Regulation of the Inflammatory Response: Enhancing Neutrophil Infiltration under Chronic Inflammatory Conditions

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Neutrophil (polymorphonuclear leukocytes [PMN]) infiltration plays a central role in inflammation and is also a major cause of tissue damage. Thus, PMN infiltration must be tightly controlled. Using zymosan-induced peritonitis as an in vivo PMN infiltration model, we show in this study that PMN response and infiltration were significantly enhanced in mice experiencing various types of systemic inflammation, including colitis and diabetes. Adoptive transfer of leukocytes from mice with inflammation into healthy recipients or from healthy into inflammatory recipients followed by inducing peritonitis demonstrated that both circulating PMN and tissue macrophages were altered under inflammatory conditions and that they collectively contributed to enhanced PMN infiltration. Detailed analyses of dextran sulfate sodium-elicited colitis revealed that enhancement of PMN infiltration and macrophage function occurred only at the postacute/chronic phase of inflammation and was associated with markedly increased IL-17A in serum. In vitro and ex vivo treatment of isolated PMN and macrophages confirmed that IL-17A directly modulates these cells and significantly enhances their inflammatory responses. Neutralization of IL-17A eliminated the enhancement of PMN infiltration and IL-6 production and also prevented severe tissue damage in dextran sulfate sodium-treated mice. Thus, IL-17A produced at the chronic stage of colitis serves as an essential feedback signal that enhances PMN infiltration and promotes inflammation. The Journal of Immunology, 2012, 188: 000–000.

The mechanisms that control PMN response and regulate their infiltration involve multiple cell types and various inflammatory factors, and studies in this area are still inadequate. Some aspects of PMN function, such as chemotaxis and degranulation, have been studied in vitro by refined assays (1–3). However, these assays do not reveal the entire design of PMN inflammatory response and infiltration during inflammation and how these processes are dynamically regulated in vivo. In the study of PMN function in diabetes, we have constantly observed that PMN isolated from diabetic patients showed impaired transmigration in the in vitro chemotaxis assays (Y. Liu and K. Zen, unpublished observations). These observations, which are consistent with other reports (4–6), indicated an important fact: that PMN from diabetic patients were different from those of healthy donors; however, they could not ascertain whether the activities of PMN of diabetes were truly abated or, on the contrary, were primed (activated to an extent). In the second possibility, the primed PMN were hypersensitive and may have become activated during the in vitro isolating procedure, resulting in their inefficient chemotaxis in the later transmigration assays (3).

To further study PMN response and infiltration, we designed a two-layered in vivo inflammatory system in which a relatively chronic inflammatory condition, such as colitis, type 1 diabetes, or other inflammatory condition, was first created in the experimental animals. After the condition was stabilized, a typical acute inflammatory response, such as zymosan-induced peritonitis, was induced, and the immediate PMN infiltration into the newly established inflammatory site was assayed within a few hours. Using this design, we found that active inflammation at the postacute/chronic phase in particular induces systemic alterations in both PMN and cells within tissue microenvironments, including macrophages. Instead of suppressing inflammation, these changes result in an enhancement of PMN infiltration, hence exaggerating the inflammatory reaction.
Materials and Methods
Mice and inflammatory mouse models
C57BL/6 mice 6–10 wk old (18–22 g) purchased from The Jackson Laboratory (Bar Harbor, ME) were housed with free access to water and food in a specific pathogen-free facility. All experiments using animals and procedures of animal care and handling were carried out following protocols approved by the Institutional Animal Care and Use Committee of Georgia State University. To establish dextran sulfate sodium (DSS)-induced colitis (7, 8), 2% DSS (MP Biomedicals) prepared in pure water was given to the mice as the drinking water. Mice were inspected daily for distress and colitis symptoms such as wet stool, diarrhea, bloody diarrhea, and weight loss. To induce hyperglycemia/type 1 diabetes (9, 10), the mice were fasted for 4 h and then injected with streptozotocin (STZ; Sigma-Aldrich) at a dose of 50 mg/kg body weight. STZ was freshly prepared in sodium citrate buffer (pH 4.5) prior to the injections, which were performed daily for 5 consecutive d. The mice were then maintained for an additional 2 wk to allow the diabetic condition to be stabilized. Serum glucose levels were tested with the One-Touch glucose monitoring system (LifeScan), and in general, >90% of injected mice developed hyperglycemia. To induce bronchitis/lung inflammation, the mice were given LPS (Escherichia coli serotype 026:B6; Sigma-Aldrich), 0.3 mg/kg body weight, in 70 µl PBS through the nares every other day for 15 d.

