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*J Immunol* 2003; 171:6198-6205; ;  
doi: 10.4049/jimmunol.171.11.6198  
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The American Association of Immunologists, Inc.,  
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



# Aberrant Inflammation and Lethality to Septic Peritonitis in Mice Lacking STAT3 in Macrophages and Neutrophils<sup>1</sup>

Akihiro Matsukawa,<sup>2\*</sup> Kiyoshi Takeda,<sup>†</sup> Shinji Kudo,<sup>\*</sup> Takako Maeda,<sup>\*</sup> Motoko Kagayama,<sup>\*</sup> and Shizuo Akira<sup>†</sup>

Stat3 is a transcription factor mediating anti-inflammatory properties of IL-10. In the present study, we demonstrate a pivotal role of Stat3 expressed in innate immune cells during septic peritonitis induced by cecal ligation and puncture (CLP). Mice with targeted disruption of Stat3 in macrophages and neutrophils were succumbed to septic peritonitis induced by CLP. The mice displayed an excessive local and systemic inflammation relative to the control mice, an event that was accompanied by substantial increases in the level of multiple cytokines. Hepatic and renal injury was significantly exacerbated in mice with Stat3 deficiency. Despite enhanced inflammatory responses, the mice failed to facilitate bacterial clearance as compared with the control mice. In addition, the mice exhibited an increased lethality after i.p. inoculation of live bacteria recovered from CLP-mice. In vitro, resident peritoneal macrophages from mice with Stat3 deficiency impaired bactericidal activity relative to the control whereas productions of inflammatory cytokines were significantly augmented when cells were stimulated with a synthetic lipopeptide, macrophage-activating lipopeptide-2 and LPS. Elicited macrophages and neutrophils with Stat3 deficiency also impaired bactericidal activity as compared with those with Stat3. Lysosomal enzyme release, an effector molecule for bacterial clearance, was significantly decreased in elicited leukocytes with Stat3 deficiency while increasing the production of inflammatory cytokines. Altogether, these results suggest that macrophage/neutrophil-specific STAT3 is crucial in not only modulating multiple organ failure associated with systemic inflammation but also intensifying the bactericidal activity, which highlight the significance of cell-specific Stat3 in the protective immunity during sepsis. *The Journal of Immunology*, 2003, 171: 6198–6205.

Sepsis is an infection-induced syndrome with multiple physiologic and immunologic abnormalities, which is commonly caused by bacterial infection (1, 2). The innate immune response is the first-line of host defense in infection, in which infiltrating leukocytes such as macrophages and neutrophils are essential, enabling the host to achieve efficient removal of invading microbes (3, 4). However, the inflammatory response primarily directing microbes can be excessive, which may in turn become pathologic, self-destructive, and fatal to the host (5, 6). The inflammatory response is regulated by a set of pro- and anti-inflammatory cytokines. An imbalance in the cytokine response may allow infection to become established or may result in an uncontrolled systemic inflammatory response (7–9).

Cytokines use complex signaling cascades to elicit their biological effects. Stat proteins are fundamental transcription factors that mediate cytokine signaling and are implicated in a variety of immune responses (10, 11). Although most of the studies to date have focused on the role of Stat proteins in T cells or T cell-related acquired immunity, it is now evident that Stat proteins are also involved in innate immunity. In a murine model of septic peritonitis induced by cecal ligation and puncture (CLP)<sup>3</sup>, we have shown that mice deficient in Stat4 and Stat6 are resistant to the lethality by balancing local type-1 and systemic type-2 cytokine responses (12). Stat3 is a key transcriptional factor for several cytokines including anti-inflammatory cytokine IL-10 (13). Recently, we have demonstrated that Stat3 activation in macrophages is indispensable for IL-10 mediated anti-inflammatory responses in a murine model of endotoxin shock (14).

To further identify the functional role of Stat3 in innate immune cells during sepsis, we here used a murine model of sepsis induced by CLP. CLP is a clinically relevant model of intraabdominal sepsis that develops slowly and simulates a polymicrobial enteric insult similar to that seen in patients with colonic perforations (15). In the present study, we demonstrate that mice with Stat3 deficiency in macrophages and neutrophils are highly susceptible to sepsis induced by CLP. The increased lethality resulted from aberrant inflammation, characterized by excessive systemic inflammatory responses with serious multiple organ failure (MOF). The event was accompanied by overzealous production of inflammatory cytokines. Furthermore, we show novel findings that Stat3 activation is fundamental for effective bacterial clearance by innate immune cells. These results suggest a new paradigm for the role of Stat3 in host defense during sepsis.

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## Materials and Methods

### Mice

Mice with a cell type-specific disruption of Stat3 gene in macrophages and neutrophils were generated as described (14). In brief, LysMcre mice in

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Received for publication July 7, 2003. Accepted for publication September 30, 2003.

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<sup>1</sup> This work was supported in part by grants from the Ministry of Education, Science, Sports and Culture, Japan, and the Ministry of Health and Welfare, Japan.

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<sup>3</sup> Abbreviation used in this paper: CLP, cecal ligation and puncture; MOF, multiple organ failure; TSA, trypticase soy agar; AST, aspartate transaminase; ALT, alanine transaminase; BUN, blood urea nitrogen; SAA, serum amyloid A; MPO, myeloperoxidase; MALP-2, macrophage-activating lipopeptide-2; APP, acute phase protein; SIRS, systemic inflammatory response syndrome.

which the *cre* cDNA was inserted into the mouse *lysozyme M* gene (a mixed 129/Ola, C57BL/6 and CB.20 background) were crossed to mice heterozygous for a Stat3 null mutation (16), leading to the generation of mice that carried the *cre* gene and the heterozygous Stat3 null mutation (LysMcre/Stat3<sup>+/-</sup> mice). LysMcre/Stat3<sup>+/-</sup> mice were further mated with a mouse in which the Stat3 gene was flanked by two loxP sites (Stat3<sup>lox/+</sup> mice) to generate LysMcre/Stat3<sup>lox/+</sup> mice. LysMcre/Stat3<sup>lox/+</sup> from these crosses were used as wild-type littermate controls in the experiments. Despite the residual STAT3 protein in peritoneal macrophages, cytokine-induced Stat3 activation was completely abolished in LysMcre/Stat3<sup>lox/+</sup> mice (14). Female mice (6–8 wk) were used in this study under specific pathogen-free conditions. The animal use committee at the Kumamoto University (Honjo, Japan) approved all studies.

