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Stat3 in Resident Macrophages as a Repressor Protein of Inflammatory Response¹

Akihiro Matsukawa,^{2*} Shinji Kudo,^{*} Takako Maeda,^{*} Kousuke Numata,^{*} Hiroyuki Watanabe,^{*} Kiyoshi Takeda,[†] Shizuo Akira,[‡] and Takaaki Ito^{*}

Inflammation is counterbalanced by anti-inflammatory cytokines such as IL-10, in which Stat3 mediates the signaling pathway. In this study, we demonstrate that resident macrophages, but not other cell types, are important targets of IL-10 in a murine model of acute peritonitis. Injection of thioglycollate i.p. induced a considerable number of neutrophils and macrophages in the peritoneum, which was significantly augmented in mice with a cell-type specific disruption of the Stat3 gene in macrophages and neutrophils (LysMcre/Stat3^{fllox/-} mice). The augmented leukocyte infiltration was accompanied by increased peritoneal levels of TNF- α , MIP-2, KC chemokine (KC), and MCP-1/CCL2. Stat3 was tyrosine phosphorylated in peritoneal resident macrophages as well as infiltrating leukocytes in the littermate controls, suggesting that Stat3 in either or both of these cells might play a regulatory role in inflammation. The peritoneal levels of TNF- α , MIP-2, KC, and MCP-1 were similarly elevated in LysMcre/Stat3^{fllox/-} mice rendered leukopenic by cyclophosphamide treatment as compared with the controls. Adoptive transfer of resident macrophages from LysMcre/Stat3^{fllox/-} mice into the control littermates resulted in increases in the peritoneal level of TNF- α , MIP-2, KC, and MCP-1 after i.p. injection of thioglycollate. Under these conditions, control littermates harboring LysMcre/Stat3^{fllox/-} macrophages exhibited an augmented leukocyte infiltration relative to those received control macrophages. Taken together, these data provide evidence that resident macrophages, but not other cell types, play a regulatory role in inflammation through a Stat3 signaling pathway. Stat3 in resident macrophages appears to function as a repressor protein in this model of acute inflammation. *The Journal of Immunology*, 2005, 175: 3354–3359.

Leukocyte infiltration into sites of infection is a fundamental host defense against invading pathogens where infiltrating leukocytes inactivate and clear the pathogens (1, 2). Thus, inflammation is primarily a beneficial host response to noxious stimuli. However, inflammation acts as a double-edged sword because an excessive and prolonged response can cause tissue damage, contributing to the pathogenesis of many disease states (3). Therefore, the response must be under the strict control of endogenous mechanism(s). Evidence indicates that inflammatory response is counterbalanced by anti-inflammatory cytokines that include IL-10 (4).

Stat proteins are transcription factors that mediate cytokine signaling and are implicated in a variety of immune responses (5). We have demonstrated in a murine model of septic peritonitis that mice deficient in Stat4 and Stat6 are resistant to the lethality by balancing local type 1 and systemic type 2 cytokine responses (6), thereby providing clear evidence that Stat proteins are important in innate immunity. Recently, we have shown that mice with targeted disruption of Stat3 gene in macrophages and neutrophils exhibit

exaggerated cytokine production and increased lethality in a murine model of endotoxin shock and septic peritonitis (7, 8). Stat3 is a transcription factor mediating anti-inflammatory effects of IL-10 (8, 9). IL-10 ameliorates the inflammatory responses by inhibiting the production of inflammatory cytokines. Although IL-10 has diverse effects on multiple cell types (4), our previous data in septic peritonitis model indicate that Stat3 associated with macrophages and neutrophils is an important regulator of inflammation (7). This suggests that resident macrophages or/and infiltrating leukocytes (macrophages and neutrophils) may be responsible for the regulation through a Stat3 signaling pathway.

To further address the role of Stat3 in inflammation, we have extended our previous work and attempted to characterize the mechanisms whereby Stat3 is involved in inflammation. For this purpose, mice lacking Stat3 in macrophages and neutrophils were injected i.p. with thioglycollate, and the subsequent inflammatory responses were investigated. In this study, we provide evidence that the Stat3 signaling pathway in peritoneal resident macrophages, but not other cell types, functions as a down-regulator of inflammation in a murine peritonitis model. Resident macrophages trigger inflammation by producing an array of cytokines and chemokines, but the production of these mediators is negatively regulated in the cells through a Stat3 signaling pathway.

