A Shift Toward Activated Receptors and Enhanced Effector Functions on NK Cells Contribute to Immune Imbalance During Sepsis

Wanxue He
Department of Pulmonary and Critical Care Medicine, Xuanwu Hospital Capital Medical University

Lin Zhang
Department of Pulmonary and Critical Care Medicine, Xuanwu Hospital Capital Medical University

Zhiming Luo
Department of Pulmonary and Critical Care Medicine, Xuanwu Hospital Capital Medical University

Jianqiao Xu
College of Pulmonary and Critical Care Medicine, Chinese PLA General Hospital, Beijing

Wei Guan
College of Pulmonary and Critical Care Medicine, Chinese PLA General Hospital, Beijing

Lixin Xie (✉ xielx301@126.com)
College of Pulmonary and Critical Care Medicine, Chinese PLA General Hospital, Beijing

Research Article

Keywords: sepsis, innate immune system, NK cells, immune imbalance, over-activated, secondary virus infection

Posted Date: August 4th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1669781/v2

License: © This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to an infection. It is a disease with a high incidence and mortality rate, which has a tremendous impact on global health and social economy. Remarkably, immune imbalance has been recognized as a key pathophysiological change in sepsis, and NK cells, one of the most important innate immune cells, are involved in this process and play a crucial role in it. However, the role of NK cells in the pathogenesis of sepsis is still controversial and there remains a gap in understanding the exactly immune responses of NK cells to sepsis and even infections secondary to sepsis. In this study, we used sepsis mouse models to investigate the alterations of NK cells, and then we subjected the septic mice to a secondary influenza A virus (H1N1) infection to characterize the anti-infection immune responses of NK cells. Here, we demonstrated that NK cells presented an activated phenotype in early phase of sepsis as illustrated by increased activated CD69 and NKG2D and reduced inhibitory KLRG1 expression on NK cells. Moreover, we observed that NK cells were excessive overall activated in the sepsis mouse model subjected to secondary infections as illustrated by cell surface receptor expression profiles, increased IFN-γ and CD107a expression after stimulation, aggravated lung inflammation and pathological injury, and increased systemic inflammation cytokines, which might be an important reason for higher mortality in PR8-infected sepsis mice. In summary, NK cells exhibit excessive activated phenotype induced by sepsis, contributing to unrestrained systemic inflammation, pathogenesis of tissue damage, physiological dysfunction, and increased mortality.

Introduction

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to an infection defined by the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) [1]. Sepsis has had an extraordinary impact throughout the world and has been recognized as a global health priority according to the World Health Organization [2]. It has been reported that there were an estimated 48.9 million cases of sepsis worldwide in 2017, and the cases of sepsis in Asia account for 47% [3]. Moreover, there were 11.0 million sepsis-related deaths in 2017 and hospitalized patients suffered from septic shock were associated with mortality rates of up to 40% [3, 4]. In addition to the high incidence and mortality rate of sepsis, individuals suffered from sepsis are more susceptible to nosocomial infections and even sepsis recidivism, resulting in poor prognosis and higher mortality rate in the long term, which has a tremendous impact on global health and social economy [5–7]. Immune imbalance has been recognized as a key pathophysiological change in sepsis [8]. And both the innate and adaptive immune systems play crucial roles in eliminating invasive pathogens and protecting the host from infections in an episode of sepsis.

Natural killer (NK) cells, a cohort of lymphocytes known as innate lymphoid cells, are one of the most important components of the innate immune system. NK cells are essential to initiate host defenses and mediate cytotoxic functions by rapidly migrating to the site of infections, killing cells infected with
intracellular pathogens and consequently mounting a rapid, nonspecific, effective innate immune response\cite{9}. In addition, NK cells also play an important role in coordinating innate and adaptive immune responses, and have long been characterized as an bridge between them\cite{10}. In detail, after migrating to the site of infections, NK cells coordinate early responses by recruiting innate immune cells and amplifying the antimicrobial functions of myeloid cells, particularly macrophages, by releasing essential cytokines, such as IFN-\gamma\cite{11}. Furthermore, NK cells also possess immunoregulatory potential\cite{12}. Therefore, NK cells play a significant role in immune processes that fight against infections.

