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Complement C5a regulates IL-17 by affecting the crosstalk between DC and γδ T cells in CLP-induced sepsis

Ruonan Xu[*1], *Renxi Wang*[*1], *Gencheng Han*[*1], *Jianan Wang*[1], *Guojiang Chen*[1], *Liyan Wang*[1], *Xia Li*[1], *Renfeng Guo*[2], *Beifen Shen and Yan Li*[1]

[1] Department of Molecular Immunology, Institute of Basic Medical Sciences, Beijing, P. R. China
[2] Department of Pathology, University of Michigan, Ann Arbor, MI, USA

Complement 5a (C5a) and Interleukin-17 (IL-17) are two important inflammatory mediators in sepsis. Here we studied the mechanisms underlying regulation of IL-17 by anaphylatoxin C5a. We found that C5a blockade increased the survival rate of mice following cecal ligation and puncture (CLP)-induced sepsis and decreased IL-17 expression *in vivo*. IL-17 was secreted mainly by γδ T cells in this model. Importantly, our data suggest that C5a participates in the regulation of IL-17 secretion by γδ T cells. Dendritic cells (DC) were found to act as a "bridge" between C5a and γδ T cells in a mechanism involving IL-6 and transforming growth factor β (TGF-β). These results imply that C5a affects the crosstalk between DC and γδ T cells during sepsis development, and this may result in a large production of inflammatory mediators such as IL-17.

Key words: Complement system · γδ T cells · Immunopathology

Introduction

Sepsis is a life-threatening medical condition caused by various microorganisms entering the human bloodstream and triggering an uncontrolled inflammatory reaction. In light of the multifactorial pathogenesis of sepsis, extensive work has been done to characterize the numerous agents and mediators that are involved in sepsis. In spite of extensive research efforts over the last 20 years, sepsis remains the leading cause of death in intensive care units [1, 2]. Specific therapies are generally unavailable because pathogenic mechanisms are still unclear.

There is abundant evidence that complement activation, production of cytokines and other inflammatory responses occur in sepsis [3]. It is generally accepted that the complement activation product complement 5a (C5a) plays an important inflammatory role in rodents following cecal ligation and puncture (CLP) [4]. C5a exerts its effects through the high-affinity C5a receptor (C5aR) and C5L2. C5L2, a putative "default" receptor, has been suggested to play important role in balancing the biological effect of C5a. For example, recent data showed that both C5aR and C5l2 cooperatively play functional parts in the setting of sepsis [5]. It has been shown that blockade of C5a or its receptor (C5aR) can inhibit the development of CLP and is associated with decreased levels of bacteria, preservation of innate immune functions of blood neutrophils, reduced thymocyte apoptosis and greatly improved survival rates [6, 7].

IL-17 (also known as IL-17A) is a proinflammatory cytokine produced by a variety of cells including $CD4^+$ Th17 cells, $CD8^+$ T cells, neutrophils and NK cells [8]. Recent reports also suggested that γδ T cells are a major source of IL-17 [9]. Although it has been demonstrated that IL-17 plays an important role in

Correspondence: Professor Yan Li
e-mail: liyan62033@yahoo.com.cn

*These authors contributed equally to this paper.

defense against extracellular pathogens such as *Klebsiella pneumoniae* [10], production and regulation of IL-17, interaction of IL-17 with other critical pro-inflammatory cytokine in sepsis, remain largely unclear.

Understanding crosstalk between critical pathogenic factors may be very important for designing treatments for sepsis. Here, we studied the crosstalk between two critical pathogenic factors, C5a and IL-17. Our results show that C5a acted on DC, which subsequently induced γδ T cells to secrete IL-17.

Results

C5a induced IL-17 in CLP-induced sepsis

Sepsis models were made by cecal ligation and puncture. Here both mild grade sepsis (with a survival rate of about 50%) and high grade sepsis (with a survival rate of about 25%) models, as described in the *Materials and methods*, were used. Unless described otherwise, CLP mice referred to mice with high grade sepsis. To prove the association between the critical inflammatory cytokines C5a and IL-17 in CLP-induced sepsis, a polyclonal anti-C5a antibody was developed and injected intravenously into CLP-induced septic mice to assess its efficiency. Control animals received a similar dose of nonspecific IgG antibody. The anti-C5a antibody-treated mice showed significantly higher survival rates compared with isotype IgG antibody-treated littermates ($p = 0.046$ Fig. 1A). These results indicate that anti-C5a antibodies can treat sepsis effectively. When we examined IL-17 production in the anti-C5a antibody-treated CLP group, we found that IL-17 significantly decreased 12 h after anti-C5a antibody blockade, but did not decrease in the nonspecific IgG antibody treated group (Fig. 1B), suggesting that C5a was involved in the IL-17 production in CLP-induced sepsis.