Zymosan-induced peritonitis
This model was used to assay the acute PMN response and infiltration in vivo (11). Briefly, mice were injected i.p. with 0.5 mg zymosan A (Sigma-Aldrich) that was prepared in 0.5 ml PBS. Mice were euthanized at varying time points after the injection. PMN that transmigrated into the peritoneum were collected by peritoneal lavage with 3 ml cold HBSS without Ca2+ and Mg2+ followed by analysis with myeloperoxidase (MPO) assay, cell counting, and immunofluorescence labeling using a PE-C5–conjugated anti-mouse Ly-6G (Gr-1) Ab (eBioscience). Macrophages were analyzed using an Alexa Fluor 488-conjugated anti-mouse F4/80 Ab (clone BM8; BioLegend). For leukocyte adoptive transfer experiments, bone marrow cells were obtained by flushing femur bone cavities followed by labeling with the fluorescent cell tracer CFSE or 1,1'-dihexadecyl-3,3',3'-tetramethylindocarbocyanine iodide [DiIC16(3)] (both from Invitrogen). In some experiments, the bone marrow cells were further separated by a Percoll (GE Healthcare) density gradient to obtain the population of mature PMN (12). In general, 5 × 106 labeled cells (∼2.5 × 106 cells were Gr-1+) were transfused into a recipient mouse via i.v. injection before inducing peritonitis using zymosan. To determine the role of IL-17A in PMN infiltration, ex vivo experiments were performed. In these experiments, freshly isolated bone marrow cells were labeled with CFSE followed by treatment with a recombinant mouse IL-17A (rIL-17A) (2 ng/ml; BioLegend), with or without neutralizing anti–IL-17A Ab (low concentrations) or rIL-17A Ab (low concentrations) or mAb anti-actin Ab (Sigma-Aldrich). The cells were then incubated for 48 h at 37°C. To directly observe IL-17A binding to PMN and macrophages, recombinant murine IL-17A was labeled with FITC using an EZ-Label FITC protein labeling kit (Pierce). The FITC-labeled IL-17A was then incubated with freshly isolated mouse bone marrow leukocytes and peritoneal macrophages for 30 min (25°C) followed by washing and analysis by fluorescence microscopy. To detect IL-17A–induced protein phosphorylation changes in macrophages, isolated murine peritoneal macrophages were treated with rIL-17A at concentrations of 0.5 and 2.0 ng/ml, or vehicle, for 48 h (37°C). These cells were then lysed in a buffer containing 100 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1:100 dilution of a protease inhibitor mixture (Roche), 20 mM HEPES, and 2 mM Ca2+, Mg2+, FBS, and the supernatants were collected by centrifugation. The macrophages were then stimulated with rIL-17A at concentrations of 0.5 and 2.0 ng/ml, or vehicle, for 48 h (37°C). To directly observe IL-17A binding to PMN and macrophages, recombinant murine IL-17A was labeled with FITC using an EZ-Label FITC protein labeling kit (Pierce). The FITC-labeled IL-17A was then incubated with freshly isolated mouse bone marrow leukocytes and peritoneal macrophages for 30 min (25°C) followed by washing and analysis by fluorescence microscopy. To detect IL-17A–induced protein phosphorylation changes in macrophages, isolated murine peritoneal macrophages were treated with rIL-17A at concentrations of 0.5 and 2.0 ng/ml, or vehicle, for 48 h (37°C). The cells were then lysed in a buffer containing 100 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1:100 dilution of a protease inhibitor mixture (product code P8340; Sigma-Aldrich), and 3 mM PMSF followed by SDS-PAGE and Western blot detection using the anti-phosphotyrosine Ab 4G10 (Millipore), an anti-phosphoserine Ab (Cell Signaling Technology), and an anti-actin Ab (Sigma-Aldrich).

Results
Analysis of the acute PMN response in vivo by zymosan-induced peritonitis
In this study, we used zymosan-induced peritonitis (11), a commonly used self-resolving acute inflammation model, to assay the PMN immediate response and infiltration in vivo. For these experiments, healthy C57BL/6 mice that were maintained in a pathogen-free animal facility were injected i.p. with zymosan A, followed by analysis for PMN recruitment into the peritoneum after various time periods. As shown in Fig. 1A and Table I, before zymosan injection, ∼2.6 × 106 cells were present in the peritoneum, and among these cells, ∼30% were F4/80+ macrophages, and the remainder contained few (∼5%) Gr-1+, which presumably were tissue granulocytes and other tissue cells. Soon after zymosan challenge, PMN began to infiltrate into the peritoneum, resulting in drastic changes in the cell composition and numbers. As shown, at 2 h, small numbers of PMN (∼1 × 106) transmigrated across the endothelium and arrived at the peritoneum. Meanwhile, the majority of macrophages were seemingly leaving the area, resulting in a significant decrease of the F4/80+ cells (Table I). Massive PMN infiltration into the peritoneum occurred after 2 h, resulting in ∼107 PMN and >1.2 × 107 PMN recruited to the location at 4 and 6 h, respectively. By 8 h, PMN infiltration slowed rapidly or stopped, resulting in a decrease in the number of...
The table shows the mean cell number staining used normal rat serum. The cells of different staining were counted and using an anti-F4/80 Ab (green) and an anti–Gr-1 (red) Ab, respectively. Control cell collection cells from peritonea and staining for macrophages and PMN/granulocytes indicated time points.