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Materials and Methods

Mice

LysMcre/Stat3^{+/-} mice and Stat3^{fllox/+} mice were provided by Dr. S. Akira (Osaka University, Osaka, Japan). These mice were mated to generate LysMcre/Stat3^{fllox/-} mice, in which Stat3 was selectively disrupted in the myeloid cells (8). LysMcre/Stat3^{+/+} mice from these crosses were used as wild-type littermate controls in the experiments. Male mice (6–8 wk) were used in this study under specific pathogen-free conditions. The animal use committee at the Kumamoto University approved all studies.

Table I. *Infiltrating leukocytes in leukopenic mice^a*

	Neutrophils ($\times 10^6$ cells/cavity)		Macrophages ($\times 10^6$ cells/cavity)	
	LysM:Stat3 ^{+/+}	LysM:Stat3 ^{flx/-}	LysM:Stat3 ^{+/+}	LysM:Stat3 ^{flx/-}
Control	9.1 \pm 1.2	19.6 \pm 2.1	2.9 \pm 0.3	5.5 \pm 0.9
Leukopenic	1.6 \pm 0.3 [¶]	1.0 \pm 0.4 [¶]	0.7 \pm 0.2 [¶]	0.9 \pm 0.4 [§]

^a Peripheral leukocytes were depleted with cyclophosphamide, as described in the text. The leukopenic mice and vehicle-treated control mice were injected i.p. with thioglycollate. After 4 h, the number of infiltrating leukocytes was counted (six to eight mice, each). §, $p < 0.001$, ¶, $p < 0.0001$, vs control mice.

Thioglycollate-induced peritonitis

Mice were injected i.p. with 1 ml of sterile 4% thioglycollate (Difco Laboratories). At different time points (0, 2, 4, 8, 24, and 48 h, 10–12 mice each) after thioglycollate injection, mice were euthanized by cervical dislocation. Peritoneal lavages were collected by washing the peritoneum with 2 ml of sterile saline containing 5 mM EDTA, the lavages were centrifuged, and cell-free peritoneal fluids were stored at -20°C for measurements of cytokines/chemokines. 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 cytopsin preparations.

Depletion of leukocytes

Peripheral leukocytes were depleted by i.p. injection of cyclophosphamide (150 mg/kg; Sigma-Aldrich) on days -3 , -2 , and -1 before thioglycollate injection (10). This resulted in peripheral blood leukopenia (<30 cells/ μl) on days 0 and 1. Due to the leukopenia, the treatment effectively inhibited the thioglycollate-induced leukocyte infiltration as compared with saline-treated control (Table I).

Adoptive transfer of resident peritoneal macrophages

Peritoneal lavages were collected from nontreated LysMcre/Stat3^{flx/-} and LysMcre/Stat3^{+/+} mice as described above. Macrophages were positively purified with anti-mouse F4/80 Ag (Serotec) and anti-rat IgG microbeads using the MidiMACS system (Miltenyi Biotec) from the lavages. The purified cells were routinely $>95\%$ F4/80⁺ cells as assessed by flow cytometry (not shown). Four million resident peritoneal macrophages from LysMcre/Stat3^{flx/-} or LysMcre/Stat3^{+/+} mice were transferred into the peritoneum of nontreated LysMcre/Stat3^{+/+} mice. The transferred cell number was determined in reference to the number of resident peritoneal cells from nontreated mice. Two hours later, the mice were injected i.p. with 1 ml of sterile 4% thioglycollate, after which the mice were killed at 4 h, and the peritoneal lavages were collected as described above.

Western blotting

Leukocytes were dissolved in Laemmli buffer ($1 \times 10^6/50 \mu\text{l}$), sonicated, boiled, fractionated on SDS-polyacrylamide gel ($10 \mu\text{l}$), 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 Technology) overnight at 4°C . After a 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 ECL system (Cell Signaling Technology).

Measurement of cytokines

Murine cytokines/chemokines were measured using a standard method of sandwich ELISA, as described (11, 12). The captured Abs, detection Abs, and the recombinant cytokines were purchased from R&D Systems. The ELISAs used in this study did not cross-react with other murine cytokines/chemokines available and constantly detected murine cytokines/chemokines above 10 pg/ml.

Statistics

Statistical significance was evaluated by an unpaired Student *t* test. $p < 0.05$ was regarded as statistically significant. All data were expressed as mean \pm SE.