It has been widely reported that NK cells participate in the propagation of systemic inflammation and pathophysiological dysfunctions in infectious diseases, including sepsis\cite{13}. Although there were multiple studies focused on the alterations of many immune cells, the role of NK cells in the pathogenesis of sepsis is still controversial. Numerous studies have investigated the effect of sepsis on NK cells, assessing changes in number, phenotype, and function. As is reported by several studies, NK cells appear to be critical to confer protection and eliminate pathogens during the early phase of sepsis, by exhibiting an activated phenotype and augmenting production of the pro-inflammatory cytokines. As sepsis persists, NK cells may exhibit a similar pattern of loss and dysfunction just like lymphocytes. Therefore, NK cell deficiency and dysfunction could contribute to sepsis-induced immunosuppression during the late phase of sepsis. Nevertheless, several studies have suggested that NK cells could be excessively activated, contributing to the amplification of systemic inflammation, and thus play a deleterious role during sepsis. Excessive activation of NK cells may result in a positive feedback loop that amplifies the infection-induced activation of NK cells and myeloid cells, leading to unrestrained systemic inflammation, concomitant multiple organ failure, and increased mortality\cite{11}. However, it remains not completely clear what alterations NK cells develop in response to sepsis and even in infections secondary to sepsis. Exploring the underlying alterations of NK cells during sepsis comprehensively is critical for better understanding of immune dysfunction following sepsis, contributing to the development of immunotherapies and improving the prognosis of sepsis patients.

Therefore, the purpose of this study was to decipher NK cell immunity of septic mice in a more comprehensive way. In the present study, we used mouse models of sepsis to investigate the numerical and phenotypic alterations of NK cells, and then we subjected the septic mouse models to a secondary viral infection to characterize the anti-infection immune responses of NK cells. Our results provide evidence showing that NK cells exhibit excessive activated phenotype induced by sepsis, contributing to unrestrained systemic inflammation, concomitant tissue damage, and increased mortality.

**Materials And Methods**

**Ethics statement**

The mouse experimental design and protocols used in this study were approved by the Regulation of the Institute of Microbiology, Chinese Academy of Sciences (IMCAS) of Research Ethics Committee (permit no. SQIMCAS2018046). All mouse experimental procedures were performed in accordance with the
Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China.

**Mice**

Female BALB/c mice were purchased from Vital River when they were 8-10 weeks old and rested at least 1 week in the specific pathogen-free (SPF) animal facility before use. For virus infection experiments, mice were transferred to a Biosafety Level 2 room in the Institute of Microbiology, Chinese Academy of Sciences. After the induction of mouse models, mice were intraperitoneally (i.p.) anaesthetized with pentobarbital sodium (50 mg/kg body weight) and euthanized by cervical dislocation at the indicated time points.

**Induction of sepsis**

Sepsis was induced according to an established protocol[6]. Briefly, sepsis in mice was induced by intraperitoneally (i.p.) injecting 0.5 mg/ml of lipopolysaccharide (LPS; serotype 055: B5, Sigma #L2880), dissolved in saline, at a dose of 10 mg/kg, and were euthanized at 12, 24 and 48 h after LPS administration. Control mice were intraperitoneally injected with saline at the same dose.

**Virus and infections**

To further explore the anti-infection immune responses, we subjected the septic mice to a secondary virus infection as previously described[14]. Briefly, the mouse-adapted influenza A/Puerto Rico/8/34 (H1N1; PR8) strain was propagated in the chorio-allantoic cavities of 10-day-old specific pathogen-free (SPF) embryonated chicken eggs (Beijing Merial Vital Laboratory Animal Technology) for 48-72 h at 37°C, then allantoic fluids were harvested and stored in aliquots at -80 °C. Virus titers were determined by plaque assays on Madin-Darby canine kidney (MDCK) cells (ATCC CCL-34). For infection experiments, septic and control mice were transferred to a Biosafety Level 2 room. Mice were intraperitoneally (i.p.) anaesthetized with pentobarbital sodium (50 mg/kg body weight) and inoculated intranasally (i.n.) with 2x10^5 pfu PR8 in 40 μl sterile PBS. Control mice were given an equal volume of PBS i.n. for mock infection. Post infections, survival of infected mice was observed daily for a period of 7-9 days.

**Isolation of splenic mononuclear cells**

Spleen tissues were collected from the sacrificed mice and processed individually. Single cell suspensions were obtained by gentle mechanical dissociation in PBS containing 2% fetal bovine serum (FBS), and passing it through a 200-mesh screen. After hemolysis with 1X RBC lysis buffer (Invitrogen, Carlsbad, CA), cells were washed and resuspended in complete RPMI 1640 medium as described previously[6]. The number of viable cells was counted by Trypan blue exclusion using a hemocytometer.