### *In vivo experimental protocol*

The mice were subjected to CLP surgery, as described elsewhere (7, 15, 17). In brief, mice were anesthetized and the cecum was exposed, ligated with a 3–0 silk suture below the ileocecal valve, and punctured through and through once with an 18-gauge needle. In the first set of experiments, CLP mice were monitored for 7 days after CLP to determine mice survival. In the second set of experiments, CLP mice were anesthetized, bled, and euthanized at appropriate intervals after CLP. The peritoneal cavities were washed with 1 ml × 2 of sterile saline, and the lavage fluids were harvested. After taking a 10- $\mu$ l aliquot for bacteria CFU, the lavage fluids were centrifuged and the resultant cell-free peritoneal fluids were stored at –50°C. Ten microliters of peritoneal lavage fluids and peripheral blood from each mouse were serially diluted with sterile saline. Five microliters of each dilution was aseptically plated on Trypticase Soy Agar (TSA) plates with 5% sheep blood and incubated overnight at 37°C, after which the number of aerobic bacteria colonies was counted. Cell pellets were resuspended in saline and the cell numbers were counted in a hemocytometer. Differential cell analysis was made after Diff-Quik staining of cyto-spin preparations. The liver and kidney were excised, snap frozen in liquid nitrogen, and stored at –80°C. A part of tissues was fixed in 4% paraformaldehyde, embedded in paraffin, and the tissue sections were stained with H&E or masson-trichrome. In other experiments, mice were i.p. inoculated with live bacteria ( $2 \times 10^5$  CFU/mouse) recovered from CLP mice, after which the survival rates were monitored. For this, peritoneal lavage fluids at 24 h after CLP were plated on TSA-blood plates and incubated overnight at 37°C. The resultant aerobic colonies were resuspended in saline and the CFU was titrated.

### *Clinical chemistry and endotoxin level*

Levels of aspartate transaminase (AST), alanine transaminase (ALT), blood urea nitrogen (BUN), creatinine, serum amyloid A (SAA), and albumin were measured with human assay systems. Fibrinogen level in plasma was measured by sandwich ELISA using anti-murine fibrinogen IgG (Nordic, Tilburg, The Netherlands) and murine fibrinogen (Molecular Innovations, Southfield, MI). Plasma endotoxin level was assessed with a QCL-1000 kit (Daiichi Pure Chemicals, Tokyo, Japan).

### *Measurement of cytokines and myeloperoxidase (MPO)*

Murine cytokines were measured using a standard method of sandwich ELISA, as described (7, 12). The captured Abs, detection Abs and the recombinant cytokines were purchased from R&D Systems (Minneapolis, MN). The ELISAs used in this study did not cross-react with other murine cytokines available. Measurement of MPO levels in liver extracts was measured by ELISA kit (Calbiochem, San Diego, CA). The livers were homogenized in PBS containing 0.1% TritonX-100 and complete protease inhibitor (Roche, Mannheim, Germany), centrifuged, and the cleared supernatants were obtained. Protein concentrations in the extracts were measured by protein-dye binding assay (Bio-Rad, Hercules, CA).

### *Western blotting*

Leukocytes were dissolved in Laemmli buffer ( $1 \times 10^6/50 \mu$ l), sonicated, boiled, fractionated on SDS-polyacrylamide gel ( $10 \mu$ l), and transferred to a nitrocellulose membrane. After blocking with TBS + 0.1% Tween 20 containing 5% skim milk for 1 h at room temperature, the membrane was incubated with Abs to STAT3 or tyrosine-phosphorylated STAT3 (Cell Signaling, Beverly, MA) overnight at 4°C. After washing with TBS + 0.1% Tween 20, the membrane was incubated with anti-HRP-linked Ab for 1 h at room temperature and visualized with an enhanced chemiluminescence system (Cell Signaling).

### *In vitro bactericidal activities of leukocytes*

Peritoneal cells were harvested from non-treated mice, cultured for 1 h at 37°C ( $3 \times 10^6$ /ml) in antibiotic-free RPMI 1640 containing 5% FCS, and the adherent cells were used as resident macrophages. Infiltrating leukocytes were harvested at 18 h after i.p. injection of 1 ml of 4% thioglycolate, washed, and placed in 24-well culture plates ( $3 \times 10^6$ /ml) in antibiotic-free RPMI 1640 containing 5% FCS. Most of the cells were alive (>97%, trypan blue exclusion) and consisted of macrophages and neutrophils. The populations were similar between LysMcre/Stat3<sup>lox/+</sup> mice and LysMcre/Stat3<sup>+/+</sup> mice (50–60% neutrophils). Lymphocytes were <5% in total leukocytes. In some experiments, neutrophils were isolated from elicited leukocytes (>94%) by Ficoll gradient centrifugation. To determine in vitro bactericidal activities of cells, a classical CFU assay was used with minor modifications (18). In brief, cells were infected with  $1 \times 10^6$  CFU of live bacteria recovered from CLP-mice. Control wells contained only bacteria. After a 4-h culture in a 5% CO<sub>2</sub> incubator, plates were placed at –80°C for 30 min and cells were lysed by thawing. This did not affect bacteria viability, as determined in control experiments. The lysates were serially diluted, plated on TSA-blood plates, incubated overnight at 37°C, and the number of aerobic colonies were counted. Bactericidal activity was expressed as the percentage of bacteria death = [CFU from control wells (without cells) – CFU from experimental wells]/[CFU from control wells (without cells)] × 100.

