Increased Oxidative Stress Contributes to Impaired CD56^{dim}CD57⁺ NK Cells in Patients With Systemic Lupus Erythematosus

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Abstract

Background: Systemic lupus erythematosus(SLE) is characterized by loss of immune tolerance and imbalance of immune cell subsets. NK cells contribute to regulate both the innate and adaptive immune response. In this study, we aimed to detect peripheral NK cell subsets in SLE patients, investigate their cytotoxic effect on activated CD4⁺ T cells, and explore intrinsic mechanisms contributing to NK cell abnormality.

Methods: Blood samples from healthy controls(HCs) and patients with SLE and rheumatoid arthritis(RA) were collected. The NK count, NK subsets(CD56^{bright}, CD56^{dim}CD57⁻, and CD56^{dim}CD57⁺), phenotypes, along with apoptosis were analysed by flow cytometry. The t-stochastic neighbor embedding (tSNE) analysis of lymphocytes based on immune cells flow cytometry markers was performed. Mitochondrial reactive oxygen species(mtROS) and total ROS levels in NK cells were detected by MitoSOX Red and DCFH-DA staining respectively. Published data(GSE63829 and GSE23695) from Gene Expression Omnibus(GEO) was analyzed by Gene Set Enrichment Analysis(GSEA).

Results: NK count was down-regulated in untreated SLE patients in comparison to untreated RA patients or HCs and the count was negatively correlated with disease activity. SLE patients exhibited a selective reduction in CD56^{dim}CD57⁺ NK cell proportion, which was negatively correlated with Systemic Lupus Erythematosus Disease Activity Index(SLEDAI) and positively correlated with complement C3 and C4. CD56^{dim}CD57⁺ NK cells exhibited increased cytotoxic effect on activated CD4⁺ T cells relative to CD56^{dim}CD57⁻ NK cells. Compared with HCs, CD56^{dim}CD57⁺ NK cells in SLE patients exhibited impaired cytotoxic function, increased apoptosis and higher levels of both mtROS and ROS. GSEA analysis indicated that ROS pathway was significantly enriched in NK cells from lupus. Compared with CD56^{dim}CD57⁻ NK cells in SLE patients, CD56^{dim}CD57⁺ NK cells showed higher levels of oxidative stress and apoptosis. Furthermore, oxidative stress levels were negatively correlated with perforin expression and positively correlated with apoptosis. CD56^{dim}CD57⁺ NK cells were more prone to undergo apoptosis when exposed to ROS in vitro.

Conclusion: Beyond the reduced NK cell number, our study demonstrated a selective loss of mature CD56^{dim}CD57⁺ NK cell subset, which was conversely correlated with disease activity. This subset showed attenuated cytotoxic function, up-regulated endogenous apoptosis and increased oxidative stress, which might contribute to NK cell abnormality.

Background

Systemic lupus erythematosus(SLE) is a multi-organ autoimmune disease driven by aberrant activation of autoreactive T and B cells and the production of autoantibodies^[1]. Patients with SLE are known to exhibit diminished antiviral immunity and heightened risk for the development of severe opportunistic infections, which represents an important cause of mortality^[2].

NK cells are innate lymphoid cells which mediate resistance against tumor cells and microbial pathogens, especially the virus, and also contribute to regulation of the adaptive immune response^[3-4]. NK cells exert cytotoxicity through CD107a degranulation, production of lytic granules(perforin and granzyme B) and CD16 engagement. They also regulate the immune response by cytokine production(IFN- γ and IL-10). In humans, NK cells have long been classified into two well-characterized subsets, known as CD56^{dim} NK cells and CD56^{bright} NK cells. The former, with stronger cytotoxic activity, is more mature and predominant in the peripheral blood. The latter, with more cytokine production, is predominant in tissues^[5]. Recent studies suggest that CD57 expression marks mature human NK cells and CD56^{dim}CD57⁺ NK cell is a newly discovered important subgroup, which is characterized by enhanced lytic granule protein contents, including perforin and granzyme B^[5-7]. Furthermore, transcriptome and protein expression data have displayed gradients in cell activities from CD56^{bright} NK cells to CD56^{dim}CD57⁻ NK cells to CD56^{dim}CD57⁺ NK cells^[7].

NK cells have been shown to play a beneficial role in the regulation of pathogenic autoreactive T cell and B cell responses^[3-4]. NK cell depletion accelerates disease activity in lupus-prone mice, whereas NK cell transfer delays disease onset^[8]. Transcriptome level study suggests NK cell cytotoxicity transcript is negatively correlated with lupus disease activity^[9]. Number reduction and phenotype alterations of NK cells in SLE patients have been described^[10-12], but the results are not consistent. Discrepancy in the above studies may be explained by differences in treatment regimen and NK cell heterogeneity, as total NK cells rather than NK cell subsets were detected in most studies.

We conducted this study with the purpose of detecting the number and subsets of NK cells in peripheral blood from SLE patients and their correlation with disease activity. Furthermore, we investigate their cytotoxic effect on activated CD4⁺ T cells, and explored the intrinsic mechanisms which may contribute to NK cell abnormality in SLE patients.

Methods

Study subjects.

All the samples were obtained from individuals fulfilling the diagnosis of SLE or rheumatoid arthritis(RA) according to the American College of Rheumatology(ACR) criteria^[13-14] in Affiliated Hospital of Nantong University and The Second Hospital of Dalian Medical University(from 2017 to 2020). Patients complicated with tumor, infections or other autoimmune diseases were excluded. Healthy controls(HCs) were chosen from age and gender matched healthy volunteers. Our study was approved by the ethics committee of the Affiliated Hospital of Nantong University and the Dalian Medical University.