**Flow cytometric analysis**
To determine the difference of the number and phenotype of NK cells between control and sepsis mice, up to $2 \times 10^6$ splenic mononuclear cells were added to a 96-well plate. The isolated cell suspension was washed with PBS, incubated with Fcγ receptor blocker CD16/32 (clone 2.4G2; BD Pharmingen), and stained with specific monoclonal antibodies at the indicated concentration in PBS solution containing 1% FBS as described previously[6]. The following antibodies were used: fluorescently-conjugated antibodies (BD Pharmingen) against mouse CD3 (145-2C11), CD49b (HMA2), NKP46 (29A1.4), CD69 (H1.2F3), NKG2D (CX5), KLRG1 (2F1). Surface antibody staining was performed at 4 °C for 30 min in the dark. After staining, the cells were washed three times with PBS, and fixed with 0.5% paraformaldehyde at 4 °C.

Further, to determine the anti-infection immune responses of NK cells in the spleen, up to $1 \times 10^6$ splenic mononuclear cells were incubated in a 96-well plate at 37°C in medium containing IL-2, monensin, and FITC conjugated anti-CD107a Ab for 2 h. Then brefeldin A was added to the wells and incubated for another 2.5 h. To determine the response of NK cells as positive controls, another 96-well plate containing $1 \times 10^6$ splenic mononuclear cells, IL-2, monensin, FITC conjugated anti-CD107a Ab, PMA, and ionomycin was incubated at 37°C for 2 h. Then brefeldin A was added to the wells and incubated for another 2.5 h. After these incubations, the cell suspension was washed with PBS, incubated with Fcγ receptor blocker CD16/32, and then stained with surface molecules, fixed, permeabilized, and stained for intracellular cytokine IFN-γ using the Cytofix/Cytoperm kit according to the manufacturer's instructions. The following antibodies were used: fluorescently-conjugated antibodies (BD Pharmingen) against mouse CD3 (145-2C11), CD49b (HMA2), IFN-γ (XMG1.2), and CD107a (1D4B). After staining, the cells were washed three times with PBS, and fixed with 0.5% paraformaldehyde at 4 °C.

The stained cells were analyzed on a FACS Aria III flow cytometer (BD Biosciences). The acquired data was analyzed with FlowJo software (Tree Star).

**Plaque assay**

Virus titers in the lung tissue were determined by plaque assay according to an established protocol[6]. The MDCK cells were resuscitated in advance, up to $2 \times 10^5$ MDCK cells were added to 12-well plates. The lung tissues with 5ml PBS of infected mice were thawed and grinded. Then 10-fold serial dilutions of the virus supernatant were prepared and used to infect confluent MDCK cells in 12-well plates for 1 h at 37 °C. The virus inoculums were removed by washing with PBS. The cell monolayers were overlaid with an agar medium (DMEM supplemented with 1% low melting point agarose and 1 μg/ml N-tosyl-L-phenylalanyl chloromethyl ketone-treated trypsin) and incubated at 37 °C for 48-72 h. The plates were fixed with 4% paraformaldehyde for 1 h, and then the agarose overlays were carefully removed. The wells were incubated with staining buffer (0.1% crystal violet and 20% ethanol in water) for at least 10 min, and subsequently aspirated. The number of plaques were counted and calculated the virus titers.

**Histopathology**
Infected mice were sacrificed at 3 days post infection (dpi). For histological analysis, the lung tissues were removed and fixed with 4 % paraformaldehyde at 4 °C for 20 h. Post fixing, the tissues were dehydrated in a series of graded alcohols and embedded in paraffin. The tissue was sectioned into 5 μm thick sections, mounted on glass slides, and stained with haematoxylin and eosin solution (H&E) as described previously\textsuperscript{[14]}. Histological sections were examined using a Zeiss Axio Imager M1 microscope equipped with an AxioCam HRc camera under control of AxioVision 4 software. The histological analysis was processed with Image J software.

**Cytokine analysis**

Mice were sacrificed at the indicated time points. For systemic cytokine detection, peripheral blood was collected from the retro-orbital venous plexus and serum were stored at -80 °C. Cytokine levels were measured using the LEGEND plex Mouse Inflammation Panel (BioLegend, CA, USA) according to the manufacturer’s instructions and read on a Luminex 100 (Bio-Rad) or a FACS LSRFortessa flow cytometer (BD Biosciences) as described previously\textsuperscript{[6]}.

**Statistical analysis**

Statistical analysis was performed using Prism software (GraphPad). All statistical analyses were performed using an unpaired two-tailed Student’s t-test or two-way ANOVA test as applicable. When applicable, results are displayed as mean ± SD. \( p \) values < 0.05 were considered statistically significant.