### *Cell culture*

Resident peritoneal macrophages and infiltrating leukocytes, prepared as described above, were cultured in RPMI 1640 supplemented with 5% FCS, glutamine and antibiotics in a 5% CO<sub>2</sub> incubator for 24 and 48 h with a TLR2-ligand, macrophage-activating lipopeptide-2 (MALP-2) (10 ng/ml, Alexis Biochemicals, Montreal, Canada) or a TLR4-ligand, *Escherichia coli* LPS (100 ng/ml, 0111, B4; Difco, Detroit, MI). The culture supernatants were used for measurements of cytokines and lysosomal enzyme release, which was assessed by measuring  $\beta$ -glucuronidase activity (19). In other experiments, spleens were excised from CLP-mice post-24 h and spleen cells were dispersed into single-cell suspensions. After lysing RBC, the cells ( $5 \times 10^6$ /ml) were suspended in RPMI 1640 supplemented with 5% FCS, glutamine and antibiotics and cultured in a 5% CO<sub>2</sub> incubator for 48 h without stimulation.

### *Statistics*

Statistical significance was evaluated by ANOVA. In case of survival curve and CFU count, the data were analyzed by the log-rank test and Mann-Whitney *U* test, respectively. A *p* < 0.05 value was regarded as statistically significant. All data were expressed as mean ± SE.

## **Results**

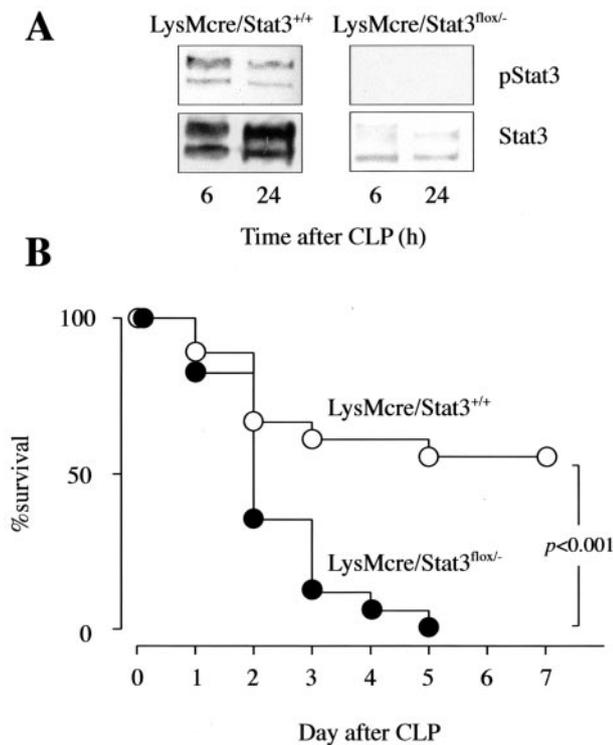
### *Increased lethality to CLP in mice lacking Stat3*

In initial investigations, we examined whether Stat3 was activated in the infiltrating leukocytes during CLP. CLP permitted the recruitment of neutrophils and macrophages in the peritoneum. Lymphocytes were minor populations within 24 h post CLP (<5%). As shown in Fig. 1A, Stat3 in infiltrating leukocytes was tyrosine-phosphorylated in Stat3<sup>+/+</sup> control littermates, indicating that the Stat3 was activated during CLP. In contrast, the phosphorylated-Stat3 was completely abrogated in mice with Stat3 deficiency in macrophages and neutrophils despite the negligible Stat3 protein in the infiltrating leukocytes (Fig. 1A).

To determine the role of the Stat3 in host defense during CLP, LysMcre/Stat3<sup>lox/+</sup> mice and the control littermates were monitored after CLP. The data in Fig. 1B demonstrated that the survival rate in LysMcre/Stat3<sup>lox/+</sup> mice was significantly lower than that in the control mice. All LysMcre/Stat3<sup>lox/+</sup> mice died within 5 days while 56% (10/18 mice) of the control mice survived for 7 days (Fig. 1B). Thus, Stat3 associated with macrophages and neutrophils was beneficial in host defense during CLP.

### *Enhanced local inflammation in mice lacking Stat3*

In an attempt to identify the mechanism underlying the increased lethality, we first investigated the leukocyte influx in the peritoneum during CLP. The data in Fig. 2 showed that the numbers of



**FIGURE 1.** Mice lacking Stat3 in macrophages and neutrophils are susceptible to CLP-induced lethality. *A*, Infiltrating leukocytes (macrophages and neutrophils) were harvested from LysMcre/Stat3<sup>lox/-</sup> mice and the control littermates (LysMcre/Stat3<sup>+/+</sup> mice) after CLP, and the lysates were immunoblotted with anti-Stat3 or anti-tyrosine-phosphorylated Stat3. *B*, The survival rates in LysMcre/Stat3<sup>lox/-</sup> mice (17 mice) and LysMcre/Stat3<sup>+/+</sup> mice (18 mice) were monitored for 7 days after CLP.

infiltrating neutrophils and macrophages were significantly augmented in LysMcre/Stat3<sup>lox/-</sup> mice, resulting in a 2.3- and 1.7-fold increase, respectively, at 24 h post-CLP as compared with the control mice. Given previous data implicating Stat3 in apoptosis (20), we examined leukocyte apoptosis at 6 and 24 h after CLP. Consequently, most of the leukocytes from LysMcre/Stat3<sup>lox/-</sup> mice and control mice were alive (>94%) as determined by trypan