Baseline Characteristics.

Related test results of the subjects enrolled were collected through review of medical records, including: age, gender, duration, white blood cell(WBC), hemoglobin(Hb), platelet(PLT), erythrocyte sedimentation

rate(ESR), C-reactive protein(CRP), complement C3 and C4, anti-double-stranded-DNA antibodies(anti-dsDNA antibodies), antinuclear antibodies(ANA), immunoglobulin G(IgG), immunoglobulin M(IgM), immunoglobulin A(IgA), and albumin(ALB). Disease activity was measured using the Systemic Lupus Erythematosus Disease Activity Index(SLEDAI) score for SLE patients^[15]. Lupus nephritis(LN) is defined as proteinuria(more than 0.5 g of protein per day or 3+ on dipstick) or the presence of cellular casts(either red blood cell, haemoglobin, granular, tubular or mixed) in urinary sediment or renal biopsy.

Total NK cell analysis in treatment-naive patients.

We conducted 156 treatment-naive SLE patients and 22 treatment-naive RA patients with no history of glucocorticoids or immunosuppressive drugs. 30 age and gender matched HCs were enrolled. Blood cells were incubated with a panel of monoclonal antibodies(anti-CD3-FITC, anti-CD56-PE)(BD Biosciences). Trucount tubes were used to determine the absolute number. The count and percentage of CD3⁺CD56⁺ NK cells were measured.

NK subset analysis by Flow cytometry.

We conducted 37 SLE patients and 30 HCs matched for age and gender. Blood samples were stained with fluorophores conjugated antibodies for phenotypic detecting. Peripheral blood mononuclear cells(PBMCs) were isolated using Ficoll density gradient centrifugation(400 g, 30 min) for cell stimulation. The NK cell subset gating strategies were depicted in Supplementary Figure 1. All antibodies for staining were shown in Supplementary Table 1. Cell suspensions were surface-labeled with antibodies for 30min. For intracellular staining, cell suspension was firstly incubated with surface antibody, then fixed and permeabilized using the Transcription Factor Fix/Perm Buffer(eBioscience). For IFN- γ and IL-10 expressions, cells were stimulated with phorbol 12-myristate 13-acetate(PMA, 50ng/ml) and ionomycin(1 μ g/ml) for 4 hours in the presence of Brefeldin A(10 μ g/ml). All the cell samples were detected by BD FACSCantoTM II or ACEA NovoCyte and the acquired data were further analyzed with BD FACSDiva software or FlowJo software.

Apoptosis detection.

Apoptosis of NK cell subsets was detected by staining with annexin V-FITC(Invitrogen) and 7-amino-actinomycin D(7-AAD)(BD Biosciences) in 19 SLE patients and 10 HCs .

Measurement of reactive oxygen species(ROS) levels

ROS levels were assessed by incubating cells with 10 μ M 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA)(AAT Bioquest) for 20 minutes at 37°C. Mitochondrial reactive oxygen species(mtROS) levels were assessed by incubating cells with 5 μ M MitoSOX Red probe(invitrogen) for 15 min at 37°C. DCFH-DA and MitoSOX fluorescence intensities in NK cells were determined by flow cytometry as mean fluorescence intensity(MFI).

Isolation of NK cell subsets.

Total NK cells(>92% purity) were negatively isolated by magnet associated cell sorting(MACS) (Biolegend). CD56^{dim}CD57⁺ NK cells and CD56^{dim}CD57⁻ NK cells (>95% purity of each subset) were isolated from fresh PBMC by fluorescence activated cell sorting(FACS) using a FACS Aria II cell sorter(BD).

Detection of CD107a degranulation.

MACS-sorted NK cells were stimulated with PMA(50ng/ml) and ionomycin(1µg/ml) for 6 hours in the presence of Brefeldin A(10µg/ml) and PE-conjugated anti-CD107a in RPMI 1640 medium(10% FBS) at 37°C in a 5% CO₂ atmosphere. CD107a expression of CD56^{dim}CD57⁺ NK cells and CD56^{dim}CD57⁻ NK cells were detected by Flow cytometry.

Cytotoxicity assay(NK cells targeting CD4⁺ T cells).

Purified CD4⁺ T cells (>95% purity)(Biolegend) were labeled with 1 µM carboxyfluorescein diacetate succinimidyl ester(CFSE)(Invitrogen) and rested or activated in RPMI 1640 medium(10% FBS) with human T-activator CD3/CD28 antibody (both 2µg/ml, eBioscience) for 72 hours. FACS-sorted NK cell subsets(CD56^{dim}CD57⁺ NK cells and CD56^{dim}CD57⁻ NK cells) were cocultured with these CD4⁺ T cells at a ratio of 1:1 for 15 hours in RPMI 1640 medium(10% FBS) at 37°C in a 5% CO₂ atmosphere. FACS analysis was performed after 15 hours of coculture. Cytotoxicity was assessed by Fixable Viability Dyes(FVD) eFluor™ 780(eBioscience). CFSE⁺ cells were gated, and FVD 780⁺ cells were measured as the cytotoxicity percentage of CD4⁺ T cells.

In vitro stimulation assays.

PBMC(1×10⁶ cells/ml) were treated with hydrogen peroxide(H₂O₂) at concentrations of 20µM, 40µM and 80µM, respectively, for 24 hour culture in RPMI 1640 medium(10% FBS) at 37°C in a 5% CO₂ atmosphere. H₂O₂ was diluted in PBS and used within minutes of preparation.

