DOI: 10.1189/jlb.3HI0517-198R

JLB JOURNAL OF LEUKOCYTE BIOLOGY

HIGHLIGHTED ARTICLE

Frontline Science: Proliferation of Ly6C$^+$ monocytes during urinary tract infections is regulated by IL-6 trans-signaling

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Abstract

Ly6C$^+$ monocytes are important components of the innate immune defense against infections. These cells have been shown to proliferate in the bone marrow of mice with systemic infections. However, the proliferative capacity of Ly6C$^+$ monocytes in infected peripheral tissues as well as the associated regulatory mechanisms remain unclear. In this study, we analyzed the proliferative capacity of Ly6C$^+$ monocytes in the urinary bladder after infection with uropathogenic *E. coli*, one of the most prevalent pathogen worldwide, and in LPS-induced peritonitis. We show that Ly6C$^+$ monocytes proliferated in the bladder after infection with uropathogenic *E. coli* and in the peritoneum after intraperitoneal injection of LPS. We identified IL-6, a molecule that is highly expressed in infections, as a crucial regulator of Ly6C$^+$ monocyte proliferation. Inhibition of IL-6 via administration of antibodies against IL-6 or gp130 impeded Ly6C$^+$ monocyte proliferation. Furthermore, repression of IL-6 *trans*-signaling via administration of soluble gp130 markedly reduced the proliferation of Ly6C$^+$ monocytes. Overall, this study describes the proliferation of Ly6C$^+$ monocytes using models of urinary tract infection and LPS-induced peritonitis. IL-6 *trans*-signaling was identified as the regulator of Ly6C$^+$ monocyte proliferation.

KEYWORDS
microscopy, immunology, innate immune cells, cytokines, local accumulation of monocytes

1 | INTRODUCTION

Monocytes are key components of the innate immune defense against infections.[1–4] These cells can be classified as inflammatory Ly6C$^+$CCR2$^+$CX$_3$CR1^{int} cells and patrolling Ly6C$^-$CCR2$^-$CX$_3$CR1^{hi} cells.[5] Ly6C$^+$ monocytes play an important role in the immune defense against infection and are especially crucial to fight urinary tract infections.[6,7] Proliferation of Ly6C$^+$ monocytes and their progenitors in the bone marrow has been shown in mice with systemic infection due to *Listeria monocytogenes*.[8] Moreover, monocyte proliferation has shown to be regulated by the growth factor M-CSF.[9–11] However, the proliferative capacity of Ly6C$^+$ monocytes during infections remains unclear.

The cytokine IL-6 has been shown to be critical in inflammatory diseases in which monocyte accumulation occurs.[4,12,13] In urinary tract infection, IL-6 is highly expressed and reduced IL-6 levels causes enhanced susceptibility to infection.[14–16] Moreover, epithelial cells have been shown to be the major source of IL-6 in the infected bladder.[17,18] Previously, two distinct modes of IL-6 signaling have been described. In classic signaling, IL-6 stimulates leukocytes by binding to a membrane bound IL-6 receptor, whereas *trans*-signaling is mediated by binding of IL-6 to a soluble IL-6 receptor. Both modes of signaling result in homodimerization of the molecule gp130.[4] While only a few cells express the receptor for IL-6, all cells display gp130 [4,19] and are responsive to a complex composed of the IL-6 protein and the soluble form of the IL-6 receptor.[20] Myeloid cells, such as monocytes and neutrophils, have been considered as a crucial source of the soluble IL-6 receptor.[21] Furthermore, inflammatory mediators released by neutrophils induce the shedding of the IL-6 receptor in an ADAM17-dependent fashion.[22–25] The binding of this complex (soluble IL-6R/IL-6) to the ubiquitously expressed gp130 dramatically widens the spectrum of the IL-6-responding cells. Soluble gp130 (sgp130) binds soluble IL-6R/IL-6 complexes and therefore specifically inhibits IL-6 *trans*-signaling.[26] Previously, *trans*-signaling has been shown to mediate a

Received: 18 May 2017 | Revised: 15 July 2017 | Accepted: 21 August 2017

proinflammatory functionality of leukocytes, whereas IL-6 classic signaling induced homeostatic and anti-inflammatory properties.[20,27,28] In this study, we assessed the role that IL-6 signaling plays in the regulation of Ly6C$^+$ monocyte proliferation during urinary tract infections, one of the most prevalent infections worldwide.

2 | MATERIALS AND METHODS

2.1 | Mice

All mice were between 8–12 wk of age. Animals were bred and maintained under specific-pathogen-free conditions in the central animal facility at the Essen University Clinic. The LysM^{Cre} (B6.129P2-*Lyz2*$^{tm1(cre)Ifo}$/J) mice were provided by Gisa Tiegs (Hamburg, Germany). IL-6Rα floxed mice (B6.SJL-*Il6ra*$^{tm1.1Drew}$/J) were obtained from Juergen Scheller (Düsseldorf, Germany), and female C57BL/6 mice were purchased from Janvier Laboratories (Genest-Saint-Isle, France). All animal experiments were approved by the local animal review boards (Bezirksregierung Köln, Landesamt für Natur, Umwelt und Verbraucherschutz NRW in Recklinghausen, Germany).

2.2 | Urinary tract infection model

Uropathogenic *E. coli* (UPEC) strain 536 (O6:K15:H31)[29] were cultured overnight at 37°C in LB medium (LB broth by Roth). Bacteria were harvested via centrifugation at 1200 *g* for 10 min and suspended in 1 ml of PBS. Female mice, between 8 and 10 wk of age, were anesthetized with a 1:1 mixture of 2% Rompun (BAYER) and 10% Ketamine (Medistar). The animals were then infected via transurethral inoculation of 5×10^8 UPECs in 0.05 ml PBS using a soft polyethylene catheter (BD Biosciences, San Jose, CA, USA).

2.3 | Blocking experiments

Blocking experiments were performed by transurethral injection (using a soft polyethylene catheter) of the indicated reagents: anti-IL-6 (clone MP5-20F3, 20 µg; eBioscience), anti-gp130 (clone 125605, 750 ng; R&D Systems, Minneapolis, MN, USA), Sgp130 (20 µg; R&D Systems) into the bladder lumen 3 h after infection. PBS and IgG1 isotype antibody (clone 2A3; Bio X Cell, NH, USA) were used as controls.