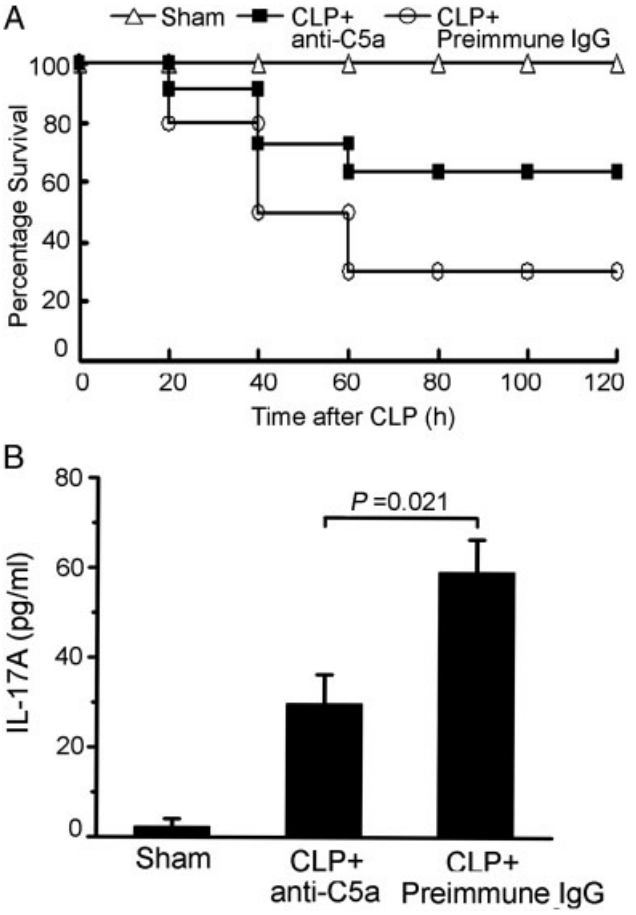

Figure 1. The effect of C5a blockade on IL-17 expression in CLP-induced sepsis. (A). Animals received either 400 μg of anti-C5a IgG (CLP +anti-C5a) or preimmune IgG (CLP+preimmune IgG) intravenously immediately after the CLP procedure. Sham animals underwent laparotomy without CLP. Data are presented as survival rates ($n = 15$/group), which are representative of those for four independent experiments. (B) Serum was obtained 12 h after sham surgery or CLP in mice treated immediately at T0 with IgG antibodies against C5a (anti-C5a) or preimmune IgG. IL-17A was measured by ELISA. Each group consisted of 6mice. *p*-values were calculated with Kruskal–Wallis H nonparametric test.

IL-17 was secreted mainly by γδ T cells in CLP-induced sepsis

γδ T cells act as an important source of IL-17 and have been known to initiate innate immune responses [11, 12]. To explore the origin of IL-17-secreting cells in CLP-induced sepsis, we first isolated γδ T cells from the splenocytes of sham and CLP mice. We found that the capacity of γδ T cells to release IL-17 was significantly higher than those of non-γδ T cells (Fig. 2A), especially in CLP mice. Cell cytometry showed that IL-17 was predominantly expressed in γδ T cells (Fig. 2B), indicating that γδ T cells were a major source for IL-17 in CLP mice.

IL-17 plays an important pathogenic role in sepsis [9]. To demonstrate the role of IL-17- expressing γδ T cells in the development of sepsis, here both mild grade sepsis and high grade sepsis models were used. γδ T cells were purified separately from sham control and CLP mice (high grade sepsis), and then transferred into mice with mild grade sepsis (with a survival rate of about 50%). We found that γδ T cells from the sham group mice did not significantly affect the survival rate. Adoptive transfer of γδ T cells derived from CLP mice resulted in a significant decrease in the survival rate in recipients, compared with adoptive transfer of γδ T cells derived from sham mice ($P = 0.039$, Fig. 3A). In addition to the capacity to promote sepsis, γδ T cells from CLP mice also secreted high levels of IL-17, while γδ T cells from sham mice only secreted a small amount of IL-17 (Fig. 2A). These results demonstrate that high levels of CLP-induced IL-17-secreting γδ T cells play an important pathogenic role.

We then evaluated the dynamic changes in γδ T cells during CLP-induced sepsis. Results showed that the percentage of γδ T cells in spleen was significantly elevated after CLP in comparison with sham controls (Fig. 4A). In addition, the capacity of γδ T cells to secrete IL-17 was much stronger than that in sham controls (Fig. 4B).

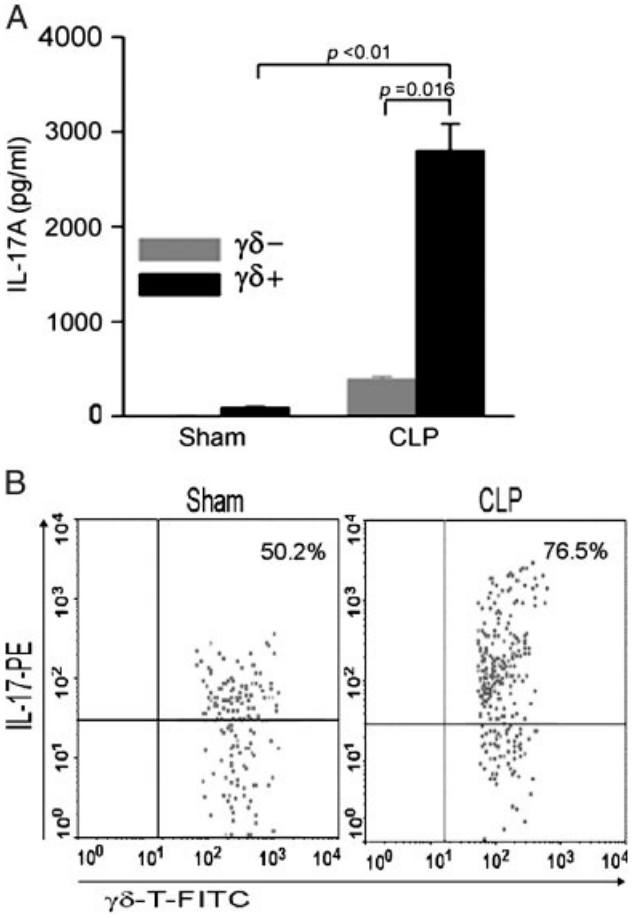

Figure 2. IL-17 secretion by purified γδ T cells. Splenocytes were collected from sham and CLP mice 12 h after the CLP operation, and γδ T cells were obtained from splenocytes by positive magnetic cell sorting. (A) Purified positive γδ T cells ($\gamma\delta^+$) and lymphocytes lacking γδ T cells ($\gamma\delta^-$were stimulated with rIL-23 (10 ng/mL) for 48 h. Amounts of IL-17A in the supernatant were measured by ELISA. (B) Representative plot showing the co-expression of IL-17 by purified γδ T cells from sham and CLP mice. Each experimental group consisted of four mice.