Mice receiving injection (i.p.) of zymosan A were euthanized at the indicated time points. A, Macrophages and PMN lavaged from the peritoneum were stained with anti-F4/80 (green) and anti–Gr-1 (red) Abs, respectively, and visualized under a fluorescent microscope. B, Serum concentrations of inflammatory cytokines during peritonitis were evaluated by ELISA. Data are expressed as means ± SEM; n = 6 mice/group. ***p < 0.01 versus respective serum cytokine levels at 0 h.

PMN in the area. After the peak of PMN recruitment, monocyte infiltration began to increase at 6 h and continued to increase thereafter, suggesting that the acute inflammation proceeded to a resolving stage. The infiltrating monocytes and their mediated clearance likely accounted for the decrease of PMN numbers in the peritoneum at later time points. Consistent with others (13, 14), we detected a robust but transient increase in IL-6 in the peritoneal fluid and in the blood serum (Fig. 1B, Table II) after zymosan challenge. As observed, the time course of IL-6 change tightly correlated with the dynamics of PMN infiltration, suggesting a potential role of this cytokine in controlling the episode of zymosan-induced peritonitis. Moderate increases in IL-1, IL-17A, and TNF-α were also detected. The dynamics of IL-1 resembled that of IL-6, but IL-17A was only increased at later time periods, when the massive PMN transmigration was nearly completed (Fig. 1B, Table II).

Table I. Leukocytes in the peritoneum and their dynamic changes during zymosan-induced peritonitis

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>F4/80+ (×10^5)</th>
<th>Gr-1+ (×10^5)</th>
<th>Total (×10^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10.4±1.4</td>
<td>2.0±0.7</td>
<td>26.4±3.7</td>
</tr>
<tr>
<td>2</td>
<td>1.5±0.2</td>
<td>10.7±1.5</td>
<td>15.3±2.2</td>
</tr>
<tr>
<td>4</td>
<td>0.9±0.1</td>
<td>96.2±6.8</td>
<td>120.5±8.5</td>
</tr>
<tr>
<td>6</td>
<td>1.5±0.2</td>
<td>124.3±10.4</td>
<td>146.2±12.2</td>
</tr>
<tr>
<td>8</td>
<td>8.2±1.1</td>
<td>115.1±6.6</td>
<td>154.3±8.8</td>
</tr>
</tbody>
</table>

Mice injected with zymosan A were euthanized at various time points followed by collection cells from peritonea and staining for macrophages and PMN/granulocytes using an anti-F4/80 Ab (green) and an anti–Gr-1 Ab (red) respectively. Control cell staining used normal rat serum. The cells of different staining were counted and analyzed by FACS. The 0 h time point was determined without zymosan injection. The table shows the mean cell number ± SEM, n = 6 mice/group.

Enhancing PMN infiltration under chronic inflammatory conditions

To investigate whether chronic inflammation affects PMN infiltration, we induced various inflammatory conditions in C57BL/6 mice before performing the zymosan-peritonitis assay. In these experiments, mice were treated with 2% DSS to induce colitis (7, 8), STZ to induce type 1 diabetes (9, 10), or LPS to induce bronchitis/lung inflammation (15). A group of mice were also treated with one dose of emulsified CFA (50% in PBS) to induce chronic inflammation. As shown in Fig. 2A, progressive body weight loss, which was associated with diarrhea and colitis, was observed in DSS-treated mice. Staining of colon tissues after treating the mice with DSS demonstrated significant intestinal inflammation that was associated with aggressive leukocyte infiltration, severe mucosal damage, and loss of epithelial structures (Fig. 2A). Treating mice with STZ and LPS also successfully induced diabetic and lung inflammatory conditions. As shown in Fig. 2B, all of the STZ-treated mice developed hyperglycemia, with the mean blood glucose level being increased to 431.7 ± 56.1 mg/dl, whereas the control group treated with vehicle (PBS) maintained a blood glucose level of 141.2 ± 14.0 mg/dl. Meanwhile, treating mice with low-dose LPS via the nares for 15 d induced evident upper respiratory inflammation that was characterized by noticeable leukocyte infiltration around bronchi, bronchioles, and in some area of the lung parenchyma (Fig. 2C).