T-distributed stochastic neighbour embedding (t-SNE) analysis.

Samples(from four SLE patients with active disease activities and four HCs) were used for t-SNE analysis using R scripts based on the 'flowCore' package and 'Rtsne' package^[16]. In R, all data were transformed using the logicleTransform function to roughly match scaling historically used in FlowJo.

Gene Set Enrichment Analysis(GSEA).

The gene expression datasets were attained from the Gene Expression Omnibus(GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>)(GSE63829 and GSE23695) using R scripts based on the 'clusterProfiler' package^[17]. A normalized enrichment score(NES) were calculated for every gene set(using p value< 0.05 and FDR < 0.25 as the threshold). The gene sets used in the GSEA belong to hallmark gene sets from Molecular Signature v7.2 Database(MSigDB).

Statistical Analysis.

Continuous variables were expressed as mean $\pm$ SD or median(interquartile range, IQR). Categorical variables are expressed as number(percent). The chi-square test or Fisher test was used to compare qualitative values according to distribution. The Student's unpaired or paired t test was performed to compare two groups for parametric data, and the Mann-Whitney U test or Wilcoxon rank sum test was performed for nonparametric data. The Pearson test or Spearman was used to determine the correlation between variables. The statistical analyses were performed using R v.3.6.1 software. And the figures were drawn using GraphPad Software. P values less than 0.05 were considered significant.

Results

1.The number and correlation analyses of peripheral NK cell in treatment-naive SLE patients.

The present study included a total of 156 treatment-naive SLE patients(median age 33, IQR:26-47 years, 90.4% female), 22 treatment-naive RA patients(median age 37, IQR: 32-47 years, 90.9% female) and 30 HCs(median age 32, IQR:27-38 years, 90.0% female). The demographic and clinical manifestations of these patients and HCs were shown in Supplementary Table 2. The percentages and counts of NK cells were remarkably down-regulated in untreated SLE patients compared with that of untreated RA patients or HCs[NK cell percentage: 6.68 $\pm$ 5.17% ,11.75 $\pm$ 8.46% and 12.72 $\pm$ 5.04% respectively; NK cell count:50(26-77)/ μ l, 104(70-215)/ μ l and 266(167-336)/ μ l respectively, Figure 1A, B, all P<0.001], suggesting that NK cell deficiency was associated with the disease itself regardless of treatment.

Correlation analysis showed that in treatment-naive SLE patients, the NK count was positively correlated with complement C3(r=0.193, P=0.017)(Figure 1C) and PLT(r=0.258, P=0.001)(Figure 1D), and negatively correlated with SLEDAI(r= -0.211, P= 0.008)(Figure 1E). Compared to SLE patients without LN[59(33-93)/ μ l, n=82], NK count was lower in SLE patients with LN[35(21-72)/ μ l, n=74](P<0.05)(Figure 1F).

2. SLE patients exhibited alterations in specific subpopulations of NK cells

We described T-SNE map of CD45⁺ lymphocyte cells on four SLE patients with active disease activities and four HCs. T-SNE map visually showed the distribution anomaly of subpopulations^[18] between SLE patients and HCs(Supplementary Figure 2). Compared with HCs, SLE patients exhibited fewer NK cell subsets, especially for CD56^{dim}CD57⁺ NK subgroup(Supplementary Figure 2). To further confirm the above results and assess the relationship between NK subsets and disease activities, three NK cell subpopulations(gating strategy shown in Supplementary Figure1; CD56^{bright} NK cells, CD56^{dim}CD57⁻ NK cells, and CD56^{dim}CD57⁺ NK cells) were studied in 37 SLE patients and 30 HCs. Our study indicated that SLE patients exhibited a selective reduction in CD56^{dim}CD57⁺ NK cells(33.9 $\pm$ 21.5% vs 50.1 $\pm$ 15.8%, P<0.01), but not CD56^{bright} NK cells(6.09 $\pm$ 4.88% vs 3.57 $\pm$ 2.44%) or CD56^{dim}CD57⁻ NK cells(59.8 $\pm$ 20.2% vs 46.1 $\pm$ 15.5%) compared with HCs(Figure2A). The three NK subset proportions in CD45⁺ lymphocytes from SLE patients were decreased(CD56^{dim}CD57⁺ NK cell percentage: 9.01 $\pm$ 6.56% and 3.32 $\pm$ 3.73% for HC and

SLE respectively; CD56^{bright} NK cell percentage: $0.49 \pm 0.21\%$ and $0.39 \pm 0.39\%$ for HC and SLE respectively; CD56^{dim}CD57⁻ NK cell percentage: $7.60 \pm 4.04\%$ and $4.15 \pm 3.38\%$ for HC and SLE respectively), and the two groups of CD56^{dim} NK cells reached the statistically significant differences ($P < 0.01$, Supplementary Figure 3).

Furthermore, correlation analysis showed that CD56^{dim}CD57⁺ NK cell proportion was negatively correlated with SLEDAI ($r = -0.425$, $P = 0.009$) (Figure 2B), positively correlated with both complement C3 ($r = 0.403$, $P = 0.016$) and C4 ($r = 0.518$, $P = 0.001$) (Figure 2C,D). In addition, PLT and anti-dsDNA antibody level were weakly associated with CD56^{dim}CD57⁺ NK cell proportion (Figure 2E-G).

Together, these findings demonstrated that SLE patients had not only a global reduction in their peripheral NK cells but also a shift in subpopulations of NK cells—a loss of mature CD56^{dim}CD57⁺ NK cells which were negatively correlated with disease activities.