C5a is involved in the regulation of IL-17 secretion by γδ T cells in CLP-induced sepsis

As CLP-induced IL-17-secreting γδ T cells play an important pathogenic role and C5a acts as an important mediator in IL-17 expression during sepsis, we postulated that C5a may affect the function of γδ T cells in CLP-induced sepsis, and that once C5a was blocked, the γδ T cells from the C5a blockade group would lose their pathogenic role. γδ T cells were collected from the anti-C5a blockade group and were then transferred into a mild sepsis model with a higher survival of 50%. The result showed that mice that received γδ T cells from anti-C5a-treated CLP mice had a similar survival rate to mice that received γδ T cells from sham control, while the mice that received γδ T cells from the nonspecific IgG antibody-treated group had a significantly lower survival rate. (P = 0.042, Fig. 3B). Furthermore, the pathogenic effect of γδ T cells derived from CLP mice can also be abolished by anti-C5a antibody (P = 0.028, Fig. 3A). The data demonstrate that the pathogenic role of γδ T cells during CLP-induced sepsis was associated with the production of C5a. In addition, blockade of C5a reduced the capacity of γδ T cells to secrete IL-17 but did not affect the percentage and absolute number of γδ T cells in mice with CLP-induced sepsis (Fig. 4C and D). These results suggest that C5a is directly or indirectly involved in IL-17 production from γδ T cells in CLP-induced sepsis.

DC play an important role in C5a-mediated IL-17 production.

Whether C5a receptor (C5aR) is present on the surface of T cells is also controversial. We postulated that the relationship between C5a and γδ T cells was indirect. As it has recently been shown that C5aR is expressed on DC [13], DC may function as a bridge between C5a and γδ T cells. As shown in Fig. 5A, C5a significantly increased IL-17 production when T lymphocytes were co-cultured with DC. By employing flow cytometric analysis, it was found that IL-17-producing cells were mainly γδ T cells in the cell population from the co-culture and that C5a treatment doubled the number of IL-17-producing γδ T cells (Fig. 5B). These results suggest that DC are involved in the regulation of IL-17 production by C5a. To further prove that DC function as a bridge between C5a and IL-17-secreting γδ T cells, DCKO mice pretreated with diphtheria toxin (DT) (depletion of $CD11c^+$ DC) were used. The efficiency of DC depletion was 85.7% in blood and 48% in spleen (data not shown). As shown in Fig. 6A, the depletion of DC resulted in a sharp decline in the survival curve, and anti-C5a treatment did not show any beneficial effect in the CLP model using DC-depleted mice. Interestingly, DC depletion had a direct influence on IL-17 levels in the circulation. As shown in Fig. 6B, serum level of IL-17 was elevated in control mice after CLP and was measurably lower in comparison to that in DC-depleted mice. These results suggest that DC may play a pivotal role in IL-17 production. Next, we studied the mechanisms underlying C5a induction of IL-17 secretion by DC. It is known that IL-6 and TGF-β can induce IL-17 in Th17 cells. Here we found that C5a promotes IL-6 production by DC (Fig. 7A); when anti-IL-6 and anti-TGF- blocking antibodies were added to the co-culture of lymphocytes and DC, C5a-stimulated IL-17 production decreased markedly (Fig. 7B and C), while the isotype control had no effect (data not shown), indicating that C5a-mediated IL-17 production is partially dependent on IL-6 and TGF-β.

Discussion

This study demonstrates that γδ T cells produce a large amount of IL-17 in the CLP-induced sepsis model and that C5a can indirectly modulate γδ T cell-secretion of IL-17 in a DC-dependent manner. Sepsis is a life-threatening medical condition caused by various microorganisms entering the human bloodstream triggering an uncontrolled inflammatory reaction. During the onset of sepsis, the inflammatory system becomes hyperactive, involving both cellular and humoral defense mechanisms. Endothelial and epithelial cells, as well as neutrophils, macrophages and lymphocytes, produce

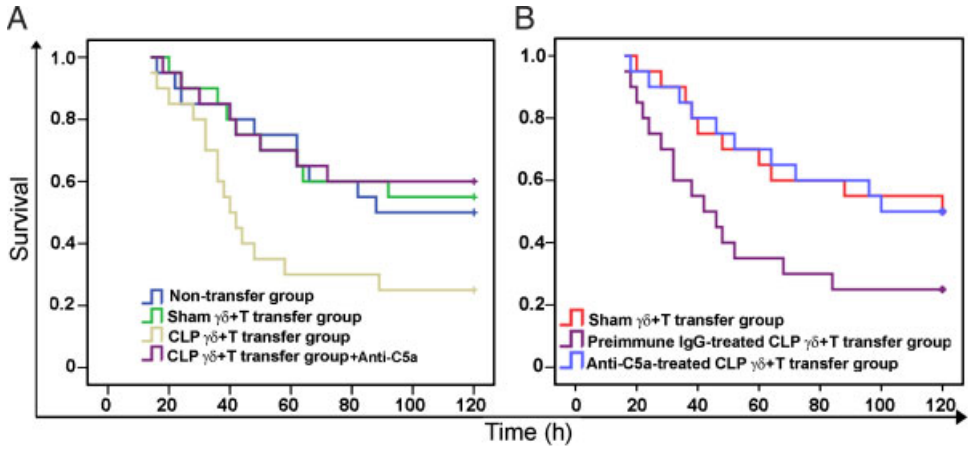

Figure 3. IL-17-overexpressing γδ T cells contribute to CLP. (A) CLP-induced γδ T cells have the pathogenic role function. γδ T cells (1.5×10^6) were isolated from sham or CLP group and then transferred into recipient CLP mice with survival rate of 50% for the mild sepsis procedure. Furthermore, 400 μg of anti-C5a IgG were given intravenously immediately after γδ T cells (1.5×10^6) from CLP group were transferred into recipient. Not-transferred CLP mice were used as controls. Each experimental group consisted of 20 mice. (B) C5a blockade cancelled the pathogenic role of γδ T cells in CLP. γδ T cells (1.5×10^6) were isolated from anti-C5a or nonspecific IgG antibody-treated CLP mice and then transferred into recipient CLP mice with survival rate of 50% for the mild sepsis procedure). γδ T cells from the sham mice transfer group were used as controls. Each experimental group consisted of 20 mice.