We then examined PMN function in these mice with various inflammatory conditions by performing zymosan-induced peritonitis. For these experiments, we used the mice that had been treated with DSS for 12 d and manifested apparent colitic condition. As shown in Fig. 2D, compared with the control healthy mice that PMN just began to infiltrate into the peritoneum at 2 h and only small numbers (~1 × 10^6 PMN) were detected, significantly increased numbers of PMN (>6 × 10^6) were recruited to the area in colitic mice at the same time point. Similar early promoted PMN infiltration into the peritoneum in response to zymosan challenge was also observed in STZ-induced diabetic mice and LPS-treated mice. These mice were used to test for PMN infiltration by peritonitis after the STZ- and LPS-induced pathological conditions were stabilized. As shown in Fig. 2D, at 2 h, 5.6 × 10^6 PMN and ~3 × 10^6 PMN were recruited to peritoneal PMN infiltration in STZ- and LPS-treated mice, respectively. We also induced peritonitis in the mice that were treated with CFA 1 mo ago. As shown in Fig. 2E, this form of chronic inflammation also significantly enhanced PMN response to zymosan, resulting in >7 × 10^6 PMN being recruited to the peritoneum by 2 h.

Kinetic analysis of PMN infiltration (Fig. 2E) in colitic mice by plotting the total numbers of PMN transmigrated as a function of time showed that the robust PMN infiltration occurred earlier than that in the control mice. Indeed, >70% of the infiltrated PMN in mice with DSS treatment for 12 d had arrived at the peritoneum by 2 h, whereas in the controls, the majority of PMN were recruited by 4 h. Significant enhanced PMN infiltration into the peritoneum at an early time point (2 h) was also observed in mice with DSS treatment for 9 d (Fig. 2E). The kinetic results also indicated that the early enhancement of PMN infiltration in colitic mice was not due to inflammation-induced increases in PMN production but to accelerated PMN response and transmigration. In fact, the accumulative PMN numbers that infiltrated into the peritoneum in colitic mice at 4 h were not higher, but tended to be lower, than those in the controls. Parallel analyses of PMN numbers in the peripheral circulation and in bone marrow indicated that despite the increase of GM-CSF in the serum (Table III), continual DSS treatment (~9 d) and colitis exhausted the
entire system, and also consumed PMN availability in the circulation (data not shown).

Inflammation-induced modulations of both circulating PMN and tissue microenvironments contribute to the promotion of PMN infiltration

The results of enhanced PMN infiltration in mice under various inflammatory conditions suggest a likelihood that alterations occurred in either PMN, which directly promote PMN transmigration, or in cells within the peritoneal vicinity, which led to an earlier local response to zymosan and/or contributed to the facilitation when PMN transmigrated through. To test these possibilities, we designed two directional, adoptive leukocyte transfer and peritonitis experiments. In these, donor PMN derived from mice with different inflammatory conditions were transfused into healthy, noninflammatory recipient mice and tested for response and infiltration in zymosan-induced peritonitis; conversely, donor PMN from healthy mice were transfused into recipient mice with ongoing inflammation and then tested for infiltration in response to zymosan challenging. The former experiments determine whether chronic inflammation converts PMN to be faster migrating cells (even in a new host environment). The latter experiments examine the peritoneal tissue microenvironment and determine whether chronic inflammation could induce changes that promote PMN infiltration.

### Table II. Time-course detection of serum cytokines during zymosan A-induced peritonitis

<table>
<thead>
<tr>
<th>Cytokine (pg/ml)</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>49.2 ± 10.3</td>
<td>118.1 ± 20.6*</td>
<td>111.8 ± 16.0*</td>
<td>96.4 ± 12.8*</td>
<td>100.7 ± 16.5*</td>
</tr>
<tr>
<td>IL-1β</td>
<td>31.0 ± 6.6</td>
<td>159.3 ± 26.5*</td>
<td>498.0 ± 58.9**</td>
<td>113.7 ± 32.5*</td>
<td>64.3 ± 22.7</td>
</tr>
<tr>
<td>IL-6</td>
<td>65.6 ± 22.8</td>
<td>2180.0 ± 387.9***</td>
<td>4713.1 ± 1333.4***</td>
<td>1023.7 ± 322.4***</td>
<td>555.0 ± 165.6***</td>
</tr>
<tr>
<td>IL-17A</td>
<td>109.6 ± 10.8</td>
<td>126.9 ± 12.7</td>
<td>370.3 ± 94.5***</td>
<td>562.0 ± 77.4***</td>
<td>517.3 ± 64.6***</td>
</tr>
</tbody>
</table>

Concentrations of inflammatory cytokines in serum during peritonitis were evaluated by ELISA. Data are expressed as means ± SEM; n = 6 mice/group.

*p < 0.05, **p < 0.01 versus respective serum cytokine levels at 0 h.