3. CD56^{dim}CD57⁺ NK cells underwent increased endogenous apoptosis level in SLE patients

To delineate the underlying mechanisms for the sharp decrease of peripheral NK cells in SLE patients, Annexin-V/7-AAD double staining flow cytometry was applied to detect the proportion of the apoptotic NK cells. Total apoptotic rate of peripheral CD56^{dim}CD57⁺ NK cells in SLE patients was increased compared with that in HCs ($57.7 \pm 16.0\%$, $n = 19$ versus $29.8 \pm 6.2\%$, $n = 10$, $P < 0.01$) (Figure 3A, D). Total apoptotic rate of peripheral CD56^{dim}CD57⁺ NK cells in SLE patients was increased compared with CD56^{dim}CD57⁻ NK cells in SLE patients ($57.7 \pm 16.0\%$, $n = 19$ versus $52.0 \pm 15.2\%$, $n = 19$, $P < 0.01$) (Figure 3A, E).

Cleaved-caspase 3 is an important regulator of apoptosis and Cleaved-caspase 9 mediates endogenous apoptosis. Compared with CD56^{dim}CD57⁺ NK cells in HCs, CD56^{dim}CD57⁺ NK cells in SLE patients showed increased expressions of Cleaved-caspase 3 (MFI: 129 ± 26.8 , $n = 4$ versus 238 ± 75.3 , $n = 6$, $P < 0.05$, Figure 3B, F) and Cleaved-caspase 9 (MFI: 181 ± 20.3 , $n = 8$ versus 262 ± 69.3 , $n = 8$, $P < 0.001$, Figure 3C, G).

Collectively, the selective reduction in CD56^{dim}CD57⁺ NK cells was due to, at least in part, increased endogenous apoptosis level in lupus microenvironment.

4. CD56^{dim}CD57⁺ NK cells showed higher oxidative stress in SLE patients

To identify cellular programs that may underlie low levels of NK activity in lupus, we analyzed a published dataset^[19] (GSE63829) by GSEA for Hallmark gene sets, which are coherently expressed signatures derived by aggregating many MSigDB gene sets to represent well-defined biological states or processes. Among 50 hallmark gene sets from MSigDB, ROS pathway was significantly enriched (NES = 1.528, $p = 0.029$, Figure 4A) in NK cells from lupus, compared with controls. All enriched pathways were summarized in Supplementary Table 3. As mitochondria are key cellular sources of ROS, total and mitochondrial ROS levels were assessed in NK cells. Both ROS (67357 ± 23137 , $n = 14$ versus 40983 ± 9046 ,

n=8 , P<0.01, Figure4B) and mtROS(601±157, n=14 versus 353±42.0, n=11 , P<0.001, Figure4C) levels of CD56^{dim}CD57⁺ NK cells were significantly increased in the SLE patients.

Interestingly, ROS pathway was highly activated(NES =1.573, p=0.018, Figure 4D and Supplementary Table 4) in CD56^{dim}CD57⁺ NK cells compared with the CD56^{dim}CD57⁻ NK cell subset when we analyzed another published dataset^[20](GSE23695). In addition, ROS levels(67357±23137, n=14 versus 63539±16767, n=14, P=0.1, Figure4E) were marginally higher and mtROS levels (601±157, n=14 versus 464±119, n=14, P<0.001, Figure4F) were much higher in CD56^{dim}CD57⁺ NK cells in SLE patients compared with CD56^{dim}CD57⁻ NK cells. Of note, we observed strong negative association between ROS level and perforin expression (r= -0.649, P= 0.012, Figure4G), and weak positive association between ROS level and total apoptosis(r=0.443, P= 0.11, Figure4H) in CD56^{dim}CD57⁺NK cells in the SLE patients. These indicators reflected the higher oxidative stress of CD56^{dim}CD57⁺NK cells in SLE patients, which may influence NK cell activities.

Therefore, we next performed a detailed analysis on the effect of ROS on the two NK subsets-analyzing the sensitivity of total PBMC to H2O2 which is a model for exposure to ROS.

We used a similar range of concentrations in our study to mimic physiological levels of ROS exposure^[21]. With increasing concentration of H2O2, apoptotic cells were increased in both two NK cell subsets(Figure4I). At concentrations of 20µM and above, apoptotic rates of CD56^{dim}CD57⁺ NK cells were higher than CD56^{dim}CD57⁻ NK cells when they exposed to the same H2O2 level, suggesting that CD56^{dim}CD57⁺ NK cells were more prone to undergo apoptosis when exposed to ROS(Figure4I).

These studies suggested that CD56^{dim}CD57⁺ NK cells were preferentially targeted for oxidative stress in lupus patients, leading to decreased activity of this NK subset.

5. Attenuated cytotoxicity and altered phenotypes of CD56^{dim}CD57⁺ NK cells in SLE patients

The distinctive characteristics of CD56^{dim} NK cells and CD56^{bright} NK cells have been well-characterized, and the former is predominant in the peripheral blood, with stronger cytotoxic function. To assess the functions of two CD56^{dim} NK cell subsets(CD56^{dim}CD57⁺ NK cells and CD56^{dim}CD57⁻ NK cells), we examined the markers associated with degranulation, activation, and cytokine production.