powerful proinflammatory mediators. Simultaneously, humoral defense mechanisms such as the complement system are activated, resulting in production of proinflammatory mediators, including C3a and C5a, which are powerful pathogenic mediators. Published data provide compelling evidence for the beneficial effects of blocking either C5a or C5aR (CD_{88}) in experimental sepsis, which is associated with attenuated coagulopathy, preservation of thymic function, rescue of lymphocytes from apoptosis and reduced levels of bacteremia [14–16].

Excessive IL-17 production has been detected in sera and target tissues of patients with various autoimmune diseases, including rheumatoid arthritis, multiple sclerosis, systemic lupus erythematous and asthma [17]. However, evidence of the impact of IL-17 on sepsis pathogenesis was quite contradictory. IL-17 may not only mediate CpG-inducible host defense during intra-abdominal sepsis [18], but may also play adverse functions in experimental sepsis [9]. Additionally, its prevalence and the mechanisms by which IL-17 is generated and regulated in sepsis are unclear. In this report, we found that blockade of C5a by anti-C5a polyclonal antibodies can not only increase CLP survival rate but can also compromise IL-17 production; thus, C5a induced IL-17 in sepsis was confirmed.

IL-17 is a proinflammatory cytokine produced by a variety of cells, including $CD4^+$ αβ T cells, γδ T cells, activated $CD8^+$ T cells, neutrophils and NK cells. However, γδ T cells are often an important source of this cytokine. In contrast to conventional T cells, γδ TCR-bearing cells constitute only a small proportion (1–5%) of the lymphocytes that circulate in the blood and peripheral organs [19], but they are widespread within epithelia-rich tissues and provide local regulation of inflammation. Regarding the source of IL-17 in sepsis, we identified γδ T cells as being an important source of IL-17 production in septic mice. After CLP operation, both the percentage and IL-17 producing capacity of γδ T cells increased significantly and were much greater than those in the WT-type sham controls. Especially, at 24h after CLP operation, the IL-17 producing capacity of γδ T cells was much higher than at 120h. This difference may be due to the natural process of sepsis in animals, with 24h representing the acute phase. By 120h, those animals that survived the acute phase demonstrated limited abscess formation (data not shown).

It has been shown that γδ T cells can mitigate the organ injury and mortality of sepsis [20]. But some studies on the role of γδ T cells in WT septic littermates have yielded divergent results, and it is likely that γδ T cells differentially regulate the inflammatory response based on the type or site of infection. In our study, the role of γδ T cells in sepsis development was investigated by adoptive transfer. In key published experimental sepsis papers, the survival rate is almost between 20 and 25%. Here we used CLP mice with a low survival rate (20%-25%) as the source of γδ T cells because γδ T cells from such severe CLP-induced sepsis had a more powerful capacity to secret IL-17. On the other hand, CLP mice with a higher survival rate (50%) were used as the recipients to identify the function of γδ T cells. For future experiments, normal γδ T cells and IL-17-over-expressing γδ T cells should be compared in function to avoid any differences between the survival models used. It was found that γδ T cells derived from different animals have entirely distinct functions. IL-17 over-expressing γδ T cells are more pathogenic, while normal γδ T cells with minimal IL-17 production had no detrimental effects. Interestingly, C5a blockade *in vivo* can influence the capacity of γδ T cells to secrete IL-17 but not the percentage and absolute number of γδ T cells. Most importantly, γδ T cells derived from the C5a blockade group lost their pathogenic role in adoptive transfer. These results suggest an intimate link between C5a, γδ T cells and IL-17.

C5aR is widely expressed on myeloid cells, but whether it is expressed on T cells, especially on γδ T cells, was not clear [21–23]. We assumed that the mechanism of C5a modulation of γδ T cells during sepsis might be indirect. Using FACS analysis, we showed that C5aR is expressed on DC. In addition, IL-17 production is significantly elevated in the presence of