2.4 | Bone marrow transfer experiments

Bone marrow cells were isolated by flushing out the bone marrow of the femur of mice by sterile PBS from C57BL/6 donor mice. 1×10^7 cells were transferred intravenously into CD45.1 recipient mice 3 h post infection. Twenty four hours post infection mice were sacrificed and bladders were harvested for flow cytometry analysis.

2.5 | BrdU incorporation assays

To label proliferative cells in vivo, 100 µl BrdU solution (10 mg/ml solution of BrdU in sterile 1× DPBS) was administered via intraperitoneal injection at 3 h postinfection. The mice were then sacrificed 24 h after infection. If later postinfection time points were analyzed (Fig. 1E), BrdU was administered 3 h after infection, followed by repetitive injections of BrdU every 24 h. After sacrificing the mice, the bladders were isolated and bladder digests were further processed for BrdU staining using the FITC BrdU Flow Kit by BD Biosciences.

2.6 | Isolation of leukocytes from the urinary bladder

The bladders were sliced into small pieces using a scalpel. The bladder tissue was then digested for 30 min at 37°C with 0.5 mg/ml collagenase and 100 µg/ml DNAse I in RPMI 1640 Medium (GE Healthcare, Waukesha, WI, USA) containing 10% heat-inactivated FCS (PAA Laboratories, Dartmouth, MA, USA), 20 mM HEPES (Roth), 0.1% β-mercaptoethanol, 1 mM L-Glutamine and antibiotics. Single-cell suspensions were filtered through a 100 µm nylon mesh and further processed for flow cytometry analysis.

2.7 | In vitro proliferation assay

Bone marrow cells were obtained by flushing the femurs with PBS. Ly6C$^+$ monocytes were isolated by a 2-step MACS protocol. 1) Untouched isolation of monocytes (Monocyte isolation KIT (Miltenyi Biotec, Gladbach, Germany) followed by 2) Ly6C-positive selection (purified Ly6C [clone ER-MP20; AbD Serotec (CA, USA); anti-biotin MACS beads (Miltenyi Biotec)]. Ly6C$^+$ monocytes were stained with CFSE and cultured in the absence or presence of Hyper-IL-6 (a fusion protein of IL-6 and the soluble IL-6R) in RPMI medium (10% FCS+ P/S+ L-Glu+ 0.5% β-mercaptoethanol) at 37°C in a 96-well plate (1×10^5 cells/well). Three days after incubation, cells were detached using 2 mM EDTA and single cell suspensions were analyzed by flow cytometry.

2.8 | Flow cytometry

Cell surface staining: Single-cell suspensions were washed with PBS containing 0.1% BSA and 0.1% NaN_3, and FcR were blocked with human immune globulin (Privigen, Kankakee, IL, USA). Titrated amount of the fluorochrome-labeled antibodies were used for staining (1×10^6 cells/sample).

For intracellular IL-6 staining, single-cell suspensions were incubated for 4 h with 1 µl/ml Golgi Plug (BD Biosciences) in RPMI 1640 Medium (GE Healthcare) containing 10% heat-inactivated FCS (PAA Laboratories), 20 mM HEPES (Roth), 0.1% β-mercaptoethanol, 1 mM L-Glutamine. After surface marker staining, the samples were fixed with 4% PFA, washed with Perm wash buffer (BD Biosciences) and incubated with anti-IL-6 antibody for 20 min at 4°C in the dark.

Cells were measured using a LSR Fortessa (BD Biosciences) and analyzed with Flow Jo software (Tristar). Absolute cell numbers were calculated by adding a fixed number of APC labeled microbeads (BD Biosciences) to each sample.

