dim}$ NK cells was decreased in active SLE patients. Correlation analysis showed that the percentages of CD158a/h/g^+ cells among $CD56^{dim}$ NK cells correlated inversely with disease activity and positively with serum C3 and C4 levels. KIR2DL1 (CD158a) polymorphisms have been identified as a risk factor for SLE [30]. The monoclonal antibody we used recognizes several CD158 proteins, also known as KIR2D, specifically KIR2DL1 (CD158a), KIR2DS1 (CD158h), KIR2DS3, and KIR2DS5 (CD158g). Thus, we were unable to accurately know which one or which specific receptors are involved. Bai et al. found that the expression levels of the KIR2DL1 were decreased, while the expression levels of the KIR2DS1 were increased in NK cells from SLE patients [31]. Hou et al. found a higher frequency of KIR2DS1 gene in patients with SLE [32]. Another study also showed decreased proportions of KIR2DL1/KIR2DS1$^+$ NK cells in SLE patients [11]. Therefore, according to our results, we hypothesize that it is most likely that CD158a expression in $CD56^{dim}$ NK cells is reduced in SLE patients, which is consistent with the relatively activated phenotype of the $CD56^{dim}$ NK cells. Some studies conducted on the association of KIR with SLE suggested that individuals with more stimulatory receptors or fewer inhibitory receptors could be more susceptible to developing lupus [33]. The absence of inhibitory CD158a could potentially lower the threshold for activating receptor-mediated $CD56^{dim}$ NK cell activation, thereby contributing to the pathogenesis of SLE. Taken together, we showed that CD158a/h/g may be used as a clinical activity index for SLE and may be relevant to disease progression.

It has been reported that activated type II interferon signaling pathway might play a role in the pathogenesis of SLE by our and other group [34]. Although $CD56^{bright}$ NK cells are more efficient at producing cytokines overall, $CD56^{dim}$ NK cells can also contribute significantly to early cytokine production when activated, which was confirmed by our results (data not shown). Our data revealed that the expression levels of IFN-γ in spontaneous and stimulated $CD56^{dim}$ NK cells were higher both from active and inactive SLE patients compared with those of cells from HDs, which was not found in $CD56^{bright}$ NK cells, suggesting that $CD56^{dim}$ NK cells in SLE patients were already activated in vivo and were able to respond to further stimulation in vitro. Our data are partially consistent with previous observations by Hervier et al. that showed a higher proportion of IFN-γ^+ NK cells in patients with active SLE than in inactive SLE and controls after IL-12 and IL-18 stimulation, but the proportion of IFN-γ^+ NK cells in inactive SLE patients was equivalent to that in controls [11]. However, our results are different from those of Ye et al., who showed no significant differences in the proportions of both spontaneous and inducible IFN-γ secretion by NK cells from SLE patients and controls [15]. The two studies above did not analyze the secretion of IFN-γ by the two NK subsets. Such differences may be due to the variable disease activity or treatments of patients.

Finally, in addition to the disease and/or its activity, phenotypic changes may be due to medication. A previous study has shown that classic therapies modulate the expression of several NK cell receptors in SLE patients who respond well to drugs [14]. Our analysis based on glucocorticoid treatment revealed that patients taking high doses of glucocorticoids showed lower frequencies of $CD16^+$ $CD56^{dim}$ NK cells compared with those of patients not receiving or receiving low to moderate doses of steroids, which might mean more activated $CD56^{dim}$ NK cells in patients taking high doses of steroids. However, it is important to note that most those patients taking high doses of steroids were on intravenous steroids or even pulse therapy, which meant that they generally had high disease activity. Our data also suggested that the effect of hydroxychloroquine on the expression of CD16 and IFN-γ cannot be excluded. Intriguingly, the frequencies of CD158a/h/g^+ $CD56^{dim}$ NK cells in patients taking IS were significantly higher than those of patients not taking IS, which indicated that IS may restore the expression of CD158a/h/g, thereby inhibiting the activation of $CD56^{dim}$ NK cells. Therefore, we should further explore the possibility that CD158a/h/g may serve as a biomarker to evaluate the therapeutic responses of SLE patients to IS.

Taken together, in SLE patients $CD56^{dim}$ NK cells showed a trend toward reduced proportion, were in activated status, and showed increased IFN-γ production. CD158 expression on $CD56^{dim}$ NK cells may be used as an indicator for evaluating disease activity in SLE patients. Exploring the detailed

mechanisms underlying the alterations in $CD56^{dim}$ NK cells and understanding whether CD158a/h/g expression on $CD56^{dim}$ NK cells drives more pathogenic or protective functions in SLE will shed light on disease pathogenesis and may help to form a basis for new therapeutic strategies.

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Compliance with ethical standards

Disclosures None.

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