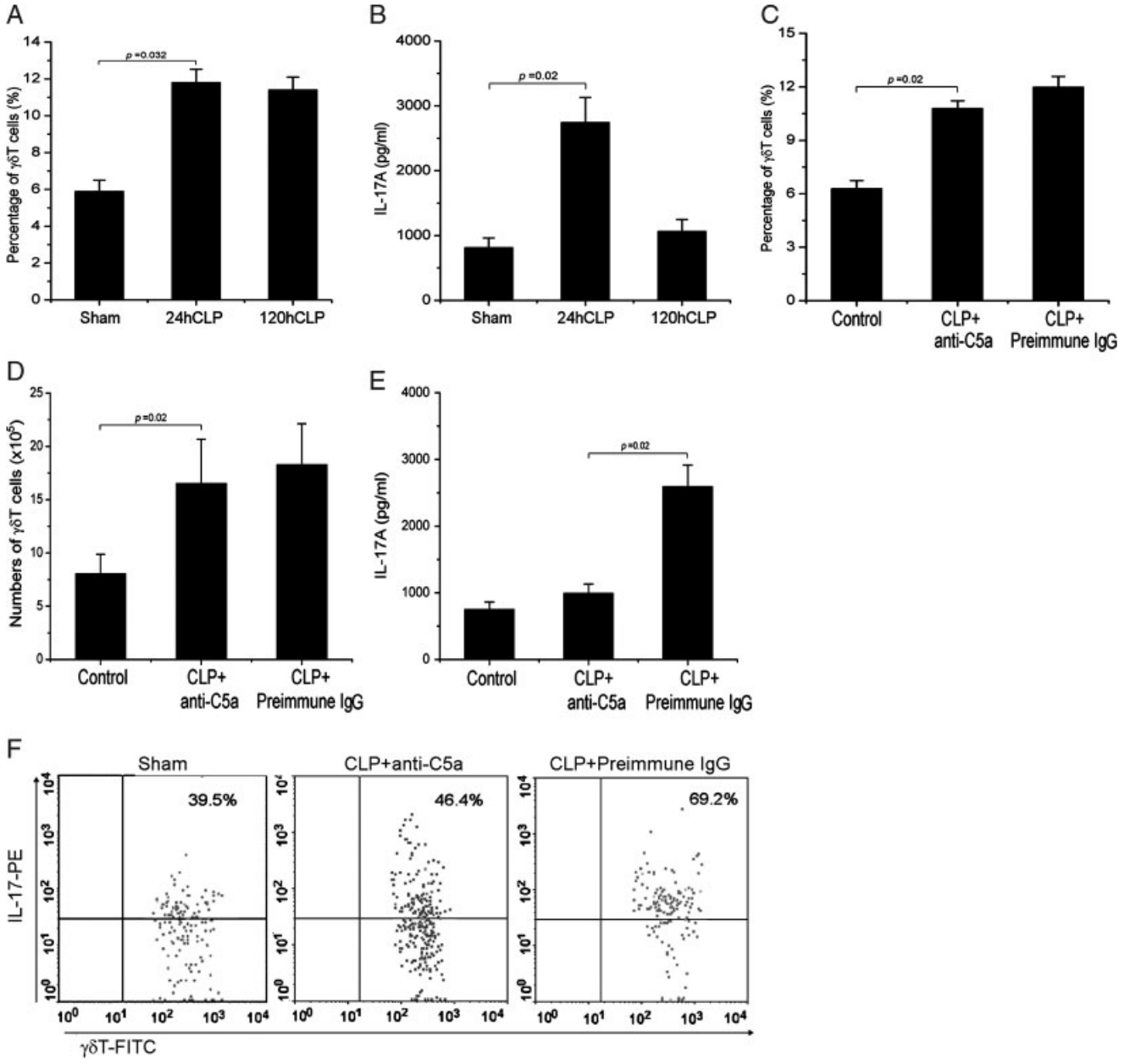

Figure 4. Effects of C5a blockade on γδ T cells during sepsis. (A) Purified γδ T cells from splenocytes were taken from CLP mice or sham controls at 24 and 120 h. Percentages represent the means ± SD of three different experiments. (B) Purified γδ T cells from different groups of mice were stimulated with rIL-23(10 ng/mL) for 48 h, and the resulting IL-17A content in culture supernatants was measured by ELISA. One set of three repeated experiments is shown, where each experimental group consisted of four or five mice. (C) Percentage increase of γδ T cells in CLP mice with intravenous infusion of anti-C5a antibody and preimmune IgG. Values represent the means ± SD of three different experiments. (D)Absolute numbers of γδ T cells were obtained separately from the sham, anti-C5a antibody and preimmune IgG-treated group after purification. Values represent the means ± SD of three different experiments. (E) Purified γδ T cells from antibody-treated mice were stimulated with rIL-23 (10 ng/mL) for 48 h, and the resulting IL-17A levels in the culture supernatants were measured by ELISA. Data are representative of three independent experiments. Each experimental group consisted of five mice. (F) Representative plot showing the co-expression of IL-17 by purified γδ T cells from the sham, anti-C5a or nonspecific IgG antibody-treated group. Each experimental group consisted of four mice. *p*-values were calculated with Kruskal–Wallis H nonparametric test.

recombinant mouse C5a in the co-culture of DC and T lymphocytes, and the depletion of DC *in vivo* markedly dwarfed serum levels of IL-17. These results suggest an important role for DC as intermediate for C5a modulation of IL-17 production. It is believed that DC determined the fate of the immune response during infection. Using CD11c–knockout mice, it has been demonstrated that maintaining DC numbers or functions may improve sepsis survival [24]. The discrepancy that naturally arises from these data is that loss of $CD11c^+$ DC on the one hand results in dramatic reductions in pathogenic IL-17, while on the other hand it leads to decreased survival. An alternative explanation is that the environment DC encountered might determine