**Results**

1. **The number of NK cells decreased in the acute sepsis and then restored.**

We i.p. injected LPS to induce the sepsis state experimentally, and collected samples at the indicated time points (Fig. 1A). Then we isolated and analyzed mononuclear cells in the spleen and found that the total splenocyte count showed a significant reduction at 24 h, which recovered by 48 h (Fig. 1B). To determine the impact of sepsis on NK cells, we compared the percentage and absolute number of NK cells in the spleen at the indicated time points using flow cytometry. We estimated the percentage and number of NK cells (CD3\(^{-}\) CD49b\(^{+}\) NKP46\(^{+}\)) in sepsis mouse models (Fig. 1C and 1D). In septic mice, we found that both the percentage and absolute number of NK cells decreased gradually. Although the decrease was significant at 12 h and 24 h, both the percentage and absolute number of NK cells presented a tendency to restore at 48 h (Fig. 1D), which is consistent with previous studies reporting that the decrease might be the reflection of sepsis-induced lymphopenia and at least partly due to apoptosis\textsuperscript{[15, 16]}

2. **NK cells were activated in early phase of sepsis.**

To evaluate the receptor expression profiles on splenic NK cells, we estimated the expression of cell surface receptors, which is important to cellular activation and inhibition, using flow cytometry. We compared the mean fluorescence intensity (MFI) of CD69 and NKG2D, and the positive percentage of
KLRG1 expressed on the gated NK cells in septic mice (Fig. 2). The expression of early activation marker CD69 increased significantly at 12 h and then declined slowly on NK cells (Fig. 2A), suggesting that NK cells were activated significantly in the early phase of sepsis. Similar to CD69, we observed the expression of NKG2D, which is a member of NKG2 receptors and has originally been described as another activating receptor for NK cells[17], increased significantly at 12 h and then declined (Fig. 2B). KLRG1, a member of the C-type lectin-like family, is widely accepted as a unique inhibitory receptor expressed on NK cells[18,19]. In septic mice, we found that the positive percentage of KLRG1 expressed on NK cells decreased significantly at 12 h and 24 h, and then recovered at 48 h (Fig. 2C). These alterations indicated that NK cells exhibited an activated phenotype in the early phase of sepsis.

3. Septic mice presented higher mortality post PR8 infection.

To further determine the alteration of anti-infection immune responses of NK cells induced by sepsis, septic mice were infected with the HIN1 PR8 strain (2×10^5 PFU/mouse; i.n.) five days after LPS injection (Fig. 3A). As shown in Fig. 3B, we observed large differences in the kinetics of survival rates. Notably, PR8-infected sepsis mice (sepsis + PR8) exhibited lower survival rates compared with PR8-infected control mice (control + PR8). However, quantification of viral titers at 3 dpi in the lung showed that there was no difference in virus loads between PR8-infected sepsis mice and PR8-infected control mice during secondary infection (Fig. 3C).

4. PR8 infection secondary to sepsis resulted in more pronounced activation of NK cells.

To estimate the activated and inhibitory receptor expression profiles of NK cells induced by virus infection secondary to sepsis, we compared the mean fluorescence intensity (MFI) of CD69 and NKG2D, and the positive percentage of KLRG1 expressed on the gated NK cells using flow cytometry (Fig. 4). In PR8-infected mice, we found that both CD69 and NKG2D increased significantly compared with their controls (Fig. 4B and 4C), suggesting that NK cells were activated by secondary virus infection. Notably, the expression of CD69, which is widely accepted as an early activation marker of NK cells, increased significantly in PR8-infected sepsis (sepsis + PR8) mice compared with PR8-infected control (control + PR8) mice (Fig. 4B). Besides, we observed that the positive percentage of KLRG1 decreased significantly only in PR8-infected sepsis mice (Fig. 4D). Together, these alterations indicated that NK cells were activated more significantly in PR8-infected sepsis mice according to the cell surface receptor expression profiles.