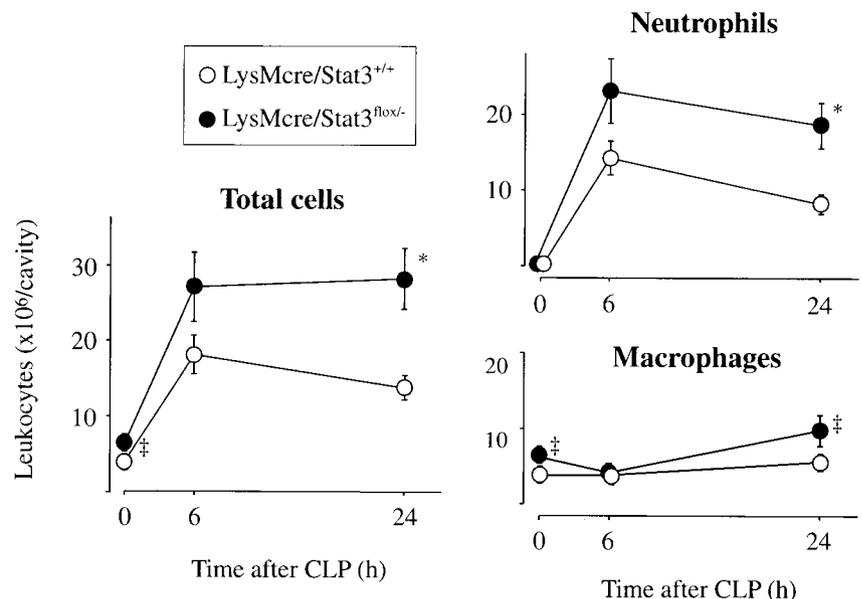
blue exclusion. DNA ladder pattern was not detected on electrophoresis (data not shown). We then measured peritoneal levels of cytokines during CLP, which showed that LysMcre/Stat3<sup>lox/-</sup> mice displayed dramatic increases in the peritoneal level of TNF- $\alpha$ , IL-1 $\beta$ , IL-12, and IFN- $\gamma$ , relative to the controls (Fig. 3). In addition, levels of IL-6 and IL-10, cytokines using Stat3 in their signaling, were elevated in LysMcre/Stat3<sup>lox/-</sup> mice. Thus, mice lacking Stat3 in macrophages and neutrophils exhibited an exaggerated local inflammation that was accompanied by increases in the peritoneal level of multiple cytokines, but not associated with apoptosis.

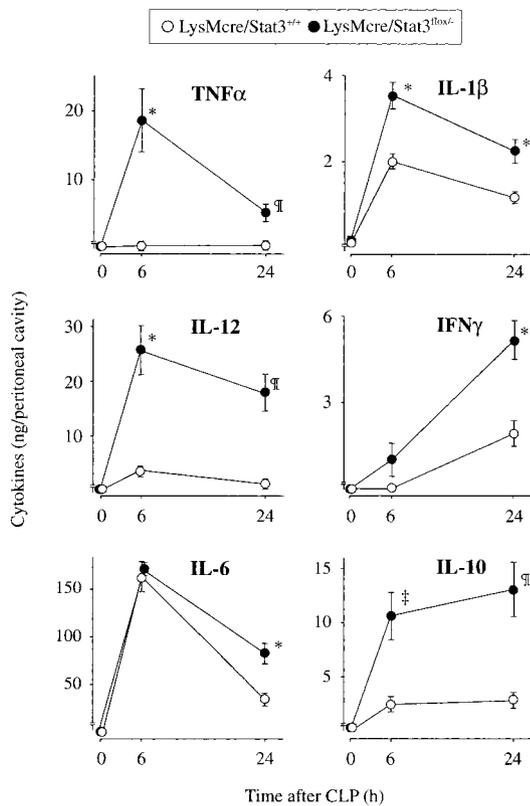
#### Exacerbated systemic inflammation and organ damage in mice lacking Stat3

Plasma cytokine level was next investigated, which demonstrated that levels of circulating TNF- $\alpha$ , IL-1 $\beta$ , IL-12, IFN- $\gamma$ , IL-6, and IL-10 were dramatically increased in LysMcre/Stat3<sup>lox/-</sup> mice relative to the controls (Fig. 4A). In addition, spleen cells from LysMcre/Stat3<sup>lox/-</sup> mice that have undergone CLP spontaneously released higher levels of IL-12 and IFN- $\gamma$  than did cells from the control mice (Fig. 4B), suggesting that systemic inflammation might be deteriorated in LysMcre/Stat3<sup>lox/-</sup> mice. Subsequently, the severity of systemic inflammation was examined. Acute phase protein (APP) is a marker of systemic inflammation, in which fibrinogen and SAA are positive APP while serum albumin is a negative APP (21). As shown in Table I, LysMcre/Stat3<sup>lox/-</sup> mice exhibited increased levels of fibrinogen and SAA while decreasing albumin levels relative to the controls, thereby indicating an exaggerated systemic inflammation in mice lacking Stat3 in macrophages and neutrophils.

Systemic inflammation during sepsis frequently causes MOF, in which the liver and kidney are major target organs (1, 2). CLP resulted in significant increases in the plasma level of AST and ALT, markers of hepatic injury, in the control mice, and the level was much higher in LysMcre/Stat3<sup>lox/-</sup> mice (Fig. 5). The liver injury during sepsis generally arises from an excessive hepatic inflammation (22). The hepatic level of MPO, an indirect means to determine the recruitment of neutrophils, was significantly increased in LysMcre/Stat3<sup>lox/-</sup> mice relative to the controls (Fig. 6A). Histologically, CLP induced neutrophil infiltration in the liver, an event that was much more severe in LysMcre/Stat3<sup>lox/-</sup>

**FIGURE 2.** The leukocyte infiltration in the peritoneum after CLP. After CLP surgery, the numbers of infiltrating leukocytes in the peritoneum were counted (6–15 mice, each point). ‡,  $p < 0.05$ , \*,  $p < 0.01$ , vs control.





**FIGURE 3.** Mice lacking Stat3 in macrophages and neutrophils augment the production of cytokines in the peritoneum. Peritoneal fluids were harvested after CLP, and levels of cytokines were measured (7–15 mice, each point). ‡,  $p < 0.05$ , \*,  $p < 0.01$ , ¶,  $p < 0.0001$ , vs control.

mice (Fig. 6B). The kidney injury was more profound in LysMcre/Stat3<sup>lox/-</sup> mice as determined by plasma level of BUN and creatinine (Fig. 5). Although CLP caused little histological change in

the control mice, epithelial cells in proximal tubules were vacuolated in LysMcre/Stat3<sup>lox/-</sup> mice (Fig. 6C). The kidney excised immediately after the death of LysMcre/Stat3<sup>lox/-</sup> mice demonstrated karyorrhexis and grainy pink cytoplasm of epithelial cells in proximal tubules, indicative of acute tubular necrosis (Fig. 6D). Taken together, these data suggest that mice lacking Stat3 in macrophages and neutrophils deteriorated organ damage associated with exacerbated systemic inflammation via overzealous productions of cytokines.