Results

Augmented leukocyte infiltration in mice lacking Stat3 in macrophages and neutrophils

To investigate the role of myeloid cell-associated Stat3 in acute inflammation, LysMcre/Stat3^{flx/-} mice and the control littermates were injected with thioglycollate, after which the number of infiltrating leukocytes was counted. As shown in Fig. 1A, the neutrophil influx in control mice reached its peak at 4 h, and the level remained elevated by 48 h. In LysMcre/Stat3^{flx/-} mice, the neutrophil infiltration also peaked at 4 h, but the level was significantly augmented, resulting in a 2.0-fold increase relative to the control. There were no differences in the number of neutrophils after 24 h between the groups (Fig. 1A). Likewise, the number of macrophages within 8 h, but not after 24 h, was augmented in LysMcre/Stat3^{flx/-} mice, reaching a 1.5-fold increase at 4 h after the injection (Fig. 1B). Thus, leukocyte influx in mice lacking Stat3 in macrophages and neutrophils was augmented in an initial phase of inflammation (within 8 h).

Augmented cytokine/chemokine production in mice lacking Stat3 in macrophages and neutrophils

Leukocyte infiltration is governed by multiple cytokines and chemokines (13). We next measured the peritoneal levels of several cytokines/chemokines known involved in inflammation in this model. The data in Fig. 2 demonstrated that levels of TNF- α , MIP-2, KC chemokine (KC),³ and MCP-1 in LysMcre/Stat3^{flx/-} mice were significantly higher than those in the control littermates, resulting in a 4.1-, 1.6-, 2.5-, and 2.3-fold increase at their peak, respectively, relative to the control. IL-10 level peaked at 2 h in control mice, whereas the peak level persisted by 4 h in LysMcre/Stat3^{flx/-} mice (Fig. 2). Interestingly, augmented levels of these cytokines/chemokines coincided well with the increased number of neutrophils and macrophages in LysMcre/Stat3^{flx/-} mice. There is a direct link between cytokine/chemokine expression and the leukocyte infiltration in acute inflammation models (14–16). Thus, mice with Stat3 deficiency in macrophages and neutrophils demonstrated augmented production of cytokines/chemokines, accounting for the increases in the number of infiltrating leukocytes.

Stat3 activation in peritoneal resident macrophages and infiltrating leukocytes

Experiments were next conducted to investigate whether Stat3 was activated in the peritoneal resident macrophages and the infiltrating leukocytes during inflammation. As shown in Fig. 3A, Stat3 in resident macrophages was tyrosine phosphorylated in control littermates, indicating that the Stat3 was spontaneously activated in the cells. The intensity of the phosphorylated Stat3 was increased

³ Abbreviation used in this paper: KC, KC chemokine.

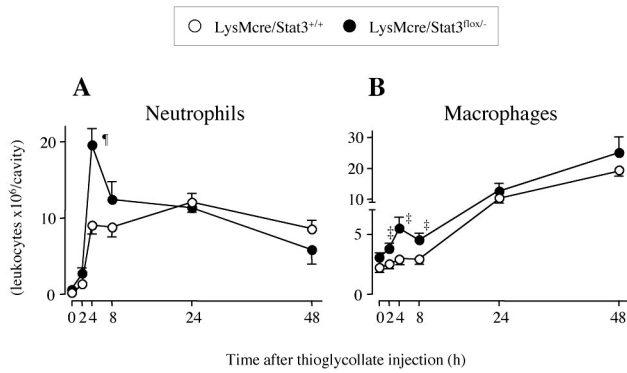


FIGURE 1. Leukocyte infiltration in the peritoneum after thioglycollate injection. At indicated time intervals after thioglycollate injection, infiltrating leukocytes were harvested from LysMcre/Stat3^{lox/-} mice and LysMcre/Stat3^{+/+} mice, and the cell number was counted (10–12 mice, each point). ‡, $p < 0.05$, and ¶, $p < 0.0001$, vs LysMcre/Stat3^{+/+} mice.

at 4 h after the injection, suggesting that Stat3 activation was augmented during inflammation (Fig. 3A). In contrast, the phosphorylated Stat3 was completely abrogated in LysMcre/Stat3^{lox/-} mice despite the negligible Stat3 protein in resident macrophages as well as infiltrating leukocytes (Fig. 3B). These results suggest that the augmented leukocyte infiltration in LysMcre/Stat3^{lox/-} mice may be ascribed to the Stat3 in resident macrophages or/and infiltrating leukocytes composed of macrophages and neutrophils.