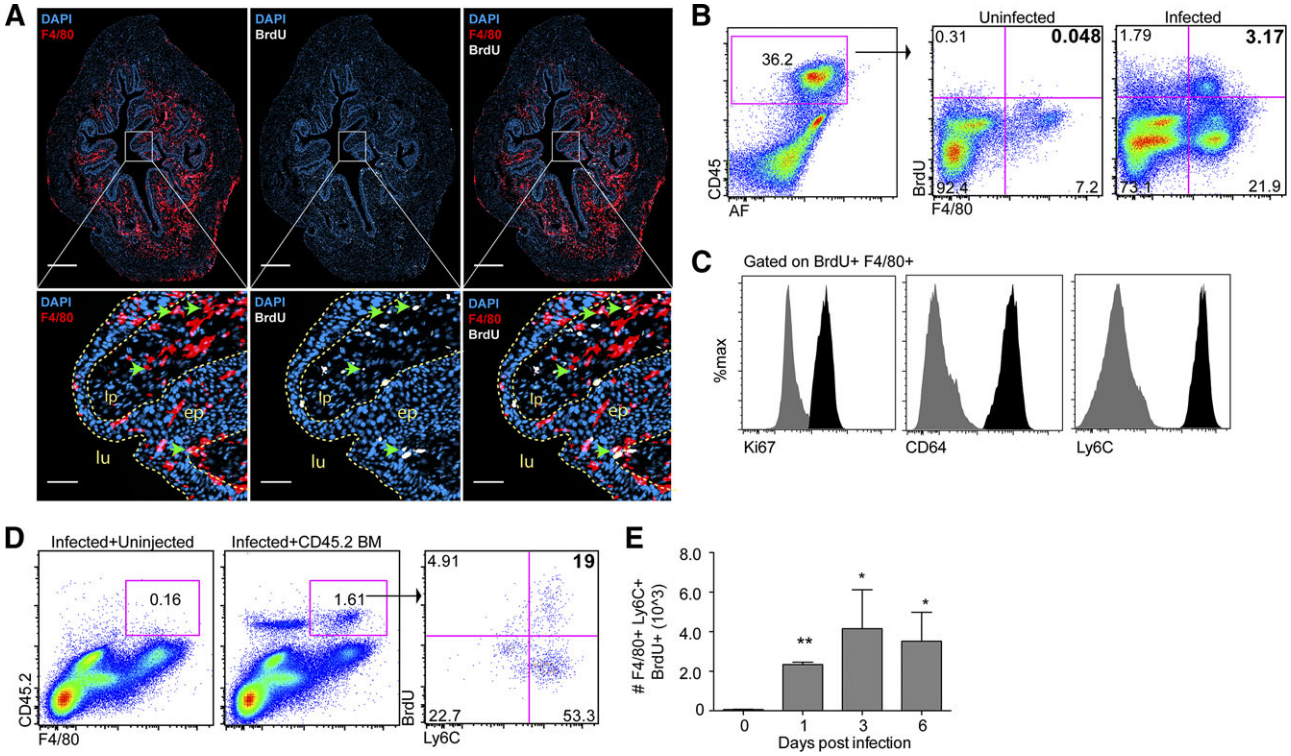

FIGURE 1 **Ly6C$^+$ monocytes proliferate in the infected urinary bladder.** C57BL/6 mice were infected with uropathogenic *E. coli* (UPEC) via transurethral injection, and BrdU was injected 3 h postinfection. (A) Bladder sections were stained for DAPI (blue), F4/80 (red), and BrdU (white) 1 d after infection. The green arrows in the detailed images (lower row) indicate DAPI$^+$F4/80$^+$BrdU$^+$ cells. The white bar is equivalent in length to 200 (top images) and 50 μm (bottom images). (B) Incorporation of BrdU by bladder leukocytes (CD45$^+$) was measured in uninfected mice and 1 d after UPEC infection via flow cytometry. (C) Flow cytometric analysis of Ly6C, CD64 and Ki67 expression levels (black; gray = isotype control) in CD45$^+$F4/80$^+$BrdU$^+$ leukocytes in the bladder 1 d postinfection. (D) CD45.2 bone marrow cells were transferred intravenously into uninfected animals (left dot plots) and into CD45.1 recipients 3 h postinfection (middle plot). BrdU incorporation by CD45.2$^+$F4/80$^+$ monocytes in the bladder of infected mice was analyzed 1 d after infection. (E) The number of F4/80$^+$Ly6C$^+$BrdU$^+$ monocytes in the bladder was measured at various time points after infection via flow cytometry. BrdU was administered 3 h postinfection, followed by repetitive injections of BrdU every 24 h. Statistical significance on days 1, 3 and 6 was tested against d 0. Data are mean ± SEM; (A and B), $n = 10$; (C–E), $n = 4$–6; $^*P < 0.05$; $^{**}P < 0.01$. ep = epithelium; lu = lumen, lp = *lamina propria*

2.9 | Histology

Bladders were fixed in PLP buffer overnight [pH 7.4, 0.05 M phosphate buffer containing 0.1 M L-lysine (pH 7.4, Roth), 2 mg/ml NaIO4 (Roth), and 10 mg/ml paraformaldehyde, final concentration of 4% (Sigma-Aldrich, St. Louis, MO, USA)]. The bladder was then equilibrated in 30% sucrose for an additional 24 h. The bladders were frozen in Tissue-Tek OCT. The bladders were then sectioned at −20°C using a cryostat. The sections (10 μm) were rehydrated using PBS containing 0.05% Triton X-100 (Roth) and then blocked for 1 h with PBS containing 1% BSA (GE Healthcare) and 0.05% Triton X-100. The sections were stained for 1 h with anti-mouse BrdU (primary antibody, 1:500), anti-rat AF647 (secondary antibody, 1:500), DAPI (1:5000), F4/80 PE (1:200), Gr1 AF488 (1:200). The stained sections were then assessed measured using the Zeiss AxioObserver.Z1 with Apotome, and the images were analyzed using ImageJ Software. ImageJ was used to generate point maps of the raw microscopy images indicating cells positive for multiple fluorescent labels. An intensity threshold was used to generate masks for each fluorescent channel. Overlapping mask regions were then used to identify cells positive for multiple labels, which were marked with a point placed at the center of the cell nucleus and color-coded according to the combination of overlapping masks.

2.10 | Antibodies

The antibodies used included the following: CD45 BV421 (clone: 30-F11; BioLegend, San Diego, CA, USA), F4/80 PE and APC (clone BM8.1; TONBO Biosciences, San Diego, CA, USA), Ly6C Per CP Cy5.5 (clone HK1.4; eBioscience), purified Ly6C (clone ER-MP2; AbD Serotec), CD64 PE (clone ×54-5/7.1; BioLegend), Gr1 AF488 (clone RB6-8C5; BioLegend;), anti-BrdU (clone BU1/75 (ICR1; Abcam Inc, Cambridge, MA, USA), Ki67 Pacific Blue (clone 16A8; BioLegend), anti-rat AF647 (Life Technologies, Carlsbad, CA, USA), purified anti-mouse IL-6 (clone MP5-20F3; eBioscience), purified anti-mouse gp130 (R&D Systems), purified IgG1 (clone 2A3; Bio X Cell).

2.11 | Reagents

BrdU Kit (FITC BrdU Flow Kit by BD Biosciences), recombinant mouse gp130 Fc chimera protein (Sgp130) Carrier Free (R&D Systems),

Monocyte isolation KIT (Miltenyi Biotec), anti-biotin MACS beads (Miltenyi Biotec).

2.12 | Cytokine array

The bladders were homogenized in the presence of complete proteinase inhibitor mix (Roche, Basel, Switzerland) in 500 µl PBS. The homogenates were centrifuged at 12,000 rpm for 20 min, and the supernatants were directly analyzed using the standard protocol provided with the MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel-Multiplex Assay (Merck Millipore, Billerica, MA, USA).

2.13 | LPS-induced peritonitis model

The mice received 1 µg/ml LPS [from *E. coli* oIII: B4; InvivoGen (San Diego, CA, USA)] via intraperitoneal injection. Peritoneal lavage was performed using chilled PBS with 2 mM EDTA to isolate the cells from the peritoneum. The peritoneal exudate was then centrifuged, and the single-cell suspension was stained for flow cytometry analysis.

2.14 | Statistical analysis

The data were analyzed using Prism software (GraphPad, La Jolla, CA, USA). Mann-Whitney and Kruskal-Wallis (Dunns) were used for statistical analysis; * indicates a *P*-value <0.05; ** < 0.01; *** < 0.001.