FIGURE 2. Mice with various chronic inflammatory conditions display enhanced PMN infiltration during zymosan-induced peritonitis. A, DSS-induced colitis. C57BL/6J mice were given 2% DSS in drinking water continuously, and development of colitis was monitored by body weight loss, diarrhea, etc., and confirmed by dissection of the colonic tissues. B, STZ-induced type 1 diabetes. Mice were consecutively injected with STZ or the vehicle (CTL) for 5 d. Serum glucose levels were assayed 3 wk after the injections. C, Inducing upper respiratory inflammation by LPS. LPS or vehicle was given to mice via the nares every other day for 15 d. The figure shows the histology images (H&E staining) of lung tissue sections after vehicle (Ctl.) or LPS treatment. D and E, Enhanced PMN infiltration in mice with systemic inflammation. Control healthy mice and mice with colitis (DSS for 12 d), hyperglycemia (3 wk post-STZ treatment), or lung inflammation (LPS) were tested for PMN infiltration by zymosan A-induced peritonitis. Mice that received one dose of emulsified CFA (50% in PBS) were also tested in the assay. D and E show the numbers of PMN infiltrated into the peritoneum at 2 h and the kinetics of PMN infiltration into the peritoneum, respectively. The data represent one of eight independent experiments and are expressed as means ± SEM; n = mice number/group.

**p < 0.01, ***p < 0.001 versus respective controls.
during peritonitis. Control experiments were performed by transfusion of PMN from healthy mice into other healthy mice followed by testing the donor PMN infiltration.

**Figure 3.** Systemic inflammation alters both PMN and tissue microenvironments. A, Testing donor PMN from inflammatory mice response in healthy recipients by zymosan-peritonitis. Bone marrow cells (2.5 × 10⁶ mature PMN) derived from colitic (DSS, 12 d), diabetic (STZ), or control healthy mice were labeled with CFSE and transfused into healthy recipients, respectively. Figure shows the microscopic analysis of infiltrated donor PMN in peritoneal lavage after inducing peritonitis for 2 h and the total numbers of donor PMN infiltrated at 2 and 4 h. The result represents one of four independent experiments. B, PMN chemotaxis in vitro. Bone marrow leukocytes from healthy control and colitic (DSS, 12 d) mice were normalized for equal PMN contents by MPO assays before being applied into the upper chambers of the transmigration setups. Chemotaxis induced by fMLF was measured at 45 min. C, Cotransfer of leukocytes from healthy and inflammatory mice and testing by peritonitis. A mixture of bone marrow leukocytes derived from healthy mice (red, DiIC16 labeling) and colitic mice (DSS, 12 d) (green, CFSE labeling) (2.5 × 10⁶ PMN each) were transferred into the same healthy recipients. The images show zymosan-induced PMN infiltration into the peritoneum at 2 h in the peritoneal lavage. The result represents one of five independent experiments. D, Transfer of PMN from healthy mice into inflammatory recipients followed by testing PMN infiltration during peritonitis. Bone marrow leukocytes from five healthy mice were combined and labeled, followed by transfusion into different types of inflammatory recipient mice (three mice per type, 2.5 × 10⁶ PMN/mouse). E, Peritoneal macrophages obtained by lavaging peritonea were stimulated in vitro using zymosan (1 mg/ml) in DMEM (containing 5% FBS) at 37°C. IL-6 secreted into the medium at different time points was assayed by ELISA. The data are expressed as means ± SEM. *p < 0.05, **p < 0.01 versus respective controls.

**Table III.** Serum cytokine levels during DSS-induced colitis

<table>
<thead>
<tr>
<th>Cytokine (pg/ml)</th>
<th>Day 0</th>
<th>Day 3</th>
<th>Day 6</th>
<th>Day 9</th>
<th>Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>52.8 ± 5.6</td>
<td>71.0 ± 5.8*</td>
<td>69.7 ± 8.1</td>
<td>57.8 ± 11.6</td>
<td>52.1 ± 9.3</td>
</tr>
<tr>
<td>IL-1β</td>
<td>35.4 ± 8.6</td>
<td>28.5 ± 6.0</td>
<td>39.4 ± 11.2</td>
<td>227.5 ± 22.7**</td>
<td>144.6 ± 16.4**</td>
</tr>
<tr>
<td>IL-6</td>
<td>70.2 ± 17.6</td>
<td>78.7 ± 10.4</td>
<td>132.2 ± 42.4*</td>
<td>430.8 ± 109.1**</td>
<td>173.4 ± 24.0**</td>
</tr>
<tr>
<td>IL-17A</td>
<td>113.6 ± 10.6</td>
<td>1089.10.9</td>
<td>249.8 ± 24.1*</td>
<td>714.1 ± 93.6**</td>
<td>2023.9 ± 172.1***</td>
</tr>
<tr>
<td>MCP-1</td>
<td>30.3 ± 8.6</td>
<td>37.2 ± 9.4</td>
<td>156.8 ± 16.2**</td>
<td>79.4 ± 18.2*</td>
<td>22.5 ± 4.3</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>51.0 ± 6.7</td>
<td>44.2 ± 7.8</td>
<td>51.1 ± 9.3</td>
<td>40.4 ± 18.0</td>
<td>41.2 ± 6.4</td>
</tr>
<tr>
<td>IL-12/23(p40)</td>
<td>55.6 ± 7.3</td>
<td>57.8 ± 6.5</td>
<td>77.4 ± 11.9*</td>
<td>67.0 ± 6.1</td>
<td>54.6 ± 7.2</td>
</tr>
<tr>
<td>IL-4</td>
<td>39.5 ± 4.2</td>
<td>43.6 ± 7.3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL-10</td>
<td>59.2 ± 20.9</td>
<td>133.6 ± 16.4*</td>
<td>35.5 ± 12.8</td>
<td>30.4 ± 11.3</td>
<td>27.2 ± 13.6</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>ND</td>
<td>65.0 ± 16.3*</td>
<td>29.4 ± 9.2</td>
<td>21.0 ± 8.4</td>
<td>ND</td>
</tr>
</tbody>
</table>