Levels of cytokines/chemokines in leukopenic mice

To examine whether Stat3 in the infiltrating leukocytes could be involved in the regulation of inflammation in this model, we compared the levels of TNF- α , MIP-2, KC, and MCP-1 in leukopenic mice, in which peripheral leukocytes were depleted with cyclo-

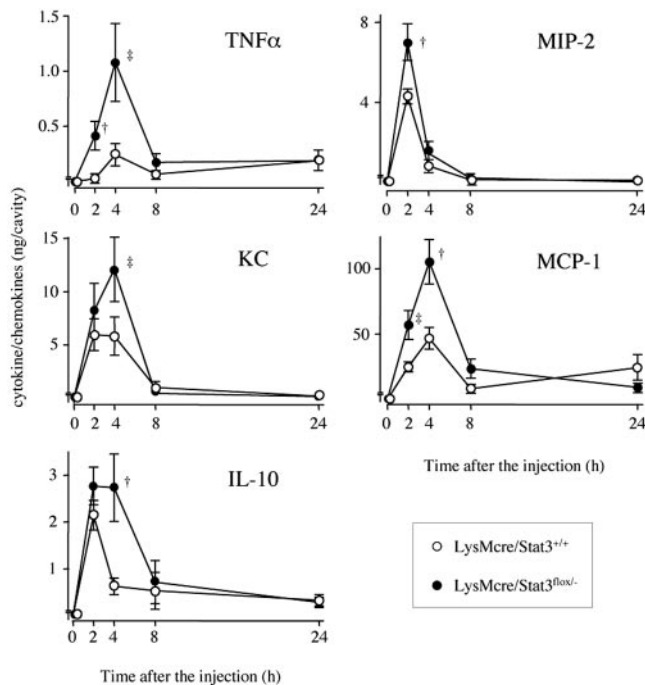


FIGURE 2. Mice lacking Stat3 in macrophages and neutrophils augment the production of cytokines/chemokines in the peritoneum. Peritoneal fluids were harvested after thioglycollate injection, and levels of cytokines/chemokines were measured (10–12 mice, each point). ‡, $p < 0.05$, and ¶, $p < 0.01$, vs LysMcre/Stat3^{+/+} mice.

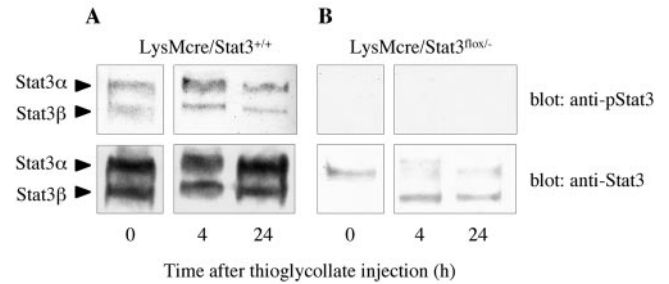


FIGURE 3. Stat3 activation in peritoneal exudates cells. Resident peritoneal cells (time 0) and infiltrating leukocytes were harvested from LysMcre/Stat3^{lox/-} mice (A) and LysMcre/Stat3^{+/+} mice (B) after thioglycollate injection, and the lysates were immunoblotted with anti-tyrosine-phosphorylated Stat3 (anti-pStat3) or anti-Stat3. Values represent data from three independent experiments.

phosphamide treatment. In leukopenic mice, the number of infiltrating leukocytes at 4 h after thioglycollate injection was significantly lower than that in saline-treated controls (Table I). As shown in Fig. 4, levels of TNF- α , MIP-2, KC, and MCP-1 in leukopenic LysMcre/Stat3^{lox/-} mice were also increased as compared with leukopenic LysMcre/Stat3^{+/+} mice. There were no differences in the level of cytokines/chemokines between control and leukopenic LysMcre/Stat3^{lox/-} mice (Fig. 4). It thus appears that Stat3 in the infiltrating leukocytes is not ascribed to the augmented productions of cytokines/chemokines.