3 | RESULTS

3.1 | Ly6C$^+$ monocytes proliferate in the infected urinary bladder

In a murine model of bacterial infection of the urinary bladder induced by uropathogenic *E. coli* (UPEC), we detected proliferating BrdU$^+$F4/80$^+$ cells in the infected bladder by microscopy (Fig. 1A, Supplemental Figure 1) and flow cytometry (Fig. 1B). We also found that these cells expressed Ki67, thus indicating in situ proliferation (Fig. 1C). Moreover, these cells expressed CD64 and Ly6C (Fig. 1C), which suggests that the cells originated from recruited circulating Ly6C$^+$ monocytes. To verify that these proliferating Ly6C$^+$ monocytes were recruited from the blood, we transferred CD45.2$^+$ bone marrow monocytes into the blood of CD45.1 animals. One day after infection, we detected proliferating CD45.2$^+$ donor monocytes in the infected urinary bladder (Fig. 1D), thus demonstrating that donor monocytes were recruited from the blood. We also observed accumulation of Ly6C$^+$ monocytes over the course of the infection (Fig. 1E).

3.2 | Proliferating Ly6C$^+$ monocytes localize to the lamina propria

We then aimed to localize proliferating Ly6C$^+$ monocytes in the infected urinary bladder via immunohistochemistry. One day after the bladder infection was induced, we localized Ly6C$^+$ monocytes by staining the bladder sections with antibodies against BrdU, F4/80, and Gr1. Gr1 detects the molecules Ly6C and Ly6G. As Ly6G is selectively expressed on F4/80-negative neutrophils, the colocalization of F4/80 and Gr1 indicates F4/80$^+$Ly6C$^+$ monocytes. One day after infection, we observed markedly elevated levels of F4/80$^+$Ly6C$^+$/Gr1$^+$ monocytes in the infected urinary bladders (Fig. 2A) compared with the uninfected bladders (S1). To better visualize proliferating Ly6C$^+$ monocytes, we generated a point map and indicated the cells that expressed BrdU, F4/80 and Ly6C/Gr1 with white quadrants (Fig. 2B, Proliferating MO). This analysis revealed that proliferating monocytes were primarily located in the lamina propria (Fig. 2B and C).

3.3 | Blocking IL-6 reduces the proliferation of Ly6C$^+$ monocytes during urinary tract infection

To further analyze the mechanism of local monocyte proliferation, we performed a comprehensive cytokine screening assay. We found that IL-6 and IL-13 were strongly upregulated 1 d after infection (Fig. 3A). As IL-6 played an important role in the infections, we aimed to determine whether this molecule also drives the proliferation of Ly6C$^+$ monocytes during urinary tract infections. To test this hypothesis, we locally inhibited IL-6 via instillation of anti-IL-6 antibody into the bladder lumen of infected mice. Administration of this antibody significantly reduced the proliferation of Ly6C$^+$ monocytes (Fig. 3B and C). We also inhibited the gp130, the signal-transducing β-receptor for IL-6 signaling. Inhibition of gp130 via local instillation of anti-gp130 antibody into the bladder completely abolished the proliferation of Ly6C$^+$ monocytes (Fig. 3B and C), thus demonstrating that IL-6-signaling plays an essential role in regulating the proliferation of these cells. Notably, we detected very limited Ly6C$^-$ Mϕ proliferation, which was unaffected by IL-6 and gp130 inhibition (Fig. 3B and C). To investigate the source of IL-6, we analyzed IL-6 production via flow cytometry. We found that IL-6 was primarily produced by CD45-negative cells, thereby indicating that non-hematopoietic cells secrete this molecule in the infected bladder (Fig. 3D and E). Ly6C$^+$ monocytes, Ly6C$^-$ Mϕs, and Ly6G$^+$F4/80$^-$ neutrophils failed to produce significant levels of IL-6 (Fig. 3D and E). These data demonstrate that IL-6 signaling plays a crucial role in regulating Ly6C$^+$ monocyte proliferation during urinary tract infections.

3.4 | IL-6 regulates the proliferation of Ly6C$^+$ monocytes during peritonitis

Next, we investigated whether the proliferation of Ly6C$^+$ monocytes also occurs in other inflammatory models and organs in addition to the bladder. Therefore, we injected LPS into the peritoneum of mice to mimic an acute bacterial infection. We observed a significantly increased frequency of Ly6C$^+$ monocytes 1 d after injection (Fig. 4A). We also found that these Ly6C$^+$ monocytes strongly incorporated BrdU, whereas Ly6C$^-$ Mϕs barely incorporated BrdU (Fig. 4B). Local inhibition of IL-6 in this in vivo setting significantly reduced the proliferation of Ly6C$^+$ monocytes (Fig. 4C and D). These data demonstrate that the proliferation of Ly6C$^+$ monocytes is also induced in the inflamed peritoneum in an IL-6-dependent manner.