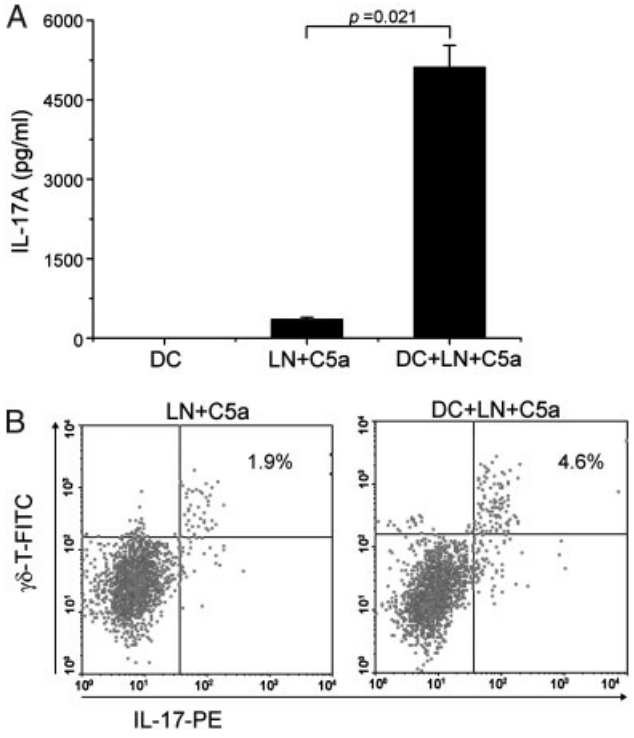

Figure 5. C5a regulation of DC-mediated IL-17 production. (A) T lymphocytes from mesenteric and groin nodes were co-cultured with DC for 3 days, C5a and anti-CD3 antibodies were added to the media and supernatants were collected for ELISA. Means and SD of IL-17 production after cell co-culture are shown for each group of six individuals. (C) Flow cytometric analysis of γδ TCR-FITC and IL-17-PE staining in T cells from DC and T lymphocyte co-cultures stimulated with C5a. Data are representative of three separate experiments. *p*-values were calculated with Kruskal–Wallis H nonparametric test.

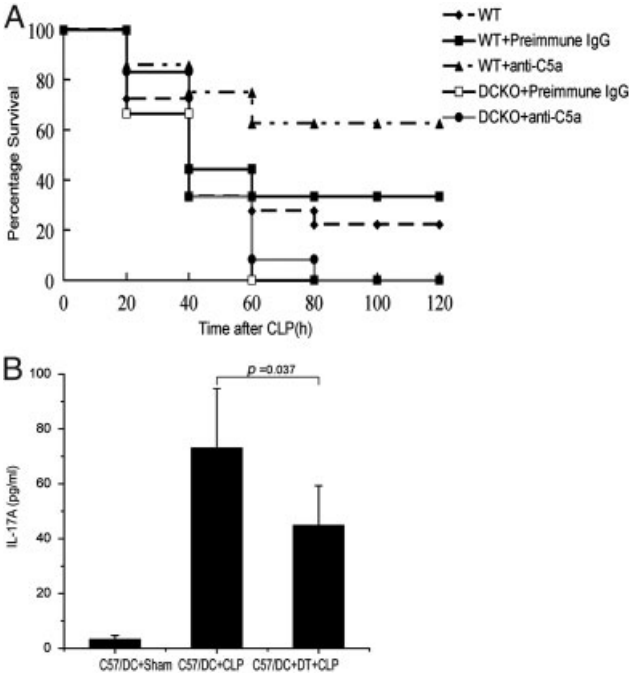

Figure 6. IL-17 in γδ T cells decreased in CLP-induced DCKO mice. (A) $CD11c^+$ DC depletion leads to high lethality of CLP. Survival was determined in DCKO *versus* WT littermates following the injection of DT (8ng/g body weight) 20h before the induction of CLP. Antibodies against C5a or preimmune IgG isotype were administered immediately after the CLP operation. Data are presented as percentage survivals ($n = 13$/group). (B) Serum was obtained from mice 24h after CLP operations, and IL-17A was measured by ELISA. Each group consisted of at least six mice. *p*-values were calculated with Kruskal–Wallis H nonparametric test.

its function. In normal condition, DC are professional antigen-presenting cells and play a guarding role in the immune system. But in septic condition, C5a treatment driven them prone to inflammatory DC, served as a mediator for IL-17 production.

Th cells producing IL-17 (Th17 cells) are a distinct subset of effector cells that differentiate from naive T cells in response to IL-6 and TGF-β [25, 26], whereas IL-23 serves as an important factor to expand previously differentiated Th17 cell populations and γδ T cells [27, 28]. Our data showed that C5a significantly increased IL-17 production by T lymphocytes in the presence of DC, while it had no influence on IL-17 production from T lymphocyte when DC were absent. This effect depended partially on the soluble cytokines, IL-6 and TGF-β, which are likely secreted by DC. How DC regulate the function of γδ T cells and how C5a influences the process are largely unknown. In addition to IL-6 and TGF-β, other mediators and/or cell-cell interactions may be involved. This line of research is currently under investigation.

In summary, we have shown that γδ T cells can release a large amount of IL-17 during experimental sepsis in a C5a-dependent manner and that IL-17-secreting γδ T cells play an important role in the pathogenesis of sepsis. DC appear to function as a bridge in C5a and γδ T cells for IL-17 production, a process dependent on IL-6 and TGF-β. These *in vitro* and *in vivo* data provide a strong evidence, for the first time, that C5a mediates IL-17 production from γδ T cells through DC during experimental sepsis. This study adds a new dimension for the research of pathogenic mechanisms in sepsis.

Materials and methods

Mice

Seven to eight-wk-old male C57BL/6 mice and conditional DC knockout mice B6.FVB-Tg (Itgax-DTR/EGFP)57Lan/J mice were obtained from the Jackson laboratory (Bar Harbor, ME, USA) and bred in our facilities under specific pathogen-free conditions. All treatment of mice in this study was in strict agreement with guidelines on the care and use of laboratory animals set out by the Institute of Basic Medical Sciences.

Production of anti-C5a antibody

The C-terminal end of mouse C5a (sequence: CTIANKIR-KESPHKPVQLGR) corresponding to amino acids 58–77 was