Adoptive transfer of resident LysMcre/Stat3^{lox/-} macrophages augments inflammation

We next asked whether Stat3 in the peritoneal resident macrophages could be responsible for the enhanced inflammatory response in LysMcre/Stat3^{lox/-} mice. As shown in Table II, resident macrophages from LysMcre/Stat3^{lox/-} mice produced significantly higher levels of TNF- α , MIP-2, KC, and MCP-1

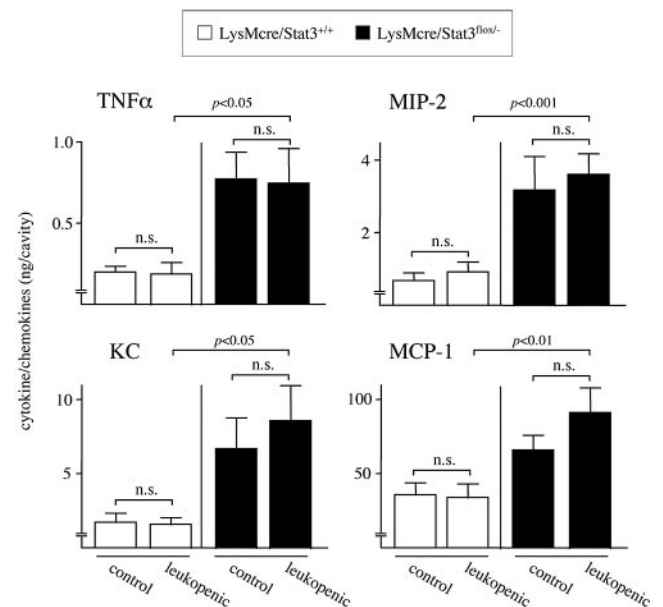


FIGURE 4. Productions of cytokines/chemokines in leukopenic mice. Peripheral leukocytes were depleted with cyclophosphamide treatment as described in the text. Sterile saline was used as a control. At 4 h after thioglycollate injection, mice were euthanized, and the peritoneal fluids were harvested (six to eight mice each). Levels of cytokines/chemokines were measured by ELISA.

Table II. Cytokine production from resident peritoneal macrophages^a

	Medium		Thioglycollate	
	LysM:Stat3 ^{+/+}	LysM:Stat3 ^{fllox/-}	LysM:Stat3 ^{+/+}	LysM:Stat3 ^{fllox/-}
TNF- α	0.03 \pm 0.01	0.26 \pm 0.01 [¶]	0.05 \pm 0.01	0.52 \pm 0.03 [¶]
MIP-2	2.8 \pm 0.1	4.7 \pm 0.2 [¶]	4.1 \pm 0.2	6.9 \pm 0.3 [¶]
KC	3.1 \pm 0.1	6.5 \pm 0.5 [¶]	6.2 \pm 0.5	11.6 \pm 1.0 [§]
MCP-1	0.8 \pm 0.1	4.5 \pm 0.5 [¶]	4.5 \pm 0.2	8.4 \pm 1.2 [§]
IL-10	0.23 \pm 0.01	1.12 \pm 0.03 [¶]	0.56 \pm 0.01	2.22 \pm 0.52 [¶]

^a Peritoneal resident macrophages were harvested from LysMcre/Stat3^{+/+} and LysMcre/Stat3^{fllox/-} mice, and the cells were stimulated with thioglycollate (0.4% v/v) for 24 h. The cytokine level in the supernatants was measured by ELISA (nanograms per milliliter, $n = 6$). Shown are representative data from three independent experiments. §, $p < 0.001$, ¶, $p < 0.0001$, vs LysMcre/Stat3^{+/+} macrophages.

relative to the control resident macrophages upon stimulation with thioglycollate in vitro. IL-10 level was also augmented in LysMcre/Stat3^{fllox/-} resident macrophages (Table II). When LysMcre/Stat3^{fllox/-} resident macrophages were transferred into LysMcre/Stat3^{+/+} mice, the mice demonstrated significant increases in the peritoneal levels of TNF- α , MIP-2, KC, and MCP-1, relative to the mice received the same number of LysMcre/Stat3^{+/+} resident macrophages, at 4 h after thioglycollate injection (Fig. 5A). Furthermore, adoptive transfer of LysMcre/Stat3^{fllox/-} macrophages resulted in a 1.8-fold increase in the number of infiltrating neutrophils. There was a trend toward increase in the number of infiltrating macrophages (1.6-fold increase) although it was not statistically significant ($p =$

0.15) (Fig. 5B). Thus, these data suggest that Stat3 in the peritoneal resident macrophages is responsible for the regulation of inflammation in this particular model.