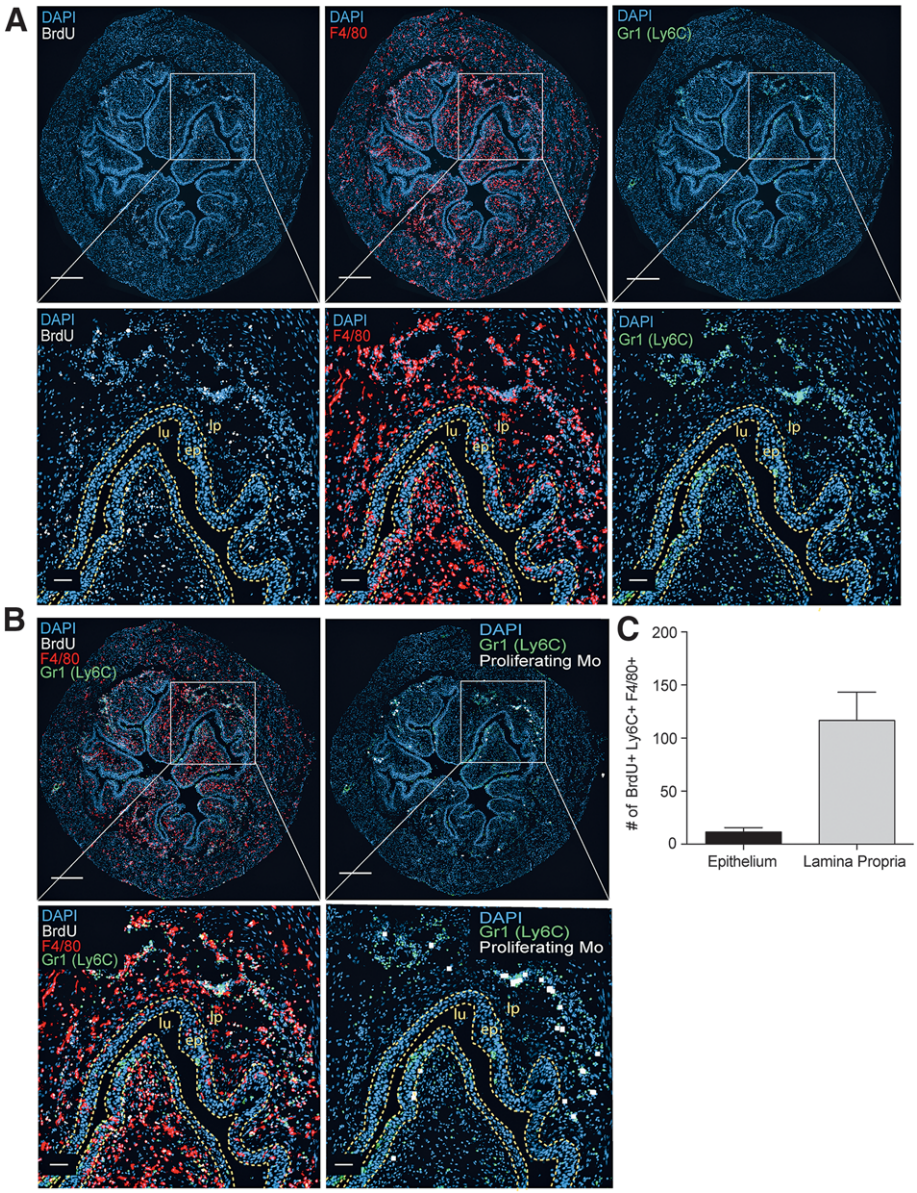

FIGURE 2 **Localization of proliferating monocytes in the infected bladder.** (A) One day after infection, bladder sections were stained for DAPI (blue), F4/80 (red), Gr1 (green), and BrdU (white). BrdU was injected into the murine peritoneum 3 h postinfection. (B) The raw microscopy images in (A) were processed to generate point maps (see Materials and Methods) that combine the fluorescent signals for DAPI, F4/80, Gr1/Ly6C, and BrdU. The white points indicate proliferating ($DAPI^+F4/80^+Gr1/Ly6C^+BrdU^+$) monocytes. The white bar indicates 200 (top images) and 50 μm (bottom images). (C) Quantification of proliferating monocytes within the epithelium and *lamina propria* of infected bladders. The quantification is based on the point maps (B), and the original images are displayed in (A). Data are mean ± SEM; (A–C), $n = 8$. ep = epithelium; lu = lumen; lp = *lamina propria*

3.5 | Proliferation of Ly6C$^+$ monocytes is regulated by IL-6-*trans*-signaling

We then investigated whether IL-6-dependent proliferation of Ly6C$^+$ monocytes is regulated by classic IL-6 signaling or IL-6 *trans*-signaling. To address this question, we employed *LysM-Cre*$^+$ *IL6r* $^{flox/flox}$ mice, which lack IL-6 receptor expression on monocytes and hence classic IL-6 signaling in Ly6C$^+$ monocytes. We found that the proliferation of Ly6C$^+$ monocytes was not reduced in *LysM-Cre*$^+$ *IL6r* $^{flox/flox}$ mice (Fig. 5A), thus demonstrating that classic IL-6 signaling is dispensable. To further investigate whether Ly6C$^+$ monocytes express the molecule gp130, which is essential for IL-6 *trans*-signaling, we stained Ly6C$^+$ monocytes from the bone marrow and from the bladder with an antibody against gp130. We found that bone marrow Ly6C$^+$ monocytes expressed gp130, whereas we could not detect gp130 expression on Ly6C$^+$ monocytes in the bladder (Fig. 5B) indicating internalization of the molecule after binding of the IL-6/IL-6R complex.[30] As the proliferation of Ly6C$^+$ monocytes might be induced by IL-6 *trans*-signaling, we stimulated Ly6C$^+$ bone marrow monocytes with Hyper-IL-6, an IL-6/sIL-6R fusion protein that mimics IL-6 *trans*-signaling, and analyzed the proliferation by the dilution of CFSE. We found that Hyper-IL-6 induced the proliferation of Ly6C$^+$ monocytes in a dose-dependent