5. Overall increased NK cell responses in PR8-infected sepsis mice.

As is known to all, NK cells are important effector cells in the innate immune responses that serve as the first line of defending against certain infections. To determine the responses post PR8 infection, we measured the IFN-γ and CD107a expression of NK cells (Fig. 5). We assessed cytokine production, an important effector function of NK cells against infection. A significant number of previous studies have confirmed the ability of NK cells to amplify the inflammatory response during infections, primarily through the secretion of IFN-γ[20]. As shown in Fig. 5B, the percentage of IFN-γ + NK cells increased
significantly in PR8-infected mice, which was more pronounced in PR8-infected sepsis mice. In addition, NK cells are cytotoxic lymphocytes of the innate immune system that are capable of killing infected cells via release of cytotoxic granules\textsuperscript{[21]}. CD107a is a lysosomal membrane protein and redistributes to the cell surface upon granule exocytosis\textsuperscript{[22]}. Thus, the expression of CD107a correlates with NK cell cytotoxicity\textsuperscript{[23]}. Therefore, we further determined whether sepsis affected the cytotoxic activity of NK cells. Similar to IFN-\gamma, although the percentage of CD107a + NK cells increased significantly in PR8-infected mice, sepsis + PR8 mice exhibited higher CD107a expression on NK cells (Fig. 5C). To be more convincing, we further determined IFN-\gamma and CD107a expression of NK cells stimulated by PMA and ionomycin as positive controls (Fig. 5D, 5E and 5F). After 4.5 h of stimulation, the expression of IFN-\gamma and CD107a was significantly enhanced in both sepsis and sepsis + PR8 mice (Fig. 5E and 5F). These results indicated that NK cell responses including both cytokine production and cytotoxicity increased significantly in PR8-infected sepsis mice.

6. Sepsis aggrivated lung inflammation and injury post PR8 infection.

As was shown in above data, the responses of NK cells increased overall in PR8-infected sepsis mice. However, there was higher mortality rate in PR8-infected sepsis mice and no significant difference in virus loads between PR8-infected sepsis mice and PR8-infected control mice, which indicated that NK cells might play a pathological role during PR8 infection secondary to sepsis. To directly evaluate the pathology of the lung, we performed histological analysis of lung sections. As shown in Fig. 6A, all PR8 infected mice showed prominent leukocyte infiltration at 3 dpi. The lung sections from PR8-infected control mice were partly filled with infiltrated leukocytes, whereas the structure of alveoli remained basically normal in non-infiltrated area. However, more severe pathological changes were exhibited in sepsis + PR8 mice. In the areas with the most severe phenotype, the lung tissues were full of leukocytes, and almost no normal alveolar structure was found in lungs of these mice. Then, we assessed quantitatively pathological alterations of the lungs. As shown in Fig. 6B, the relative alveoli area in the lungs of PR8-infected mice was lower than that of their own control mice.Remarkably, the relative alveoli area of PR8-infected sepsis mice was significantly lower compared with PR8-infected control mice (Fig. 6B). Further, we found that the sepsis + PR8 mice showed an aggravation of pathological features compared with that of control + PR8 mice (Fig. 6C). Collectively, these results indicated that over-activated NK cells aggravated lung inflammation and pathological injury, which might be an important reason for the higher mortality rate in PR8-infected sepsis mice.

7. Increased systemic inflammation cytokines in PR8-infected sepsis mice.

Additionally, we further estimated the levels of typical inflammation cytokines in the peripheral blood at 3 dpi to explore the systemic inflammatory levels. As shown in Fig. 7, IL-6, TNF-\alpha, IFN-\gamma and IL-12p70 levels were significantly increased in PR8-infected mice compared to those in uninfected mice. Remarkably, these proinflammatory cytokine levels increased much more significantly in sepsis + PR8 mice compared with those in control + PR8 mice. The cytokine level alterations suggested that the systemic proinflammatory responses increased in PR8-infected sepsis mice, which was consistent with the
aggravated lung inflammation and pathological injury, and the excessive inflammation might contribute to their higher mortality rate.

Discussion

Despite the incidence and mortality of sepsis patients have increased significantly in recent years, individuals suffered from sepsis are more susceptible to secondary infections, resulting in poor prognosis and higher mortality rate in the long term. Remarkably, immune imbalance has been recognized as a key pathophysiological change in sepsis, and NK cells, one of the most important innate immune cells, are involved in this process and play a crucial role in it. Although numerous studies have investigated the effects of sepsis on NK cells, the role of NK cells in the pathogenesis of sepsis is still controversial. NK cells have been reported to be beneficial or deleterious in fighting against sepsis in experimental murine models, either by rapidly eliminating infections resulting in resolved inflammation, or by propagating excessive systemic inflammation. However, it remains not completely clear what alterations NK cells develop in response to sepsis and even in infections secondary to sepsis. In this study, we used sepsis mouse models to investigate the alterations of NK cells, and then we subjected the septic mice to a secondary influenza A virus (H1N1) infection to characterize the anti-infection immune responses of NK cells. Our results provided evidence showing that NK cells exhibited excessive activated phenotype induced by sepsis, contributing to unrestrained systemic inflammation, concomitant tissue damage, and increased mortality.