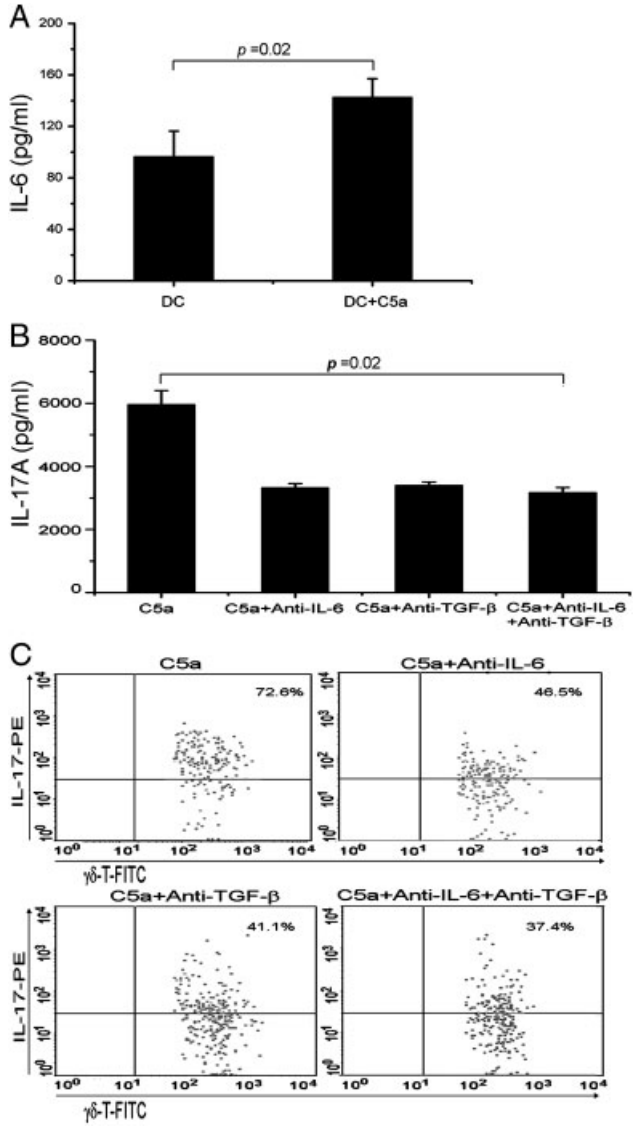

Figure 7. C5a-activated DC induced IL-17 production from T lymphocytes *via* IL-6 and TGF-β. (A) Effects of C5a on IL-6 production by DC was determined by ELISA assay 3 days after C5a was added to DC. Data are shown as means ± SD. (B) Effects of neutralizing anti-IL-6 or anti-TGF-β, or a mixture of both mAb, on IL-17 production, were determined by ELISA in DC cultures in the presence of C5a and T lymphocytes. T lymphocytes and DC were co-cultured with anti-IL-6 and anti-TGF-β antibodies. On day 3, the co-cultured supernatant was collected and IL-17 levels were evaluated. Data are representative of three separate experiments. All data are presented as means ± SD. (C) IL-17 expression in γδT after treatment with anti-IL-6, anti-TGF-β or both. FACS profiles show the percentage of IL-17$^+$γδ T cells among gated CD3$^+$γδ$^+$ T cells. The data shown are representative of those for three independent experiments. *p*-values were calculated with Kruskal–Wallis H nonparametric test.

chosen for peptide synthesis. The peptide was coupled to keyhole limpet and used for the immunization of rabbits and production of anti-C5a. The polyclonal antibody was purified by protein A chromatograph, and its reactivity with recombinant mouse C5a (Hycult biotechnology b.v, uden, The Netherlands) was confirmed by ELISA.

Depletion of DC

To deplete CD11c$^+$ DC, DCKO mice were treated with an i.p. injection of 8 ng/g body weight of DT (Sigma-Aldrich, St. Louis, MO, USA). As controls, DCKO mice were i.p. injected with saline vehicle alone. Eighteen to 20 h after i.p. injections, mice underwent CLP operations or were sacrificed for harvesting of spleen and blood samples.

Induction of sepsis by CLP

Specific pathogen-free 7-8 week old male B6.FVB-Tg (Itgax-DTR/EGFP) 57Lan-J mice treated with DT 18 to 20 h before CLP operations and C57BL/6 mice were used for studies as indicated. Sepsis was induced by Cecal Ligation and Puncture (CLP) and severity of sepsis was highly dependent on the extent of cecal ligation. Here both mild-grade sepsis and high-grade sepsis were used in our experiments as described (29). Briefly, for the induction of mild-grade sepsis, which resulted in survival rates of ∼50%, the cecum is ligated at half the distance between distal pole and the base of the cecum. While high-grade sepsis (25% survival rates) involves ligation of 75% of the cecum. After the bowel was repositioned, the abdomen was closed in layers, using a 4.0 surgical suture and metallic clips. Sham-operated mice were handled in the same manner, except that the cecum was not ligated and punctured. After CLP operations, the animals received 400 g of anti-C5a IgG in 400 l Dulbecco's phosphate buffered saline solution (DPBS) intravenously immediately after CLP. Control animals received similar amounts of normal rabbit IgG (Jing Mei Biotechonogy, Beijing, China).

Adoptive transfer

For adoptive transfer, γδ T cells purified from splenocytes by MACS from sham controls, anti-C5a-treated and nonspecific IgG antibody-treated CLP mice were transfused into recipient mice with each mouse receiving 1.5×10^6 γδ T cells. Recipient CLP mice were used as controls. To effectively reflect the role of γδ T cells, we employed a mild sepsis model for this study. During CLP procedure, 1/3 instead of 2/3 of cecum was ligated, and the rest of CLP procedure are similar.

Collection of serum samples

After induction of sepsis, animals were sacrificed after CLP or sham operations, and blood was drawn from the inferior vena cava. Blood samples were allowed to clot at room

temperature and were centrifuged at 5000 rpm for 10 min. Serum was collected and frozen immediately at −80°C until used for ELISA analysis.

In vitro generation of DC from bone marrow

Mice were sacrificed and all muscle tissues were removed with gauze from the femurs. Bones were placed in a 50-mm dish with 75% alcohol for 1 min, washed twice with PBS and transferred into a fresh dish with PBS. Both ends of the bones were cut with scissors, and the tissue was suspended and passed through a nylon mesh to remove small pieces of bone and debris. The cell suspension was centrifuged and washed twice with RPMI 1640 supplemented with 10% FBS, 50 μM 2-ME, 100 U/mL penicillin, 100 μg/mL streptomycin (all from Gibco, Grand Island, NY, USA). Aggregates were cultured in fresh medium with GM-CSF (R&D Systems, Minneapolis, MN, USA) in 6-well plates (Corning Costar, Corning, NY, USA) at a cell density of 1×10^7/mL/well. After 4 days of culture, large numbers of typical DC were released. The supernatant was then discarded and the medium was refreshed by adding new GM-CSF for subsequent experiments.