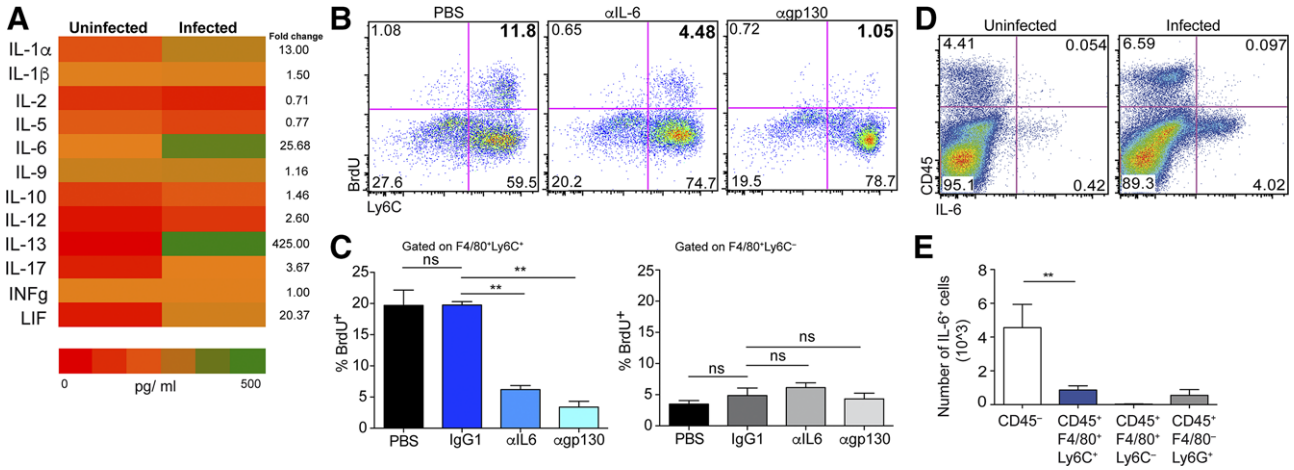

FIGURE 3 **Blocking IL-6 leads to reduced proliferation of Ly6C$^+$ monocytes.** C57BL/6 mice were infected with UPEC via transurethral injection. BrdU was administered into the peritoneum 3 hs postinfection. (A) One day postinfection, the bladders were mechanically homogenized and the levels of the indicated proteins in the supernatant were measured via Luminex assay. The concentrations of the measured proteins are depicted as a heat map; the numbers indicate fold increase in infected vs. uninfected samples. (B) Flow cytometry analysis of CD45$^+$ cells in bladder homogenates of infected mice. Mice were treated with anti-IL-6 (αIL-6) or anti-gp130 (αgp130) via transurethral injection 3 h after UPEC infection. Flow cytometric analysis was performed 1 d postinfection. (C) Quantitative analysis of (B); PBS and isotype control antibody were used as controls. (D) Representative flow cytograms show intracellular IL-6 expression by CD45$^+$ and CD45$^-$ cells in uninfected and infected animals 1 d after infection. (E) Quantification of (D). *$P < 0.05$; ** < 0.01. Data are mean ± SEM; (A), $n = 10$; (B and C), $n = 13$; (D and E), $n = 8$

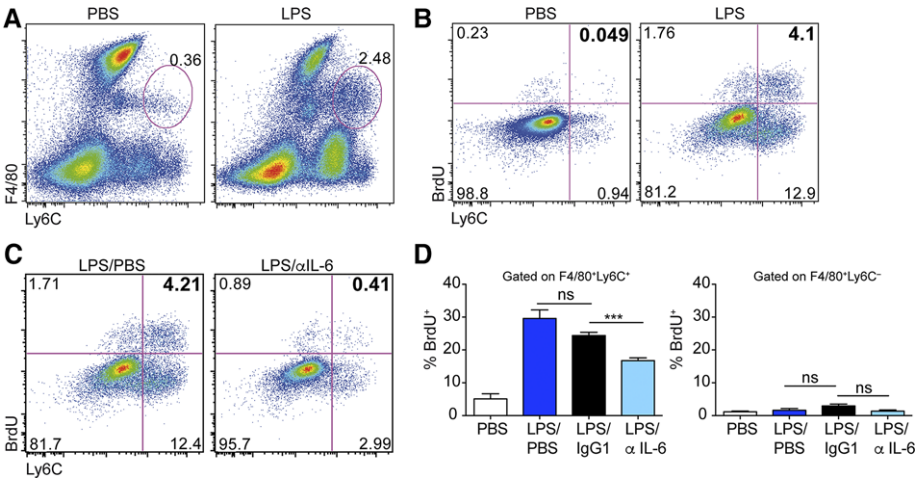

FIGURE 4 **Proliferation of Ly6C$^+$ monocytes in the inflamed peritoneum depends on IL-6.** C57BL/6 mice received LPS via intraperitoneal injection; PBS was used as a control. One day after injection, the frequency and proliferation of F4/80$^+$Ly6C$^+$ monocytes in the peritoneal exudate were analyzed via intraperitoneal injection of BrdU 3 h after LPS or PBS injection. (A) Representative flow cytograms show leukocytes (CD45$^+$) in the bladder homogenates of mice injected with PBS (left) or LPS (right) 1 d after injection. Ly6C$^+$ monocytes are encircled. (B) BrdU incorporation and Ly6C expression by F4/80$^+$ cells in the peritoneum of PBS- or LPS-injected animals. (C) BrdU incorporation by F4/80$^+$ cells in the inflamed peritoneum (+LPS) after αIL-6 antibody treatment. (D) Quantification of (C): Frequency of proliferating F4/80$^+$Ly6C$^+$ monocytes (left bar graph), F4/80$^+$Ly6C$^-$ Mϕs (right bar graph); PBS and isotype control antibody were used as controls. **$P < 0.01$. Data are mean ± SEM; (A and B), $n = 8$; (C and D), $n = 13$

manner (Fig. 5C). To analyze the function of IL-6 *trans*-signaling in vivo, we instilled sgp130 into the bladder to specifically block IL-6 *trans*-signaling.[26] We found significantly reduced incorporation of BrdU by Ly6C$^+$ monocytes in these animals (Fig. 5D and E), thereby indicating that IL-6 *trans*-signaling regulates the proliferation of Ly6C$^+$ monocytes. Interestingly, the number of Ly6C$^+$ monocytes was slightly impacted by the obstruction of IL-6 *trans*-signaling (Fig. 5F), whereas the expression of MCP-1 was unaltered (Fig. 5G). The expression levels of the growth factor M-CSF were unchanged following the inhibition of IL-6 *trans*-signaling (Fig. 5H), thus indicating that local proliferation of Ly6C$^+$ monocytes in the infected bladder is regulated by IL-6 *trans*-signaling, independent of M-CSF.