In our research, flow cytometry analysis confirmed the [discrepancy](#) of CD56^{dim}CD57⁺ NK cells and CD56^{dim}CD57⁻ NK cells. The expression of CD107a on the cell membrane correlates with NK cell killing activity. CD56^{dim}CD57⁺ NK cells exhibited higher expression of CD107a, perforin, Granzyme B, CD16 and active receptor NKG2D in HCs(CD107a: 60.4±18.6% versus 49.9±13.7%, n=4; perforin: 97.5±3.01% versus 92.2±6.50%, n=10; Granzyme B: 97.3±4.47% versus 90.0±4.69%, n=6 ; CD16: 97.7±3.01% versus 93.7±4.30%, n=12; NKG2D: 97.5±2.65% versus 94.8±3.49%, n=10, Figure 5A, B, all P<0.05) compared with CD56^{dim}CD57⁻ NK cells. With respect to inhibitory receptors specific for MHC class I, NKG2A expression was decreased on CD56^{dim}CD57⁺ NK cells relative to that on CD56^{dim}CD57⁻ NK cells in HCs(8.54±7.20%

versus $42.9 \pm 10.4\%$, $n=7$, $P<0.001$, Figure 5A, B). The expression of IFN- γ ($25.7 \pm 19.4\%$ versus $36.4 \pm 16.1\%$, $n=5$) and IL-10 ($5.27 \pm 3.17\%$ versus $5.14 \pm 2.51\%$, $n=5$) did not differ between CD56^{dim}CD57⁺ NK cells and CD56^{dim}CD57⁻ NK cells in HCs (Supplementary Figure 4). The discrepancies between CD56^{dim}CD57⁺ NK cells and CD56^{dim}CD57⁻ NK cells in SLE patients were similar (CD107a: $31.3 \pm 5.46\%$ versus $22.5 \pm 4.30\%$, $n=4$; perforin: $82.2 \pm 15.1\%$ versus $69.1 \pm 20.1\%$, $n=24$; Granzyme B: 72.5 ± 23.6 versus $65.3 \pm 22.6\%$, $n=9$; CD16: $79.3 \pm 17.5\%$ versus $75.2 \pm 16.8\%$, $n=17$; NKG2D: $92.5 \pm 5.90\%$ versus $89.8 \pm 8.87\%$, $n=10$; NKG2A: $26.7 \pm 17.3\%$ versus $43.2 \pm 23.8\%$, $n=11$; IFN- γ : $16.3 \pm 10.6\%$ versus $24.1 \pm 8.19\%$, $n=7$; IL-10: $8.51 \pm 3.64\%$ versus $10.5 \pm 3.82\%$, $n=6$ Figure 5A, B), but to a lesser extent than that in HCs. Our research demonstrated that CD56^{dim}CD57⁺ NK cells exhibited enhanced cytotoxic function.

We also observed a significant impairment in CD107a degranulation by CD56^{dim}CD57⁺ NK cells from SLE patients relative to that from HCs ($31.3 \pm 5.46\%$, $n=4$ versus $60.4 \pm 18.6\%$, $n=4$, $P<0.05$, Figure 5A, B). Compared with CD56^{dim}CD57⁺ NK cells in HCs, CD56^{dim}CD57⁺ NK cells in SLE patients showed decreased expressions of perforin ($97.5 \pm 3.01\%$, $n=10$ versus $82.2 \pm 15.1\%$, $n=24$, $P<0.01$), Granzyme B ($97.3 \pm 4.47\%$, $n=6$ versus $72.5 \pm 23.6\%$, $n=9$, $P<0.05$), CD16 ($97.7 \pm 3.01\%$, $n=12$ versus $79.3 \pm 17.5\%$, $n=17$, $P<0.01$) and NKG2D ($97.5 \pm 2.65\%$, $n=10$ versus $92.5 \pm 5.90\%$, $n=10$, $P<0.05$), along with increased NKG2A expression ($8.54 \pm 7.20\%$, $n=7$ versus $26.7 \pm 17.3\%$, $n=11$, $P<0.05$) (Figure 5A, B). There were no significant differences between the two groups in terms of IFN- γ ($25.7 \pm 19.4\%$, $n=5$, versus $16.3 \pm 10.6\%$, $n=7$) and IL-10 ($5.27 \pm 3.17\%$, $n=5$ versus $8.51 \pm 3.64\%$, $n=6$) expression (Supplementary Figure 4). Together, CD56^{dim}CD57⁺ NK cells in SLE patients exhibited not only lower proportion, but also the impaired function, especially for the cytotoxic function.

6. CD56^{dim}CD57⁺ NK cells exhibited stronger cytotoxicity in response to activated CD4⁺ T cells.

Activated CD4⁺ T cells are involved in multiple aspects of SLE pathogenesis^[1]. NK cells can negatively regulate CD4⁺ T cell responses to protect from immunopathology, and most reports have found that this process is dependent on direct cytotoxicity^[3-4]. We observed specific lysis of autologous activated CD4⁺ T cells by NK cells, but no killing of resting CD4⁺ T cells (Supplementary Figure 5, Figure 6A), which is consistent with the findings of other investigators^[4,22]. In addition, sorted CD56^{dim}CD57⁺ NK cells manifested significantly enlarged cytotoxicity relative to CD56^{dim}CD57⁻ NK cells in response to autologous activated CD4⁺ T cells from HCs ($27.1 \pm 1.19\%$, $n=3$ versus $17.7 \pm 2.24\%$, $n=3$, $P<0.05$, Figure 6A). As there are differences in CD4⁺ T cells between HCs and lupus, we chose the same allogeneic CD4⁺ T cell as the target cells. CD56^{dim}CD57⁺ NK cells from SLE patients showed significantly attenuated cytotoxicity compared with that from HCs when co-cultured with the same allogeneic activated CD4⁺ T cells ($14.5 \pm 0.75\%$, $n=3$ versus $20.7 \pm 2.38\%$, $n=3$, $P<0.05$, Figure 6B).