Sepsis mouse models are induced by the i.p. administration of LPS, a constituent of the bacterial cell wall. Although the pathophysiological process of the LPS model is not accurate enough to closely mimic the clinical course of sepsis compared with the cecal ligation and puncture (CLP) model, one of the “gold standard” in experimental sepsis research, it is widely accepted that the LPS induced septic mouse model exhibits similar symptomatology, including weight loss, piloerection, and lethargy and permits the long-term study of immune system responses using this reproducible and minimally invasive method.

We observed a significant decrease in the number of mononuclear cells in the spleen at 24 h in sepsis (Fig. 1B), corroborating previous reports found by Sharma et al. and Yoon et al. This suggests that both CLP and LPS induce immune cell depletion, which at least partly due to sepsis-induced apoptosis and is an important characteristic of sepsis-induced immune dysfunction.

Although almost all components of both the innate and the adaptive immune system are affected significantly by sepsis, the changes associated with NK cells are particularly noteworthy, since they play an essential role in immune processes that fight against infections. Our findings showed that both the percentage and number of NK cells decreased gradually and the decrease was significant at 12 h and 24 h in septic mice (Fig. 1D), which is consistent with previous reports of sepsis-induced lymphopenia due to the trafficking of NK cells to the sites of infection, or to increased apoptosis observed by Venet F et
al. and Etogo AO et al.\cite{15,16,33,34}. It is worth noting that both the percentage and absolute number of NK cells presented a tendency to restore at 48 h (Fig. 1D), which could possibly due to enhanced recruitment from secondary lymphoid tissue and self-proliferation in response to the stimulation of infections\cite{11}.

Despite the numerical alteration of NK cells, receptors expressed on the surface of NK cells which reflect their function are also significantly altered during sepsis\cite{35}. Expression of CD69, an early activation indicator of immune cells, increased significantly at 12 h and then declined slowly (Fig. 2A), suggesting that NK cells are activated in the early phase of sepsis, paralleled to the results of Joel et al.\cite{36}. Expression of NKG2D, a member of NKG2 receptors, has originally been described as another activating receptor of NK cells\cite{17}. Several studies involving sepsis patients have reported decreases in the expression of NKG2D on NK cells and even been considered as a biomarker of sepsis and septic shock patients\cite{23,37}. However, our findings showed that the expression of NKG2D increased in septic mouse models just like CD69 (Fig. 2B). This is probably due to that the LPS model we choose is not as severe as the CLP model or sepsis patients. And the LPS-induced sepsis mainly induces acute inflammatory responses and activates NK cells to up-regulate NKG2D expression to fight against infections. Despite these activating markers, we have also estimated a unique inhibitory receptor expressed on NK cells, KLRG1, to decipher the phenotypic alteration of NK cells in a more comprehensive way\cite{18,19}. And we found that the expression of KLRG1 decreased significantly in septic mice (Fig. 2C). Based on these alterations of receptors expressed on the surface of NK cells, our data suggests that NK cells exhibited an activated phenotype in the early phase of sepsis.