*Bacterial clearance in mice lacking Stat3*

Bacterial burden is tightly associated with the mortality in this model (17, 23). We next investigated bacterial load in these mice, which demonstrated that LysMcre/Stat3<sup>lox/-</sup> mice contained comparable levels of viable bacteria in the peritoneum as compared with the controls (Fig. 7A). Mice with bacteremia at 24 h post-CLP were 4/13 in LysMcre/Stat3<sup>lox/-</sup> mice and 3/19 in the control mice (not significant). Plasma endotoxin level released from bacteria was then quantitated, which showed that there was no statistical difference between the groups (Fig. 7B). Furthermore, i.p. inoculation of live bacteria recovered from CLP-mice resulted in 56% lethality in LysMcre/Stat3<sup>lox/-</sup> mice (9/16 mice) whereas none of the control mice died (0/15 mice) by the treatment (Fig. 7C). Under the conditions, the number of infiltrating leukocytes and levels of cytokines in the peritoneum were much higher in LysMcre/Stat3<sup>lox/-</sup> mice as compared with the controls (data not shown). Thus, mice lacking Stat3 in macrophages and neutrophils failed to facilitate effective bacterial clearance despite increased numbers of infiltrating leukocytes and enhanced cytokine production. The data also suggest that impaired bacterial clearance is not likely the cause of increased lethality in LysMcre/Stat3<sup>lox/-</sup> mice.

*Impaired innate immune response in leukocytes lacking Stat3*

We hence examined bactericidal activity of leukocytes. As shown in Fig. 8A, bactericidal activity in resident peritoneal macrophages from LysMcre/Stat3<sup>lox/-</sup> mice was significantly lower than those

**FIGURE 4.** Enhanced systemic cytokine production in mice lacking Stat3 in macrophages and neutrophils. *A*, Mice were bled after CLP, and plasma levels of cytokines were measured (7–15 mice, each point). Cytokines from untreated mice were below detection level. *B*, Spleen cells undergone CLP (eight mice each) were cultured for 48 h without stimulation, and IL-12 and IFN-γ level in the supernatants was measured. ‡,  $p < 0.05$ , \*,  $p < 0.01$ , §,  $p < 0.001$ , vs control.

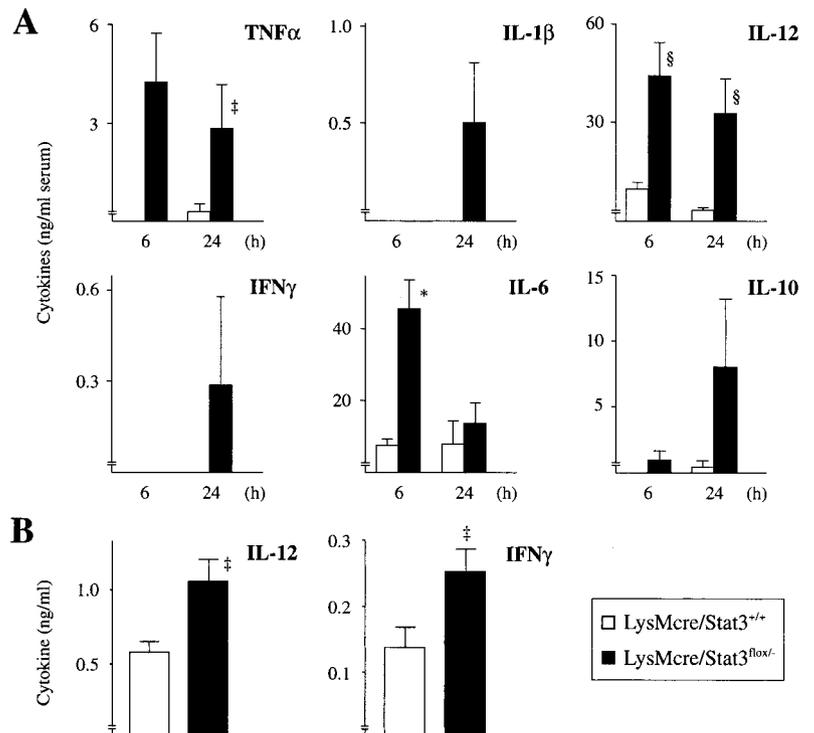


Table I. Acute phase proteins in mice lacking STAT3 in macrophages and neutrophils<sup>a</sup>

|                   | Before CLP                   |                                | After CLP                    |                                |
|-------------------|------------------------------|--------------------------------|------------------------------|--------------------------------|
|                   | LysMcre/Stat3 <sup>+/+</sup> | LysMcre/Stat3 <sup>lox/-</sup> | LysMcre/Stat3 <sup>+/+</sup> | LysMcre/Stat3 <sup>lox/-</sup> |
| Fibrinogen (g/dl) | 0.4 ± 0.1                    | 0.3 ± 0.1                      | 1.7 ± 1.4                    | 10.6 ± 3.0 <sup>‡</sup>        |
| SAA (μg/ml)       | <2.5                         | <2.5                           | 4.8 ± 0.5                    | 9.6 ± 1.1 <sup>§</sup>         |
| Albumin (g/dl)    | 4.3 ± 0.1                    | 4.1 ± 0.6                      | 3.2 ± 0.1                    | 2.9 ± 0.1 <sup>‡</sup>         |

<sup>a</sup> Levels of fibrinogen in plasma (6 h), SAA, and albumin (24 h) in sera after CLP were measured (6–10 mice, each point).