Inducible T cell costimulator (ICOS) and programmed death 1 (PD-1) are essential activation markers of CD4⁺ T cells for T-B-cell co-stimulation, which are key pathogenetic features of SLE^[1,23]. Our study also confirmed that both ICOS% ($5.24 \pm 5.04\%$, $n=10$ versus $1.36 \pm 0.64\%$, $n=9$, $P<0.05$) and PD-1% ($18.3 \pm 8.13\%$, $n=10$ versus $8.71 \pm 4.65\%$, $n=10$, $P<0.05$) were higher in SLE patients than in HCs (Figure 6C). We further

tested whether the levels of NK cell subset distribution correlated with activation makers of CD4⁺ T cells in SLE. Interestingly, lower level of CD56^{dim}CD57⁺ NK cell percentage strongly correlated with higher active maker ICOS% ($r = -0.649$, $P = 0.042$, Figure 6D) and marginally correlated with PD-1% ($r = -0.430$, $P = 0.214$, Figure 6E), suggesting a regulation effect of NK cell subset distribution to activated CD4⁺ T cells.

These data indicated that NK cells, especially CD56^{dim}CD57⁺ NK cells exclusively targeted activated CD4⁺ T cells. And selective reduction in CD56^{dim}CD57⁺ NK cells and impaired function in SLE patients might impede their ability to eradicate activated pathogenic CD4⁺ T cells which are key cells that cause lupus disease.

Discussion

In this study, we demonstrated that the number and percentage of NK cells decreased substantially in the peripheral blood of treatment-naive SLE patients compared with those of HCs and treatment-naive RA patients. Beyond reduced numbers, NK cells in SLE patients had a selective loss of mature CD56^{dim}CD57⁺ NK cell subset, which was conversely correlated with disease activity. CD56^{dim}CD57⁺ NK cells exhibited increased cytotoxic effect on activated CD4⁺ T cells. Additionally, attenuated cytotoxicity function, up-regulated endogenous apoptosis and increased oxidative stress were observed in peripheral CD56^{dim}CD57⁺ NK cells from SLE patients.

Previous studies have shown significantly down-regulated counts and abnormal phenotypes of peripheral NK cells in SLE patients^[10-12]. To exclude the influence of glucocorticoids or immunosuppressive drugs, we enrolled treatment-naive patients. We identified an extremely reduced NK count (median 50/ μ L) in SLE patients, which was less than a quarter of HCs (median 266/ μ L). Our study confirmed that compared with both HCs and untreated RA patients, numbers and percentages of NK cells were remarkably down-regulated in SLE patients, suggesting that NK cell deficiency was mainly due to immune disorder underlying lupus itself regardless of treatment.

Beyond being important innate immune cells, NK cells may regulate adaptive immune responses in many aspects^[3,4,24]. NK cell can directly eliminate activated CD4⁺ T cells, and can also exhibit potent immunosuppressive capacity that repress antigen-specific B cell and immunoglobulin somatic mutations, both through a perforin-dependent pathway^[3,24]. In some immune-related diseases, including atopic dermatitis and multiple sclerosis, NK cells exhibited reduced numbers and impaired function^[4,25,26]. NK cell depletion can lead to the enhanced development of experimental autoimmune encephalomyelitis (EAE) due to increased numbers of auto-reactive CD4⁺ T cells^[27]. Expansion of NK cells are associated with decreased T cells and clinical amelioration in multiple sclerosis, indicating that restoring NK cell activity is important for protection from immoderate autoimmunity^[25]. Hemophagocytic lymphohistiocytosis (HLH), a disease linked to genetic defects in perforin mediated cytotoxicity, has been associated with impaired NK cells and increased proliferation of T cells^[28]. Activated CD4⁺ T cells are involved in multiple aspects of SLE pathogenesis^[1,23]. ICOS are vital markers of T cell activation and also

essential costimulatory molecule to help B cell^[23,29]. Our research demonstrated that CD56^{dim}CD57⁺ NK cells exhibited higher expression of CD107a, perforin, granzyme B, CD16 and NKG2D, along with lower NKG2A expression compared with CD56^{dim}CD57⁻ NK cells. Furthermore, CD56^{dim}CD57⁺ NK cells exhibited stronger cytotoxicity in response to activated CD4⁺ T cells relative to CD56^{dim}CD57⁻ NK cells. Our study also indicated that SLE patients exhibited a selective reduction in CD56^{dim}CD57⁺ NK cells which were negatively correlated with SLEDAI, positively correlated with both complement C3 and C4, and weakly associated with anti-dsDNA antibody level, suggesting that CD56^{dim}CD57⁺ NK cell deficiency might be an important intrinsic mechanism for the pathogenesis of SLE. SLE patients had not only a global reduction in NK cells but also a shift in NK cell subsets with attenuated cytotoxicity function, indicating that altered NK cell subsets and impaired function might impede their ability to eradicate activated pathogenic CD4⁺ T cells .

On the other hand, SLE patients are susceptible to infections which have also been shown to play a role in the development and exacerbation of SLE^[2,30,31]. NK cell deficiency and its blunted cytotoxicity function may be an important risk factor for infections in SLE patients, especially the opportunistic infections. And restoration of NK cell activity could potentially help regain immune balance and alleviate both systemic autoimmunity and the risk of infection.