These above alterations of NK cells suggested that they were activated significantly in early phase of sepsis. However, there remains a gap in understanding the exactly immune responses of NK cells to infections secondary to sepsis. As reported by Hotchkiss et al., reactivation of some certain viruses, such as cytomegalovirus, was common in sepsis induced immunocompromised patients\cite{38,39}. In addition, using experimental “two-hit” mouse models, septic mice have been reported to be more susceptible to secondary opportunistic and non-opportunistic infections, such as Aspergillus, Listeria, Candida, Pseudomonas aeruginosa, and lymphocytic choriomeningitis virus, as a result of the post-sepsis immunosuppressive state\cite{40–43}. Furthermore, as observed by Jensen IJ et al., CLP sepsis impaired host immunity of NK cells to secondary murine cytomegalovirus (MCMV) challenge, including the impairment in NK-cell-mediated cytotoxicity and pathogen control\cite{44}. Nevertheless, according to the study of Toft P et al., NK cells exhibited an increased cytotoxicity and excessive inflammatory response in mice suffered from surgery followed by endotoxin adminstration\cite{45}. In addition, Stegemann-Koniszewski S et al. observed that NK cells in both lung and spleen were activated after infected by respiratory influenza A virus\cite{46}. Despite these findings, it remains complicated about the immune response of NK cells post sepsis and there is still a lack of studies investigating the immune response to secondary influenza A virus (H1N1) infection. Therefore, we subjected septic mice to secondary influenza A virus (H1N1, PR8 strain) infections to explore the immune responses of NK cells.
Our results showed that sepsis + PR8 mice exhibited higher mortality rate compared with control + PR8 mice after being infected with PR8 virus, while there was no difference in virus loads between them (Fig. 3). In addition, we found that the expression of activating indicators of NK cells, such as CD69, increased and the inhibitory receptor KLRG1 decreased much more significantly in sepsis + PR8 mice (Fig. 4), suggesting PR8 infection secondary to sepsis resulted in more pronounced activation of NK cells. In addition to these receptor expression profiles on the surface of NK cells, we further estimated their responses that fight against infections, including cytokine production and cytotoxicity, and IFN-γ and CD107a expression represented these two functions, respectively. Our results showed that the increase of the percentage of IFN-γ + NK cells was more pronounced in sepsis + PR8 mice (Fig. 5B), suggesting NK cells display an increased capacity to produce IFN-γ in PR8 virus infected mice secondary to sepsis. In addition, we estimated the cytotoxicity of NK cells by monitoring CD107a expression, and we observed that the alteration of the percentage of CD107a + NK cells was just paralleled to that of IFN-γ + NK cells. In detail, the percentage of CD107a + NK cells increased much more significantly in sepsis + PR8 mice (Fig. 5C). Furthermore, we found that the expression of both IFN-γ and CD107a was significantly enhanced in both sepsis and sepsis + PR8 mice in positive controls stimulated with PMA and ionomycin (Fig. 5D, 5E and 5F), which reinforced our supposition of increased overall responses of NK cells in PR8-infected sepsis mice. And these results we observed were consistent with previous studies reported by Guo Y et al., prompting that activated NK cells might play an essential role in augmenting acute inflammation during sepsis by producing excessive IFN-γ, which is known to potentiate inflammation by activating macrophages, dendritic cells, and other immune cells[34, 47, 48]. Nevertheless, Souza-Fonseca-Guimaraes F et al. reported that NK cells underwent a profound reduction of their capacity to release IFN-γ in sepsis patients[15]. And this difference might be caused by the original difference in the pathophysiological process between the LPS septic mouse models and clinical septic patients. Moreover, it cannot be ignored that dynamic changes in microbial, cellular and biomedical components constantly influence functional characteristics of NK cells during sepsis[11].

Although the response of NK cells increased overall, sepsis + PR8 mice still presented higher mortality rate, which indicates that NK cells might play a pathological role during PR8 infection secondary to sepsis. Notably, as shown in Fig. 6, the prominent leukocyte infiltration and more severe pathological changes were exhibited in sepsis + PR8 mice, indicating that sepsis aggravated lung inflammation and pathological injury, which might be an important reason for the higher mortality in PR8-infected sepsis mice. Additionally, we further estimated systemic inflammatory responses by detecting typical inflammation cytokines in the peripheral blood. As shown in Fig. 7, IL-6, TNF-α, and IFN-γ levels increased much more significantly in sepsis + PR8 mice, showing excessive systemic inflammatory responses. Moreover, the IL-12p70 level, which plays an important role in promoting NK cell-mediated cytotoxicity, also increased significantly in sepsis + PR8 mice[49]. Therefore, these increased cytokine levels were consistent with the aggravated lung inflammation and pathological injury, and the excessive inflammation might contribute to their higher mortality in sepsis + PR8 mice, which could be induced by excessive activation of NK cells.
There are several limitations in our study worth mentioning. Although the LPS-induced sepsis mouse model is widely accepted and generally used to study immune system responses for a long term, pathophysiological changes induced by LPS remain quite different from the clinical course of sepsis patients, and may contribute to our conclusions were inconsistent with real clinical situations. Further, we primarily explored the alterations of NK cells in response to sepsis and secondary viral infections. Although NK cells are one of the most important components of the innate immune system, our study was not comprehensive enough to reflect immune status. Remarkably, immune cells not only perform different functions themselves, but also interact each other and form large regulatory networks. And NK cells’ role during sepsis could be influenced by other immune cells as well. Thus, we plan to perform additional investigations to understand the contribution of other immune cells that interact with NK cells to sepsis and secondary viral infections. Moreover, future studies investigating the therapeutic implications of excessive activation of NK cells in order to regulate immune function, alleviate organ injury and improve long-term survival in sepsis patients need to be executed.

**Conclusion**

In summary, using a unique “two-hit” model, we provided further evidence that NK cells exhibited excessive activated phenotype induced by sepsis, contributing to unrestrained systemic inflammation, pathogenesis of tissue damage, physiological dysfunction, and increased mortality. Combined with previous studies, NK cells might function as a dual-edged sword, either by eliminating pathogens resulting in resolved inflammation, or by propagating excessive systemic inflammation, correlating with either improved or worsened prognosis, respectively. Expanding our knowledge of the underlying immunological alterations accompanying sepsis comprehensively and how they in turn affect the pathophysiology of sepsis will light the way of the development of immunotherapies and improving the prognosis of sepsis patients.