Inflammatory cytokine levels in serum during DSS-induced colitis were evaluated by ELISA. Data are expressed as means ± SEM; n = 6-8 mice/group. ND, undetectable. *p < 0.05, **p < 0.01, ***p < 0.001 versus respective controls.
host mice had no prior inflammation, donor PMN from colitic and diabetic mice (Fig. 3A, labeled with CFSE, green) still displayed much faster infiltration, resulting in more cells transmigrated into the peritoneum at 2 h than control donor PMN from healthy mice. In parallel, we also performed in vitro chemotaxis assays and compared the transmigration of PMN derived from DSS-treated mice and from healthy mice. To avoid activation of PMN, especially those from mice with inflammation, we directly applied the total bone marrow leukocytes without further isolation. As shown in Fig. 3B, greater numbers of PMN transmigrating across matrix-coated filters toward the chemoattractant fMLF were observed in samples derived from DSS-treated mice than those from healthy mice. In Fig. 3C, we further labeled donor PMN or total bone marrow cells derived from DSS-treated mice and from healthy mice with different dyes (green and red) and simultaneously transmigrated into the same recipient mice (at a 1:1 ratio of the PMN number). As shown, zymosan induced more infiltration of PMN from colitic mice (green) than those from noncolitic, healthy mice (red) at 2 h, confirming that PMN in chronic inflammatory mice had an enhanced ability to respond and migrate toward inflammatory stimuli.

To investigate whether the tissue microenvironment also contributed to the enhanced PMN infiltration under inflammatory conditions, we transferred the PMN of healthy mice into DSS- and STZ-treated chronic inflammatory mice (recipients). Inducing peritonitis subsequently showed significantly faster infiltration of donor PMN in mice with ongoing colitis or diabetes than in control healthy recipients (Fig. 3D). These results suggest that considerable alterations had occurred in the peritoneal tissue environment in inflammatory mice and that these changes promoted PMN infiltration. Because peritoneal macrophages play an important role in the development of peritonitis, we collected these macrophages and directly tested their responses to zymosan in vitro. As shown in Fig. 3E, in the cell-culture dishes, zymosan induced increased IL-6 production in macrophages collected from DSS- and STZ-treated mice than those isolated from noninflammatory controls. Interestingly, we observed significantly increased numbers of macrophages in the peritoneum of STZ-induced diabetic mice (data not shown). Thus, these results confirmed tissue environment changes under chronic inflammation. Increases in cytokine production from the local macrophages certainly contributed to enhanced PMN infiltration.

Role of IL-17 in colitis-associated inflammatory modulation

The findings that circulating PMN and peritoneal macrophages had altered responsiveness under colitis and diabetes were striking given that both cells were distal to the original inflammatory sites in those conditions. We thus hypothesized that critical inflammatory factors were released into the circulation, through which it reached and regulated cells at a distance. To identify these important factors, especially those that might contribute to enhance PMN infiltration, we analyzed inflammatory cytokines associated with DSS-induced colitis in the serum. As shown in Table III, whereas most of cytokines tested were reported in colonic tissues (7, 16–19), these cytokines stayed at low or moderate levels in the serum throughout the course of the DSS treatment. The only cytokine that we found significantly elevated in the serum was IL-17A (Fig. 4A), which started to increase at days 6–8 of DSS treatment and continued increasing, eventually reaching >2 ng/ml. We found that not only was its elevation in the serum extraordinary, but also that the dynamics of IL-17A tightly correlated with the enhancement of PMN infiltration observed in peritonitis. As shown in Fig. 4B, inducing peritonitis in mice that were treated with DSS for different days revealed that significant promotion of PMN infiltration occurred only after DSS treatment for 6 d and continued to be enhanced thereafter. Supporting this observation, assay of peritonitis-associated IL-6 release also demonstrated that enhancement of IL-6 release upon zymosan challenging occurred in mice with DSS treatment for >6 d (Fig. 4C). Interestingly, significant body weight decrease in DSS-treated mice also started at approximately the same time (Figs. 2A, 5C). These results suggest that, although DSS-induced colitis started much earlier than day 6, as elevations of inflammatory cytokines (Table III) and leukocyte infiltration into the colonic tissues were apparent on