Discussion

In the present study, we have demonstrated that LysMcre/Stat3^{fllox/-} mice exhibit an enhanced inflammatory response in a sterile peritonitis model, as evidenced by increased number of leukocytes and augmented cytokine production, affirming the importance of the Stat3 signaling pathway as a down-regulator of inflammatory response. Stat3 was tyrosine phosphorylated in peritoneal resident macrophages and infiltrating leukocytes in control littermates, thereby suggesting that either or both of these cell types might be important in the regulation of inflammation. Our present data have suggested that Stat3 in resident macrophages is responsible for the regulation. The LysMcre/Stat3^{fllox/-} mice rendered leukopenic by cyclophosphamide treatment demonstrated increased levels of TNF- α , MIP-2, KC, and MCP-1 relative to the leukopenic LysMcre/Stat3^{+/+} mice, suggesting that elevation of cytokines/chemokines was independent of infiltrating leukocytes. Adoptive transfer of LysMcre/Stat3^{fllox/-} resident macrophages into LysMcre/Stat3^{+/+} mice resulted in increases not only in the peritoneal level of cytokines/chemokines but also in the number of leukocyte infiltration in the peritoneum. Stat3 is a transcription factor mediating anti-inflammatory effects of IL-10 (9). Thus, resident macrophages, but not other cell types, appear to be an important target of IL-10 in this particular model.

Resident macrophages are regarded as sentinels in inflammation, as the cells are indigenous to tissues and readily recognize invading pathogens via pattern recognition receptors such as Toll-like receptors (17). Resident macrophages are capable of producing inflammatory cytokines/chemokines (18), thereby playing a central role in the recruitment of leukocytes (16, 19–21). A recent investigation provides clear evidence that peritoneal resident macrophages are essential in the orchestration of acute inflammation through macrophage-dependent cytokine/chemokines' production (22). Meanwhile, as shown in this study and elsewhere (23), resident macrophages produce a potent anti-inflammatory cytokine IL-10. IL-10 inhibits inflammatory response in a variety of experimental models including peritonitis by down-regulating the production of inflammatory cytokines/chemokines (4, 24). Ajuebor et al. (25) have demonstrated a cytokine/chemokine inhibitory feedback loop based on IL-10 released from peritoneal resident macrophages. In our model of acute inflammation, IL-10 production in LysMcre/Stat3^{+/+} mice was parallel with TNF- α , MIP-2, KC, and MCP-1. Levels of TNF- α , MIP-2, KC, and MCP-1 in LysMcre/Stat3^{fllox/-} mice were significantly higher than those in the control littermates (Fig. 2). Although the peak IL-10 level in LysMcre/Stat3^{fllox/-} mice persisted, the IL-10 does not appear to mediate its

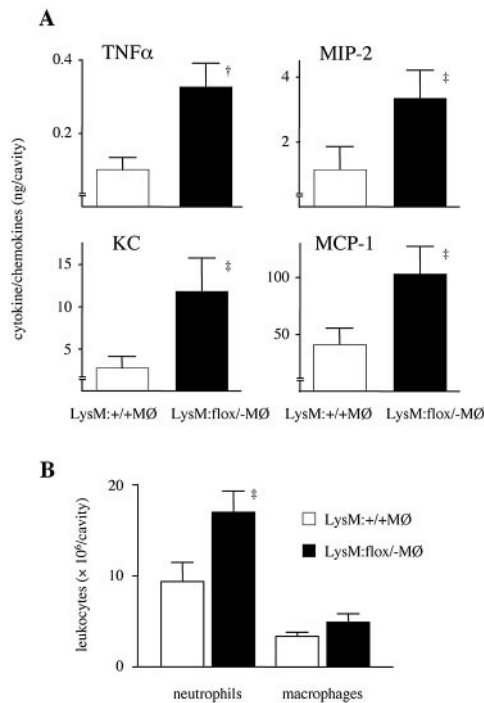


FIGURE 5. Augmented inflammatory response after adoptive transfer of LysMcre/Stat3^{fllox/-} resident macrophages. LysMcre/Stat3^{+/+} mice were injected i.p. with LysMcre/Stat3^{+/+} macrophages (LysM:+/+M ϕ) or LysMcre/Stat3^{fllox/-} macrophages (LysM:fllox/-M ϕ) (4×10^6 cells/mouse) 2 h before thioglycollate injection. At 4 h after thioglycollate injection, the mice were euthanized, and the peritoneal fluids were harvested (11 mice each). **A**, Levels of cytokines/chemokines were measured by ELISA. **B**, The number of infiltrating leukocytes was counted. Three independent experiments were performed, and the data were pooled. Each experiment showed similar results. ‡, $p < 0.05$, and †, $p < 0.01$, vs mice received LysMcre/Stat3^{+/+} macrophages.