Elevated formation of the ROS, prominently including the mitochondrial electron transport chain, and NADPH oxidases leads to molecular damage, denoted as oxidative distress^[23,32-34]. Oxidative stress characterizes the lupus immune system and promotes its pathogenesis at multiple levels^[35,36,37]. Treatment with MitoTempo, an inhibitor of mtROS production, delays disease progression in lupus mice^[36]. And treatment with the ROS scavenger N-acetyl-cysteine(NAC) relieves disease activity of SLE patients from a randomized, double-blind, placebo-controlled trial study^[38]. ROS facilitates metastasis of melanoma cells by downmodulating functions of NK cells, and ROS inhibition may restore NK cell-mediated clearance of these malignant cells^[34]. Our study confirmed that both the levels of ROS and mtROS were increased in CD56^{dim}CD57⁺ NK cells from lupus patients, and were associated with increased apoptosis and decreased function of NK cells. Previous research has demonstrated that CD56^{dim} NK cells, but not CD56^{bright} NK cells, are likely to succumb to apoptosis when undergoing oxidative stress^[39]. Consistent with this, CD56^{bright} NK cells did not change significantly in our study. Our study further indicated that, in the CD56^{dim} NK cell population, CD57⁺ cells suffered from higher oxidative stress and more cell apoptosis, which may explain the selective reduction of CD56^{dim}CD57⁺ NK cells seen in lupus patients.

We recognized that there were several limitations. The total number of patients in our study was somewhat small and larger prospective cohorts are required to perform further analyses. As NK cells were only assessed in peripheral blood in our study, further investigations are needed to study tissue resident NK cells for their specific role in different target organs. And the molecular pathways employed in cross-talk between CD4⁺ T cells and NK cells need further research.

Conclusion

In summary, we demonstrated decreased number and impaired function of CD56^{dim}CD57⁺ NK cells in SLE patients. This NK cell subset was negatively correlated with the severity of SLE patients. The selective reduction may due to preferential apoptosis of CD56^{dim}CD57⁺ NK cells related to increased oxidative stress in SLE patients. More importantly, CD56^{dim}CD57⁺ NK cells exhibited stronger cytotoxicity in response to activated CD4⁺ T cells. Altered NK cell subsets and impaired function might impede their ability to eradicate activated pathogenic CD4⁺ T cells which are key cells that cause lupus disease.

Abbreviations

SLE: systemic lupus erythematosus; RA: rheumatoid arthritis; ACR: American College of Rheumatology; HC: healthy control; PLT: blood platelet; C3: serum concentrations of complement C3; C4: serum concentrations of complement C4; anti-dsDNA antibodies: anti-double-stranded-DNA antibodies; ANA: antinuclear antibodies; IgG: immunoglobulin G; IgM: immunoglobulin M; IgA: immunoglobulin A; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index; LN: lupus nephritis; PBMCs: peripheral blood mononuclear cells; PMA: phorbol 12-myristate 13-acetate; 7-AAD: 7-amino-actinomycin D; ROS: reactive oxygen species; mtROS: Mitochondrial reactive oxygen species; DCFH-DA: 2',7'-Dichlorodihydrofluorescein diacetate; MFI: mean fluorescence intensity; MACS: magnet associated cell sorting; FACS: fluorescence activated cell sorting; FBS: Foetal Bovine Serum; CFSE: carboxyfluorescein diacetate succinimidyl ester; FVD: Fixable Viability Dyes; H₂O₂: hydrogen peroxide; t-SNE: t-distributed stochastic neighbour embedding; GSEA: Gene Set Enrichment Analysis; GEO: Gene Expression Omnibus; NES: normalized enrichment score; MSigDB: Molecular Signature v7.2 Database; IQR: interquartile range; ICOS: Inducible T cell costimulator; PD-1: programmed death 1

Declarations

Ethics approval and consent to participate

This study complies with the Declaration of Helsinki, and the design of the work was approved by the ethics committee of Affiliated Hospital of Nantong University and the ethics committee of the Dalian Medical University.

Consent for publication

Not applicable.

Data Availability

The data used and/or analyzed in the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author Contribution

XL had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. XL, JZ, YZ and ZL conceived of and designed the study. ZL, YT, ZB, JL, YZ, JQ, MJ and JZ acquired the data. ZL, YT, ZB, JL, JQ and MJ analyzed and interpreted the data. All authors were involved in drafting the article or making critical revisions for important intellectual content, and the authors read and approved the submitted manuscript.

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Figures

Figure 1

The number and correlation analyses of peripheral NK cells in patients and healthy controls (HCs). A. The percentage of NK cells in untreated SLE patients ($n=156$) untreated RA patients ($n=22$) and HCs ($n=30$). B. The absolute number of peripheral NK cells in untreated SLE patients ($n=156$) untreated RA patients ($n=22$) and HCs ($n=30$). C. Correlation of the NK count with C3. D. Correlation of the NK count with PLT. E. Correlation of the NK count with SLEDAI. F. Comparison of the absolute number of peripheral NK cells in patients with ($n=74$) and without LN ($n=82$). (* $P < 0.05$, ** $P < 0.01$). C3: serum concentrations of complement C3; PLT: blood platelet; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index; LN: lupus nephritis