<sup>‡</sup>, *p* < 0.05, <sup>§</sup>, *p* < 0.001, vs LysMcre/Stat3<sup>+/+</sup> mice.

from the controls (40 and 63%, respectively), although productions of TNF- $\alpha$ , IL-1 $\beta$ , IL-12, and IFN- $\gamma$  were augmented when cells were stimulated with MALP-2 and LPS (Table II). Similarly, the activity in elicited leukocytes from LysMcre/Stat3<sup>lox/-</sup> mice was significantly reduced to 25% while it was 52% in the controls (Fig. 8B). When neutrophils were isolated from elicited leukocytes (>94%), the bactericidal activity in cells from LysMcre/Stat3<sup>lox/-</sup> mice was also lower than the controls (65.3 ± 9.7% vs 86.5 ± 0.9%, *p* < 0.01). Thus, bactericidal activity appeared to be impaired in both macrophages and neutrophils with Stat3 deficiency. Although the elicited leukocytes lacking Stat3 produced significantly higher levels of inflammatory cytokines than those with Stat3 (not shown), Stat3-deficient leukocytes did not release  $\beta$ -glucuronidase (lysosomal enzyme) in response to MALP-2 and LPS whereas it was augmented in the control leukocytes (Fig. 8C).  $\beta$ -glucuronidase level in resident macrophages with Stat3 deficiency was also lower than the controls (not shown).

## Discussion

Inflammatory response is a fundamental host response in infection. Recently, we have demonstrated that Stat6 deficient mice augment local inflammatory response during CLP, which successfully restrict the invading pathogens to the peritoneum, resulting in reduced systemic response that can cause organ damage (12). Thus, augmented local inflammation is beneficial to the host. However, despite enhanced bacterial clearance due to an augmented local inflammation, IL-10 deficient mice were susceptible to infection resulting from an uncontrolled systemic inflammatory response (24–26). Therefore, unrestrained systemic inflammatory response appears to be more fatal than the original inciting pathogens. Because Stat3 is a transcription factor mediating anti-inflammatory effects of IL-10 (14), we had assumed together with an importance of macrophages and neutrophils in infection that mice lacking Stat3 in these cells would be deleterious during CLP.

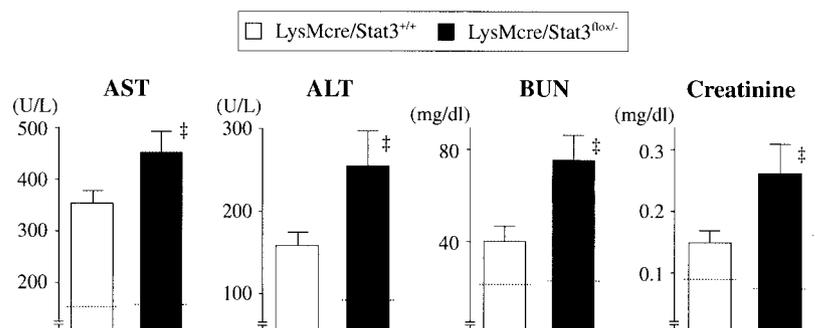
In the present study, we have demonstrated that mice with Stat3 deficiency in macrophages and neutrophils are highly susceptible to septic peritonitis induced by CLP. An aberrant inflammation is likely the mechanism underlying the increased lethality. The number of infiltrating leukocytes in the peritoneum was significantly

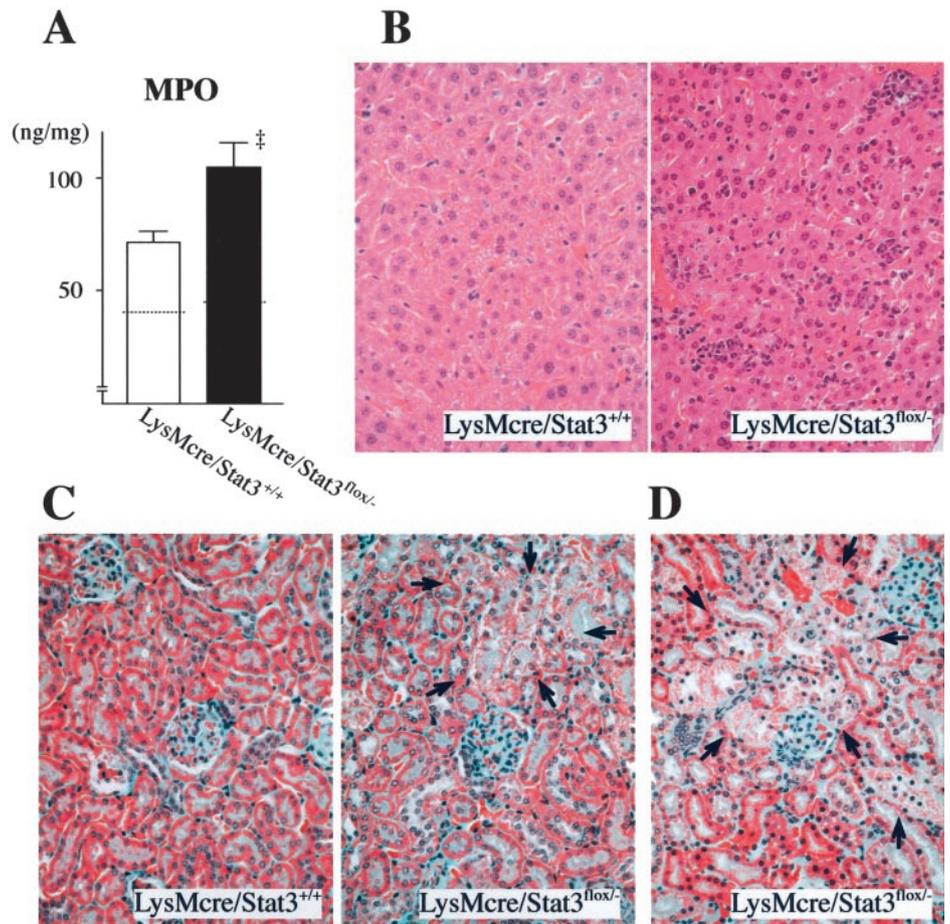
augmented in mice lacking Stat3, which was accompanied by significant increases in peritoneal level of inflammatory cytokines, accounting for the exaggerated local inflammatory response. Furthermore, the mice exhibited dramatic increases in plasma level of multiple cytokines (hypercytokinemia) that include IL-6. IL-6 level is well correlated with a fatal outcome in patients with sepsis, reflecting the clinical severity (27). Hypercytokinemia has been implicated in the development of systemic inflammatory response syndrome (SIRS) or MOF (8). In this model, the mice displayed exaggerated systemic inflammation and organ damage as evidenced by clinical chemistry and histopathology.