![Figure 4](image-url)

**FIGURE 4.** Enhancement of PMN infiltration in DSS-induced colitis is correlated with IL-17A production. A, Time-course analysis of serum cytokines during DSS-induced colitis (also see Table III). B, Analysis of PMN infiltration by zymosan-induced peritonitis in mice treated with DSS for different days. Note that significantly enhanced PMN infiltration occurs after 6 d of DSS treatment and in parallel with increases in IL-17A. C, Analysis of peritonitis-associated IL-6 production in mice treated with DSS for different days. D, Tissue section of colon intestines and labeling for infiltrated PMN using an anti-Gr-1 Ab. The data represent one of at least three independent experiments and are expressed as means ± SEM with at least five mice per group. *p < 0.05, **p < 0.01, ***p < 0.001 versus respective controls.
days 2–5 (Fig. 4D), the quick collapse of the pathological condition at the later stage was associated with the enhancement of PMN infiltration and the consequent severe tissue damage.

To further explore the role of IL-17A in the enhancement of PMN infiltration, we treated the DSS mice with a functional neutralization Ab against IL-17A (20, 21) (i.v.). As shown in Fig. 5, suppression of IL-17A function by the Ab in DSS-treated mice nearly completely eliminated the enhanced PMN infiltration at 2 h during zymosan-induced peritonitis (Fig. 5A) and such event-associated increases in IL-6 (Fig. 5B). The colitic symptoms in these DSS-treated mice, such as body weight loss, diarrhea, and intestinal tissue damage, were also largely alleviated (Fig. 5C). However, IL-17A neutralization had no effect on the basal level of PMN infiltration, as large numbers of PMN were still recruited into the peritoneum at 4 h during peritonitis even with the Ab administration (Fig. 5A). PMN infiltration into intestinal mucosa was also present in DSS-treated mice with anti–IL-17A administration, but remained at a low level (data not shown). We further performed ex vivo experiments and tested whether exposure to IL-17A directly modulates PMN function. In these experiments, bone marrow leukocytes isolated from healthy mice were ex vivo treated with a functional rIL-17A and then transferred into other healthy mice (recipients) followed by testing by zymosan-induced peritonitis. As shown in Fig. 6A, PMN treated with rIL-17A demonstrated markedly accelerated infiltration into the peritoneum during peritonitis compared with nontreated control PMN that were cotransferred into the same recipients. As expected, the presence of the anti–IL-17A Ab in the treatment completely eliminated the enhancement of PMN infiltration. Similarly, PMN that were treated with the serum from colitic mice (DSS treatment for 12 d) also manifested accelerated infiltration in recipient mice during peritonitis. Consistent with these results, assaying PMN chemotaxis in vitro (Fig. 6B) observed significantly augmented chemotactic transmigration for PMN treated with rIL-17A, whereas neutralization of IL-17A function erased such augmentation. Because tissue macrophage responses were also altered under inflammatory conditions, we tested whether IL-17A also directly modulates macrophage function. As shown in Fig. 6C, treatment of peritoneal macrophages isolated from healthy mice with rIL-17A markedly elevated IL-6 production upon the subsequent stimulation with zymosan. Western blot analyses of IL-17A–treated macrophages indicated significantly increased protein tyrosine phosphorylations (Fig. 6D), suggesting that active signaling events were elicited by IL-17A. In addition, we also observed direct binding of rIL-17A to the cell surfaces of PMN and macrophages (not shown), presumably to the receptors. In conclusion, these results strongly suggest that IL-17A, which was produced at a large amount in the postacute/chronic stage of colitis, serves as a critical feedback factor and contributes to reinforcement of the inflammatory reaction through systemically modulating leukocytes in the circulation and tissues.

**Discussion**

PMN, which comprise more than two-thirds of peripheral leukocytes, are the first-tier responsive cells during inflammation. As shown in zymosan-induced peritonitis, when a stimulatory signal is effectively produced, PMN rapidly transmigrate through the vasculature and tissues and arrive at the inflammatory site within a few hours. After a short period and vigorous activity, PMN infiltration is promptly ceased, provided that the inflammatory causes are largely cleared, to confine and limit tissue damage. However, when the inflammatory causes linger, as those in DSS-induced colitis, PMN infiltration continues, and the inflammatory reaction prolongs and becomes chronic. In this study, we employed inflammation mouse models and studied PMN infiltration under various chronic conditions. We found that animals that were preconditioned with nonresolving colitis, type 1 diabetes, or other inflammatory conditions had significantly enhanced PMN infiltration during zymosan-induced peritonitis. Cross-transfer of leukocytes from mice with ongoing inflammation to healthy recipients, or from healthy mice to inflammatory recipients, further revealed that both circulating PMN and tissue microenvironments were
altered under chronic inflammatory conditions and that these changes collectively contributed to the promotion of PMN infiltration during peritonitis.