Figure 2

SLE patients exhibited alterations in specific subpopulations of NK cells. A. Representative flow cytometric plot for NK cell subsets (including CD3-CD56^{dim}CD57⁺ NK cells, CD3-CD56^{bright} NK cells, and CD3-CD56^{dim}CD57⁻ NK cells) from a HC and a SLE patient (Left). The percentage of three NK cell subsets in total NK cells from patients ($n = 37$) and HCs ($n = 30$) (Right). B. The correlation of CD56^{dim}CD57⁺ NK% with SLEDAI C. The correlation of CD56^{dim}CD57⁺ NK% with C3 D. The correlation of CD56^{dim}CD57⁺ NK% with C4 E. The correlation of CD56^{dim}CD57⁺ NK% with IgG, IgM and IgA F. The correlation of CD56^{dim}CD57⁺ NK% with PLT G. The correlation of CD56^{dim}CD57⁺ NK% with anti-dsDNA antibodies (* $P < 0.05$, ** $P < 0.01$). SLEDAI: Systemic Lupus Erythematosus Disease Activity Index; C3: serum concentrations of complement C3; C4: serum concentrations of complement C4; IgG: immunoglobulin G;

IgM:immunoglobulin M; IgA:immunoglobulin A;PLT: blood platelet; anti-dsDNA antibodies: anti-double-stranded-DNA-specific antibodies

Figure 3

CD56^{dim}CD57⁺ NK cells underwent increased endogenous apoptosis level in SLE patients. A. Representative flow cytometric plot for apoptosis of NK cells (CD56^{dim}CD57⁺ NK cell subset and CD56^{dim}CD57⁻ NK cell subset) in one HC and one SLE patient. B. Representative flow cytometric plot for Cleaved-caspase 3 level of CD56^{dim}CD57⁺ NK cells in one HC and one SLE patient. C. Representative flow cytometric plot for Cleaved-caspase 9 level of CD56^{dim}CD57⁺ NK cells in one HC and one SLE patient. D. Comparison of total apoptotic rate of CD56^{dim}CD57⁺ NK cells between HCs (n=10) and SLE patients (n=19). E. Comparison of total apoptotic rate of CD56^{dim}CD57⁺ NK cell subset and CD56^{dim}CD57⁻ NK cell subset in SLE patients (n=19). F. Comparison of cleaved-caspase 3 level of

CD56dimCD57+ NK cells in SLE patients(n=6) and HCs(n=4) G. Comparison of cleaved-caspase 9 level of CD56dimCD57+ NK cells in SLE patients(n=8) and HCs(n=8) (* P< 0.05, **P< 0.01, ***P< 0.001).

Figure 4

CD56dimCD57+ NK cells showed higher oxidative stress in SLE patients. A. Gene set enrichment analysis(GSEA) plots showing an increase in ROS signature (enrichment plot: HALLMARK_REACTIVE_OXYGEN_SPECIES_PATHWAY) in lupus NK cells compared with normal NK cells(GSE63829). B. Production of reactive oxygen species(ROS) in CD56dimCD57+NK cells from SLE patients(n=14) and HCs(n=8). C. Production of mitochondrial reactive oxygen species(mtROS) in CD56dimCD57+NK cells from SLE patients(n=14) and HCs(n=11). D. Gene set enrichment analysis(GSEA) plots showing an increase in ROS signature (enrichment plot: HALLMARK_REACTIVE_OXYGEN_SPECIES_PATHWAY) in CD57+NK cells compared with CD57-NK cells(GSE23695). E. Comparison of ROS level of CD56dimCD57+ NK cell subset and CD56dimCD57- NK cell subset in SLE patients(n=14). F. Comparison of mtROS level of CD56dimCD57+ NK cell subset and CD56dimCD57- NK cell subset in SLE patients(n=14). G. The correlation of ROS level and perforin expression in CD56dimCD57+ NK cell subset in SLE patients(n=14). H. The correlation of ROS level and total apoptosis in CD56dimCD57+ NK cell subset in SLE patients(n=14). I. Comparison of apoptosis level of CD56dimCD57+ NK cell subset and CD56dimCD57- NK cell subset from HC upon exposure to H2O2 for 24 hour culture at concentrations of 20μM, 40μM and 80μM, respectively(n=5). (* P< 0.05, **P< 0.01, ***P< 0.001).

Figure 5

Attenuated cytotoxicity and altered phenotypes of CD56^{dim}CD57⁺ NK cells in SLE patients A. Representative flow cytometric plot for expression of CD107a, perforin, Granzyme B, CD16, NKG2D and NKG2A on NK cell subsets (CD56^{dim}CD57⁺ NK cell subset and CD56^{dim}CD57⁻ NK cell subset) in HC and SLE patient. B. Comparison of CD107a, perforin, Granzyme B, CD16, NKG2D and NKG2A expression on NK cell subsets. (* P < 0.05, ** P < 0.01, *** P < 0.001).

Figure 6

CD56^{dim}CD57⁺ NK cells exhibited stronger cytotoxicity in response to activated CD4⁺ T cells. A. Cytotoxicity assay of CD4⁺ T cells in response to NK cell subsets (n=3). B. Cytotoxicity assay of CD4⁺ T cells in response to CD56^{dim}CD57⁺ NK cells from SLE patients and HCs (n=3). C. Comparison of active makers in CD4⁺ T cells from SLE patients (n=10) and HCs (n=9-10). D. The correlation of ICOS% in CD4⁺ T cells and CD56^{dim}CD57⁺ NK cell percentage in SLE patients (n=10). E. The correlation of PD-1% in CD4⁺ T cells and CD56^{dim}CD57⁺ NK cell percentage in SLE patients (n=10). ICOS: Inducible T cell costimulator; PD-1: programmed death 1 (* P < 0.05).

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