Of interest are the findings that Stat3 deficiency exclusively in macrophages and neutrophils has significant impact on the regulation of inflammatory responses during CLP. Stat3 is ubiquitously expressed in other cell types such as endothelial cells, mast cells, dendritic cells and lymphocytes, cells capable of producing and responding to IL-10 (14, 28–30), and IL-10 level in mice lacking Stat3 was much higher than that in the control. These results suggest that Stat3 expressed in macrophages and neutrophils, but not other cells types, play a key role in modulating local and systemic inflammatory responses during sepsis. Macrophages and neutrophils are major cell populations producing cytokines in various types of inflammation and play pivotal roles in the protective immunity in infection (3, 8). Thus, the present data highlight evidence that these innate immune cells appear to be essential not only for the initiation and development of inflammatory response but also the resolution of the response, playing multifunctional roles in innate immunity during sepsis.

Effective bacterial clearance depends on the accumulation and activation of macrophages and neutrophils (17, 23, 31). Recently, we demonstrated that Stat6 deficient mice facilitated bacterial clearance due to enhanced local inflammatory response (12). We had initially hypothesized that the mice lacking Stat3 in macrophages and neutrophils would exhibit enhanced bacterial clearance, as the mice displayed a polarized immune response toward type-1 response, which is beneficial to infection. In contrast to the assumption, the bacterial clearance was not augmented in mice with Stat3 deficiency, suggesting a possibility that bactericidal activity of leukocytes may be impaired in the mice. Interestingly, the

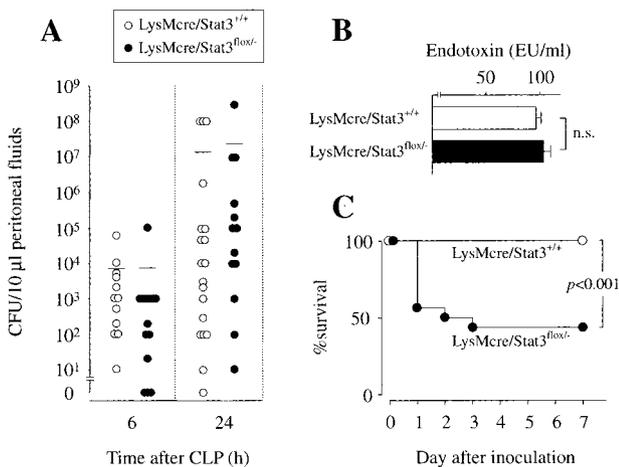
**FIGURE 5.** Exacerbated organ damage in mice lacking Stat3 in macrophages and neutrophils. At 6 h after CLP, mice were bled and euthanized. AST, ALT, BUN, and creatinine level in plasma was measured (9–10 mice, each point). <sup>‡</sup>, *p* < 0.05, vs control. Dotted line represents the mean data from untreated mice (six mice each).



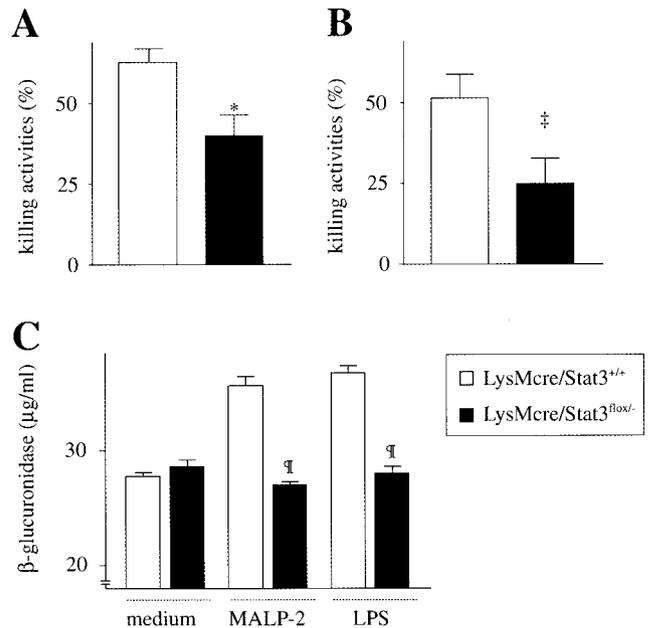


**FIGURE 6.** Hepatic and renal injury during CLP. At 24 h after CLP, mice were bled and euthanized. *A*, MPO level in liver extracts at 24 h after CLP (eight mice each). ‡,  $p < 0.05$ , vs control. Dotted line represents the mean data from untreated livers (six mice each). *B* and *C*, Shown are representative histological sections of the liver (*B*, H&E) and kidney (*C*, masson-trichrome) at 24 h after CLP. Original magnification  $\times 200$ . *D*, Representative histological sections with masson-trichrome staining of kidneys from dead mice lacking Stat3 in macrophages and neutrophils. Original magnification  $\times 200$ .

activity was significantly decreased in Stat3-deficient macrophages and neutrophils, which was accompanied by a significant decrease in the release of an effector molecule for bacterial clearance from the cells (lysosomal enzyme) although productions of cytokines were amazingly augmented. Similar data were recently reported, in



**FIGURE 7.** Bacterial clearance in mice lacking Stat3 in macrophages and neutrophils. *A*, Peritoneal fluids and blood were harvested after CLP (8–19 mice, each point). Five microliters of samples was serially diluted and plated on TSA blood agar plates. Line represents mean CFU count. *B*, Endotoxin level in plasma at 24 h after CLP was measured (eight mice each). *C*, Mice were i.p. inoculated with live bacteria ( $2 \times 10^8$  CFU/mouse), and the survival rates in LysMcre/Stat3<sup>flox/-</sup> mice (16 mice) and LysMcre/Stat3<sup>+/+</sup> mice (15 mice) were monitored.