4 | DISCUSSION

Ly6C$^+$ monocytes have been increasingly recognized as important protagonists in inflammation and infection.[2,3] During infection,

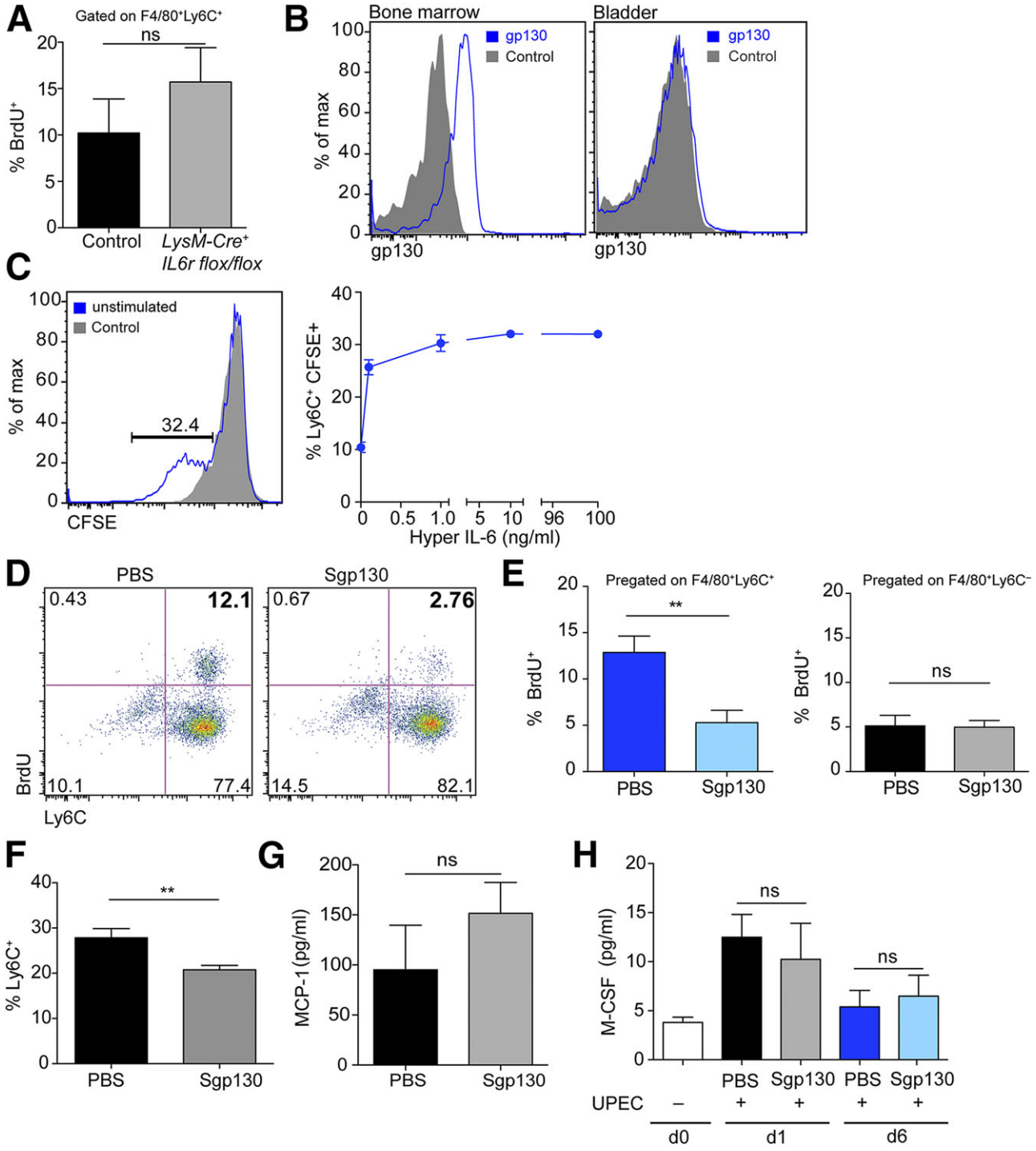

FIGURE 5 **Proliferation of Ly6C$^+$ monocytes depends on IL-6 *trans*-signaling.** (A) UPEC-infected *LysM-Cre*$^+$ *IL6r* $^{flox/flox}$ and C57BL/6 mice (control) were analyzed for BrdU incorporation via flow cytometry. The frequency of BrdU incorporation by F4/80$^+$Ly6C$^+$ monocytes is depicted. (B) Expression of gp130 was determined on Ly6C$^+$ monocytes from the bone marrow or the infected bladder by flow cytometry 1 d after infection. (C) Ly6C$^+$ bone marrow monocytes were cultured with or without hyper IL-6 (hIL-6) for 3 d and CFSE dilution was measured. (D–H) Three hours after infection, sgp130 or PBS (control) was instilled via transurethral injection into C57BL/6 animals. (D) F4/80$^+$ monocytes were analyzed for Ly6C expression and BrdU incorporation via flow cytometry. (E) Frequency of proliferating (BrdU$^+$) F4/80$^+$Ly6C$^+$ monocytes (left bar graph) and F4/80$^+$Ly6C$^-$ Mϕs (right bar graph). (F) The percentage of F4/80$^+$Ly6C$^+$ monocytes (among CD45$^+$ leukocytes) in the bladder homogenates was measured via flow cytometry. (G) MCP-1 levels in the supernatants of bladder homogenates were measured 1 d after infection. (H) M-CSF levels were measured in the bladder homogenates of uninfected (– UPEC) and infected mice (+ UPEC). Infected mice were treated with PBS or sgp130 3 h postinfection, and M-CSF levels were measured at days 1 and 6 postinfection. $^*P < 0.05$; $^{**}P < 0.01$. Data are mean ± SEM; (A), $n = 5$; (B), $n = 2$ (bone marrow) $n = 5$ (bladder); (C), values represent duplicates; (D and E), $n = 8$; (F), $n = 13$; (G), $n = 5$; (H), $n = 5$

circulating Ly6C$^+$ monocytes are rapidly recruited, and local accumulation of these cells has been considered crucial for the immune response against pathogens.[1,6,31,32] The efficient recruitment of these cells led to the paradigm that local proliferation of these cells might be dispensable and cell cycle arrest cannot be overcome. In this study, we show that recruited Ly6C$^+$ monocytes vigorously proliferated in an IL-6 *trans*-signaling dependent manner following bacterial infection.

Local proliferation of tissue-resident Mϕs has been demonstrated in Th2-mediated diseases.[10,33] We detected only slight proliferation of Ly6C$^-$ tissue Mϕs after urinary tract infection, thereby suggesting that the proliferation of these cells might not be relevant in this acute infection model. Instead, we observed rapid proliferation of cells that expressed intermediate levels of F4/80 and high levels of CD64 and Ly6C, thereby indicating that these proliferating cells are monocyte-derived.