**Declarations**

**Funding**

This work was supported by China National Key Research Program (2018ZX09201013), and China PLA Key Research Program (BLB18J008).

**Competing Interests**

The authors have no relevant financial or non-financial interests to disclose.

**Author Contributions**

Lixin Xie conceived and coordinated the study. Wanxue He performed the study and wrote the manuscript. Lin Zhang, Zhiming Luo, Jianqiao Xu and Wei Guan gave advices about concept and revised manuscript. All authors read and approved the final manuscript.
Ethics approval

Experiments and protocols involving animals were approved by the Regulation of the Institute of Microbiology, Chinese Academy of Sciences (IMCAS) of Research Ethics Committee (permit no. SQIMCAS2018046). And experiments and protocols involving animals were in accordance with ARRIVE guidelines.

Consent for publication

Not Applicable.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Acknowledgements

Not Applicable.

References


Figures

Figure 1

The number of NK cells decreased in the acute sepsis and then restored.

(A) Schematic figure of the induction of septic mouse models. (B) The number of total splenocytes in septic mouse models at the indicated time points. (C) Flow cytometry analysis of CD3⁻ CD49b⁺ NKP46⁺ NK cells (gated on single cells). (D) The percentage (left) and number (right) of NK cells in septic mouse models at the indicated time points. Data are from at least three independent experiments with more than 5 mice per group in each experiment. Data points indicate means±SD. *p<0.05, ** p<0.01, *** p<0.001.
**Figure 2**

NK cells were activated in early phase of sepsis.

Flow cytometry analysis of the mean fluorescence intensity (MFI) of CD69 (A) and NKG2D (B), and the positive percentage of KLRG1 (C) expressed on the gated NK cells in septic mice at the indicated time points. Data are from at least three independent experiments with more than five mice per group in each experiment. Data points indicate means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure 3

Septic mice presented higher mortality post PR8 infection.

(A) Experimental design. Mice received sepsis stimulation followed by i.n. infection with $2 \times 10^5$ pfu of PR8 virus. (B) Survival of infected mice was monitored daily after influenza virus infection. (C) Viral titers in lung tissues of infected mice were monitored at 3 dpi. Data are from at least three independent experiments with more than five mice per group in each experiment. Data points indicate means ± SD. **p < 0.01.
Figure 4

PR8 infection secondary to sepsis resulted in more pronounced activation of NK cells.

(A) Flow cytometry analysis of CD3⁻CD49b⁺ NK cells (gated on single cells). Flow cytometry analysis of the mean fluorescence intensity (MFI) of CD69 (B) and NKG2D (C), and the positive percentage of KLRG1 (D) expressed on the gated NK cells in the control, sepsis, PR8-infected control (control+PR8) and PR8-infected sepsis (sepsis+PR8) mice. Data are from at least three independent experiments with more than five mice per group in each experiment. Data points indicate means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 5

Overall increased NK cell responses in PR8-infected sepsis mice.

Representative flow cytometry plots (A) and statistical results of the percentage of IFN-γ+ NK cells (B) and CD107a+ NK cells (C) in experimental control, sepsis, PR8-infected control (control+PR8) and PR8-infected sepsis (sepsis+PR8) mice groups. Representative flow cytometry plots (D) and statistical results of the percentage of IFN-γ+ NK cells (E) and CD107a+ NK cells (F) in control, sepsis, PR8-infected control (control+PR8) and PR8-infected sepsis (sepsis+PR8) mice groups stimulated by PMA/ionomycin. Data are from at least three independent experiments with more than five mice per group in each experiment. Data points indicate means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure 6

Sepsis aggravated lung inflammation and injury post PR8 infection.

(A) Hematoxylin-eosin staining (H&E) of lung tissue sections at 3 dpi in the control, sepsis, PR8-infected control (control+PR8) and PR8-infected sepsis (sepsis+PR8) mice (original magnification×10, as labeled; scale bars, 100um). Statistical analysis of relative alveoli area (B) and histological scores (C) in lung tissue sections from control, sepsis, control+PR8, sepsis+PR8 mice. Data are from at least three independent experiments with more than five mice per group in each experiment. Data points indicate means ± SD. **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure 7

Increased systemic inflammation cytokines in PR8-infected sepsis mice.

Levels of IL-6 (A), TNF-α (B), IFN-γ (C) and IL-12p70 (D) at 3 dpi in the control, sepsis, PR8-infected control (control+PR8) and PR8-infected sepsis (sepsis+PR8) mice. Data are from at least three independent experiments with more than five mice per group in each experiment. Data points indicate means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.