**FIGURE 8.** Bactericidal activities in leukocytes lacking Stat3 in macrophages and neutrophils. Resident peritoneal macrophages and elicited leukocytes (macrophages and neutrophils) were harvested, as described in the text. *A* and *B*, Resident peritoneal macrophages (*A*) and elicited leukocytes (*B*) were infected with bacteria and the killing activity was examined. *C*, Elicited leukocytes were cultured with MALP-2 (10 ng/ml) or LPS (100 ng/ml) for 48 h. Lysosomal enzyme release in the culture supernatants were examined by measuring  $\beta$ -glucuronidase. ‡,  $p < 0.05$ , \*,  $p < 0.01$ , ¶,  $p < 0.0001$ , vs control.

Table II. Cytokine production from resident peritoneal macrophages<sup>a</sup>

|        | TNF- $\alpha$                | IL-1 $\beta$                 | IL-12                        | IFN- $\gamma$    |
|--------|------------------------------|------------------------------|------------------------------|------------------|
| Medium | 0.06 $\pm$ 0.02              | 0.08 $\pm$ 0.04              | 0.01 $\pm$ 0.01              | <0.01            |
|        | 0.20 $\pm$ 0.03              | 0.12 $\pm$ 0.04              | 0.42 $\pm$ 0.05 <sup>‡</sup> | <0.01            |
| MALP-2 | 0.09 $\pm$ 0.01              | 0.27 $\pm$ 0.02              | 0.04 $\pm$ 0.01              | 0.03 $\pm$ 0.03  |
|        | 0.61 $\pm$ 0.02 <sup>¶</sup> | 0.33 $\pm$ 0.01 <sup>‡</sup> | 1.96 $\pm$ 0.09 <sup>¶</sup> | 0.19 $\pm$ 0.02* |
| LPS    | 0.11 $\pm$ 0.01              | 0.24 $\pm$ 0.01              | 0.04 $\pm$ 0.01              | 0.10 $\pm$ 0.04  |
|        | 0.80 $\pm$ 0.02 <sup>¶</sup> | 0.35 $\pm$ 0.01 <sup>¶</sup> | 2.17 $\pm$ 0.01 <sup>¶</sup> | 0.23 $\pm$ 0.01* |

<sup>a</sup> Peritoneal resident macrophages were cultured with MALP-2 (10 ng/ml) or LPS (100 ng/ml) for 24 h. Cytokine level in the supernatants was measured (nanograms per milliliter,  $n = 6$ ). Upper/Lower; LysMcre/Stat3<sup>+/+</sup>/LysMcre/Stat3<sup>lox/-</sup> macrophages. Shown are representative data from three independent experiments.

<sup>‡</sup>,  $p < 0.05$ , \* $p < 0.01$ , <sup>¶</sup>,  $p < 0.0001$  vs LysMcre/Stat3<sup>+/+</sup> macrophages.

which NADPH oxidase activity of neutrophils that involved in antimicrobial response was reduced in mice with Stat3 deletion in bone marrow cells (32). Thus, Stat3 appears to be a key mediator for bactericidal activity of innate immune cells. Increased numbers of these cells in mice lacking Stat3 appeared to compensate for the lack of host defense ability of these cells, resulting in an unchanged bacterial clearance in mice during CLP.

The precise mechanism whereby macrophages and neutrophils lacking Stat3 derivate bactericidal activity is unknown. We and others have shown that IFN- $\gamma$ , a cytokine with powerful bactericidal activity, also activates Stat3 in neutrophils (33) and macrophages (14), suggesting a possibility that the lack of anti-microbial activity in Stat3-deficient macrophages and neutrophils may be due to the absence of Stat3. However, we have demonstrated that IFN- $\gamma$ -mediated signaling is not impaired in peritoneal macrophages lacking Stat3 (14). Stat1 is a transcription factor primarily activated by IFN- $\gamma$  (34), and Stat1-deficient mice have been shown highly sensitive to microbial pathogens with loss of NO production, an effector molecule for killing pathogens, by macrophages after the stimulation with IFN- $\gamma$  (35). These results suggest that the bactericidal activity of IFN- $\gamma$  appears to be mediated by Stat1. Possible mechanisms may involve suppressor of cytokine-signaling-1 and -3, which are activated by bacterial component LPS and multiple cytokines including TNF- $\alpha$ , and in turn may suppress the activity of several cytokines that include IFN- $\gamma$  (36, 37). Studies are ongoing to more fully characterize the mechanism(s).

Taken together, these results clearly demonstrate Stat3 associated with macrophages and neutrophils as an important regulator of innate immunity during sepsis. Lacking Stat3 in these cells caused aberrant inflammation, resulting in an increased lethality. The treatment of sepsis and septic shock remains a clinical conundrum (38). The present results in addition to a murine model of endotoxin shock (14) suggest that increasing Stat3 expression in macrophages and neutrophils at the right time may represent a promising new approach for the treatment of sepsis, especially in a fatal condition known as SIRS. An attractive question is how Stat3 is regulated in patients with SIRS. Stat may be involved in the later phase of infection, as over- or prolonged production of IL-10 accounts for compensatory anti-inflammatory response syndrome (9), a fatal condition in the later stage of sepsis. Another question is whether the Stat3 deletion would affect the innate and adaptive immunity to secondary bacterial infection. Further study will produce important insights into the pathogenesis of sepsis and its treatment strategy.

## Acknowledgments

We thank Dr. Irmgard Förster (Institute for Medical Microbiology, Immunology and Hygiene, Technical University of Munich, Munich, Germany) for providing LysMcre mice.

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