anti-inflammatory effect in these mice, given that Stat3 is a key transcriptional factor mediating anti-inflammatory properties of IL-10 (9). The persistence of IL-10 level is possibly due to the enhanced production by resident peritoneal macrophages, given that IL-10 production by resident macrophages was augmented on stimulation with thioglycollate (Table II). The expression of IL-10 receptor 1 (IL-10R1) in LysMcre/Stat3^{flox/-} resident macrophages was induced 2^{1.9}-fold more than control macrophages as assessed by gene expression analysis array (data not shown). Thus, resident macrophages trigger inflammation by producing inflammatory cytokines and chemokines, but the production of these mediators appears to be negatively regulated in the cells through a Stat3 signaling pathway that is most likely activated in response to IL-10 released by cells that include resident macrophages.

Several mechanisms might contribute to the up-regulation of cytokines/chemokines in the absence of Stat3 in macrophages. Yu and Kone (27) have shown that Stat3 interacts physically with NF- κ B, a ubiquitous transcription factor that controls the gene expression of many proinflammatory mediators (26), thereby inhibiting NF- κ B-mediated *trans*-activation of the inducible NO synthase gene. Defective Stat3 activation in IL-10^{-/-} mice exhibited enhanced NF- κ B activation (28). In addition, Stat3-deficient bone marrow cells displayed increased I κ B phosphorylation and enhanced DNA-binding activity of NF- κ B family members after stimulation with LPS (29). Thus, Stat3 appears to down-regulate cytokine/chemokine response in part by inhibiting NF- κ B activation. Recently, cross-talk between transcription factors has become a commonly recognized mode of gene regulation. Therefore, interactions with different pathways also should be taken into consideration to further understand the biological significance at the molecular level.

Of interest were the findings that TNF- α and MIP-2 levels in LysMcre/Stat3^{+/+} mice harboring LysMcre/Stat3^{flox/-} macrophages were lower than those in LysMcre/Stat3^{flox/-} mice, whereas KC and MCP-1 levels were comparable between these groups (Figs. 4 and 5). This suggests that Stat3 in other cell types could contribute to the production of TNF- α and MIP-2. Mast cells are a rich source of cytokines/chemokines that include TNF- α and MIP-2 (30). In addition, peritoneal mesothelial cells are capable of producing TNF- α and MIP-2 (31, 32). Stat3 is ubiquitously expressed in a variety of cell types that include mast cells and peritoneal mesothelial cells (33, 34). Thus, Stat3 in these cell types may play a role in the production of TNF- α and MIP-2. Despite this, however, it is important that resident macrophages, but not other cell types, play a major role in down-regulating inflammatory response.

The recruitment of leukocytes after 24 h was not altered in LysMcre/Stat3^{flox/-} mice. The numbers of infiltrating leukocytes at 24 and 48 h after thioglycollate injection were unchanged when LysMcre/Stat3^{flox/-} resident macrophages or -infiltrating leukocytes were transferred into LysMcre/Stat3^{+/+} mice (data not shown). These results suggest that the late stage of inflammatory response (after 24 h) is functionally different from that in the early phase (before 8 h) in this experimental model. Judging from the production kinetics of cytokines/chemokines in this model, other cytokines/chemokines and their signaling pathways may be involved in the late phase. Further studies are necessary to explore the molecular basis.

We conclude that resident macrophages are important target of IL-10 in inflammation. The findings together with the primarily function of macrophages as triggering inflammation suggest that resident macrophages play a central role in an initial phase of inflammation. Excessive inflammation is harmful to the host. The

present data highlight an inhibitory role of peritoneal resident macrophages in which Stat3 mediates the anti-inflammatory effects of IL-10, suggesting a new paradigm that the inflammatory response appears to be on an inhibitory basis by resident macrophages through a Stat3 signaling pathway.

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Disclosures

The authors have no financial conflict of interest.

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