In vitro induction of T lymphocyte secretion of IL-17

DC derived from bone marrow were cultured *in vitro*. On day 5, 1×10^7 T lymphocytes from mesenteric and inguinal lymph nodes, anti-CD3 antibody (0.05 μg/mL, clone 145-2C11, eBioscience, San Diego, CA, USA) and/or recombinant mouse C5a (100nM) were added to the cultured DC. After another 3 days of co-culture, supernatants were collected for ELISA assay.

Detection of cytokine, IL-6 and TGF- production by DC *via* C5a stimulation

Briefly, 5 days after typical DC were obtained, recombinant mouse C5a (100 nM) was added to the culture, and after incubation for 3 days, cell-culture supernatants were collected for ELISA assay of IL-23, IL-6 and TGF-β.

Anti-IL-6 and anti-TGF-β Antibodies

Anti-IL-6 antibodies (1.8 ng/mL, antigen:antibody (M) = 1:2) (R&D, Clone MP520F3) and anti-TGF-β antibodies (14 ng/mL, antigen:antibody (M) = 1:2) ((R&D, Clone MAB1835) were added to the DC-cell and T-cell co-cultures immediately after anti-CD3 antibodies (0.05 μg/mL) and C5a (100 nM) stimulation. Same dose of rat IgG1 (R&D, Clone MAB005) and mouse IgG1 (R&D, Clone MAB002) antibodies were separately used as isotype control. Supernatants were collected after 3 days for assaying IL-17 production.

Isolation of γδ T cells

γδ T cells were sorted from mice splenocytes according to the manufacturer's protocol. Briefly, spleens were collected and lymphocytes were enriched by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). Lymphocytes were washed twice with PBS buffer containing 0.5% BSA and 2 mM EDTA. For 1×10^7 cells, 50 μL PBS buffer and 5 μL PE-conjugated anti-γδ T antibody (clone GL3, eBioscience) were added and incubated at 4°C for 10 min. Subsequently, unconjugated antibodies were washed twice with PBS buffer and cells were re-suspended in 50 μL buffer *per* 1×10^7 cells. Ten-microliter anti-PE-Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) was added and incubated at 4°C for 15 min. Finally, after two washes, cells were sorted using MACS Separator columns (Miltenyi Biotec) and separated into bound and un-bound fractions. The positive fraction was collected and purity was measured by flow cytometry. Data collection and analysis were performed on a FACScaliber flow cytometer using CellQuest software (Becton Dickinson, San Jose, CA, USA).

Cytometric analysis and intracellular cytokine staining

Ex vivo intracellular cytokine staining was performed, as previously reported. Briefly, 1 μg/mL brefeldin A, 50 ng/mL PMA and 1 μg/mL Ionomycin (all from Sigma-Aldrich) were added for the final 5–6 h of the incubation period. T lymphocytes incubated with DC were collected and stained with PE-conjugated anti-IL-17 (BD Biosciences, San Jose, CA, USA) and FITC-conjugated anti-γδTCR (eBioscience), while γδ T cell positives and negatives were stained with PE-conjugated anti-γδTCR (eBioscience) and FITC-conjugated anti-IL-17 (BD Biosciences). Cell were stained in PBS with 2% heat-inactivated FBS and 0.2% sodium azide and fixed using PBS with 1% paraformaldehyde. For intracellular staining, cells were first stained with antibodies specific to γδ TCR for 30 min and then fixed for 20 min with 1 mL fixation buffer (Fix and Perm cell permeabilization kit, eBioscience). After washing, the fixed cells were incubated with anti-mouse-IL-17 antibody for 30 min. PE-conjugated anti-CD88 (Abcam, Camdridge, UK) and FITC-conjugated anti-γδTCR (eBioscience) were used to detect C5aR expression on γδ T cells. Data collection and analysis were performed on a FACS Calibur flow cytometer using CellQuest software.

Cytokine analysis by ELISA

The concentration of IL-6, IL-17A and TGF-β1 was measured by ELISA kits (eBioscience). Absorbance was measured on an automatic plate reader. For measurement of the IL-17A level secreted by γδ T cells, bound and un-bound fractions collected after column separation were seeded into round-bottomed 96-well plates (Costar). Cytokine IL-23 (10 ng/mL) (R&D) was added, and supernatants were collected after 2 days for ELISA analysis.

Statistical analysis

Data analysis was performed with SPSS version 13.0 for Windows software (SPAA, Chicago, IL, USA) and expressed as mean ± SD for percentages. Significance of difference between the two groups was determined by applying the Mann–Whitney nonparametric *U* test. Multiple comparisons with Kruskal–Wallis H nonparametric test was applied with Bonferroni step-down (Holm) correction. Actuarial overall survival rates were analyzed by the Kaplan–Meier method. Values of $p < 0.05$ were considered significant.

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Abbreviations: **C5a:** complement 5a · **C5aR:** complement 5a receptor · **CLP:** cecal ligation and puncture · **DT:** diphtheria toxin

Full correspondence: Professor Yan Li, Department of Molecular Immunology, Institute of Basic Medical Sciences, Taiping Road, No.27, Beijing 100850, P. R. China
Fax: +8610-68159436
e-mail: liyan62033@yahoo.com.cn

Additional correspondence: Renfeng Guo Department of Pathology, University of Michigan, Ann Arbor, MI, USA
E-mail: grf@med.umich.edu

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