Detailed analysis of DSS-induced colitis found that PMN infiltration into intestinal mucosa occurred shortly after the starting of DSS treatment (days 2 to 3); concomitantly, a panel of inflammatory cytokines associated with colitis, including low levels of IL-17A, which are presumably released from the local γδ T cells and infiltrated PMN (22), were also produced (Table III). However, when the DSS treatment continued over 6–8 d, we observed extraordinarily high levels of IL-17A in the blood serum. Our studies suggest that this late-produced strong wave of IL-17A, which was likely through the activation of the second-tier inflammatory cells such as Th17 cells, is capable of reaching and regulating cells distant from the original inflammatory site through circulation, leading to systemic modulations of the entire inflammation and homeostasis. Indeed, our data showed that both macrophages and PMN in distant tissues are regulated by IL-17A, which were identified in bone marrow myelocytes, especially mature PMN. Strikingly, the time course of elevation of IL-17A in serum during DSS treatment tightly correlated with the significant enhancement of PMN infiltration observed in zymosan-induced peritonitis. Subsequent in vitro and ex vivo experiments confirmed that IL-17A directly modulates PMN and significantly accelerated PMN chemotactic transmigration toward inflammatory stimuli. Not only modulating PMN, IL-17A also enhances the responses and sensitivities of macrophages around the body. In vitro exposure of macrophages to IL-17A prior to stimulation with zymosan resulted in much increased production of the proinflammatory cytokine IL-6. Peritoneal macrophages isolated from late-stage colitic mice also demonstrated enhanced responses upon inflammatory challenge. All of these results together indicate that when inflammation prolongs, feedback signals are produced and trigger a systemic regulation of the entire inflammatory reaction. In the case of DSS-induced colitis, large amounts of IL-17A produced at the postacute stage circulating PMN and tissue macrophages and enhance their inflammatory functions. These regulations by IL-17A, which are presumably designed to accelerate the removal of the original inflammatory cause, nevertheless lead to exaggerated inflammation-associated tissue damage. Suppression of IL-17A-mediated propagation of inflammation mitigates the disease condition.

Priming PMN under inflammatory conditions and by inflammatory factors has been observed in research through the years and has been considered to be a major factor responsible for the pathogenesis of organ failure occurred under postinjury conditions such as trauma, burn, and surgery and other conditions such as adult respiratory distress syndrome, sepsis, cystic fibrosis, blood transfusion, etc. (25–32). Reported agents that prime PMN include platelet-activating factor (33, 34), G-CSF (35, 36), GM-CSF (37), IL-1β (38), IL-6 (39), IL-8 (35), etc. In this study, our data suggest that IL-17A is likely to be a critical priming agent for PMN and macrophages under inflammatory conditions, yet the mechanism underlying its effect and the phenotypic characteristics of PMN (40) after exposure to IL-17A need to be further determined. Although many studies have shown that IL-17A production is regulated by IL-6 and IL-23 (and other cytokines) (41, 42), our study in DSS-induced colitis only detected slight increases of these cytokines in the serum, implicating that IL-6– and IL-23–mediated activation of Th17 cells may be mainly restricted in the local intestinal tissues. In addition, previous studies by Schwar-
exaggerated reactions can prevail and cause overstated challenge, such as viral/bacterial infection or cancer, during which markedly enhanced inflammatory status also renders the chronic inflammations that regulate the dynamics of inflammation. Our results suggesting that incessant colitis has consumed majority of PMN mature PMN in bone marrow in mice treated with DSS for ≥9 d, suggesting that incessant colitis has consumed majority of PMN and largely depleted the reservoirs.

Thus, through this study, we have learned of important mechanisms that regulate the dynamics of inflammation. Our results imply that when an inflammatory condition becomes unrelenting, such as in inflammatory bowel disease, diabetes, and rheumatoid arthritis, significantly enhanced inflammatory responses and PMN infiltration can occur and lead to severe tissue injury and deteriorated pathophysiological condition. In addition, systemically enhanced inflammatory status also renders the chronic patients highly vulnerable when they face a new inflammatory challenge, such as viral/bacterial infection or cancer, during which exaggerated reactions can prevail and cause overestimated consequences.

Disclosures

The authors have no financial conflicts of interest.

References