A broad cytokine array revealed strong upregulation of IL-6 during urinary tract infections. This molecule has been shown to be highly upregulated in patients with severe and febrile urinary tract infection.[14] Flow cytometry revealed that IL-6 was mainly produced by CD45-negative non-hematopoietic cells. Given that IL-6 has been shown to be produced by epithelial cells,[17,18] the proliferation of Ly6C$^+$ monocytes might be regulated by the crosstalk of these two cell types. Global inhibition of the IL-6 pathway, using antibodies against IL-6 or gp130, reduced the proliferative capacity of Ly6C$^+$ monocytes demonstrating that this cytokine plays a crucial role in monocyte proliferation. Over the last decade, significant progress has been made to elucidate the pleiotropic function of the potent proinflammatory molecule IL-6,[4] and studies of IL-6 inhibition in a variety of inflammatory settings have provided significant insight into the these conditions. In cases of arthritis, inhibition of IL-6 prevents disease progression [1] as well as the accumulation of monocytes.[34] By contrast, overexpression of IL-6 signaling during herpes virus 8 infections in humans promotes monocyte accumulation and development of Kaposi's sarcoma,[35–37] which indicates that IL-6 plays an important role in monocyte accumulation in a variety of inflammatory conditions. Our study reveals that IL-6 drives the proliferation of Ly6C$^+$ monocytes during urinary tract infection. We found that the percentage of Ly6C$^+$ monocytes in the infected urinary bladder was slightly decreased after blocking *trans*-signaling. These data suggest that the number of Ly6C$^+$ monocytes is regulated by local proliferation. This finding does not exclude a role of IL-6 *trans*-signaling in regulating the recruitment of Ly6C$^+$ monocytes from the circulation. However, we show that the expression of the chemokine MCP-1 that mainly induces the recruitment of Ly6C$^+$ monocytes, is similarly expressed after blocking IL-6 *trans*-signaling suggesting that the impact of IL-6 *trans*-signaling on the local proliferation might be dominant over recruitment.

An important component of IL-6 *trans*-signaling is the shedding of the IL-6 receptor, which binds to IL-6 and initiates IL-6 signaling via the gp130 molecule. Myeloid cells, such as monocytes and neutrophils, have been considered as a crucial source of this shed receptor.[21] Furthermore, inflammatory mediators released by neutrophils induce the shedding of the IL-6 receptor in an ADAM17-dependent fashion.[22–25] However, proliferation of Ly6C$^+$ monocytes was unchanged in *LysM^{cre}/IL6r$^{flox/flox}$* animals, thus indicating that shedding of the IL-6 receptor by cells other than LysM$^+$ monocytes and neutrophils facilitates IL-6 *trans*-signaling in this infection model. These data also indicate that the IL-6 receptor expressed on Ly6C$^+$ monocytes is dispensable indicating that classic IL-6 signaling does not initiate monocyte proliferation. Accordingly, we found strong expression of gp130 on the surface of bone marrow Ly6C$^+$ monocytes, the direct progenitor of Ly6C+ monocytes in infected organs. Notably, the expression of gp130 was undetectable on Ly6C$^+$ monocytes in the infected urinary bladder indicating that IL-6 *trans*-signaling and internalization of the complex might have occurred.[30] Moreover, administration of sgp130, which blocks *trans*-signaling by inhibiting the binding of the soluble IL-6R – IL-6 complex to gp130 on the cell surface, strongly reduced Ly6C$^+$ monocyte proliferation.

Previously, IL-6 *trans*-signaling has been shown to mediate a proinflammatory functionality of leukocytes.[20,27,28] In murine models, overexpression of sgp130 or administration of sgp130 to reduce IL-6 *trans*-signaling blocked the inflammatory processes demonstrating a major role of IL-6 *trans*-signaling in regulation the proinflammatory properties of leukocytes.[38,39] In this study, we demonstrate that IL-6 *trans*-signaling regulates the proliferation of Ly6C$^+$ monocytes. As this subset of monocytes has been considered proinflammatory in a variety of infection models,[3] reduced proliferation of these cells might impact on the inflammatory milieu in infected organs. Moreover, Ly6C$^+$ monocytes also have the potential to differentiate into Mϕs [40,41] and these cells might be directly involved in the healing process. Hence, blocking the proliferation of Ly6C$^+$ monocytes might alter the resolution of the infection and the infection-induced fibrosis development.

This study shows that IL-6 *trans*-signaling initiates the proliferation of Ly6C$^+$ monocytes in experimental models of urinary tract infection and LPS-induced peritonitis. Moreover, stimulation of Ly6C$^+$ monocytes with Hyper-IL-6 induced the proliferation of these cells. Further studies are required to investigate the impact of Ly6C$^+$ monocyte proliferation vs. monocyte recruitment during urinary tract infection and the role of these proliferating monocytes during the progression and resolution of the disease.

In conclusion, this study describes the local proliferation of recruited Ly6C$^+$ monocytes during bacterial infections. We found that IL-6 *trans*-signaling regulates local monocyte proliferation. These findings provide new insights into the role that IL-6 *trans*-signaling plays in the regulation of Ly6C$^+$ monocytes during urinary tract infections and LPS-induced peritonitis.

AUTHORSHIP

A.D. designed the experiments and wrote the paper. J.B., A.-L.B., J.S., S.T., A.B., and A.S. performed the experiments, analyzed the data, and provided assistance concerning data imaging. C.G., S.R.-J., and H.-W.M. provided crucial reagents and discussed the data; D.R.E. conceived the study and wrote the paper.

ACKNOWLEDGMENTS

We acknowledge support by the Central Animal Facilities of the Medical Faculty Essen; This work was supported by Grants of the

Deutsche Forschungsgemeinschaft to D.R.E (EN984/5-1, EN984/6-1 and SFBTR57), the Marga and Walter-Boll Foundation (220-06-16), Mercur (An-2015-0066) and intramural research funds of the Medical Faculty of the University Duisburg-Essen (IFORES to D.R.E.) and SFB841. We thank Judith-Mira Pohl for critically reading the manuscript.

DISCLOSURES

The authors declare no conflicts of interest.

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How to cite this article: Dixit A, Bottek J, Beerlage A-L, et al. Frontline Science: Proliferation of Ly6C$^+$ monocytes during urinary tract infections is regulated by IL-6 trans-signaling. *J Leukoc Biol*. 2018;103:13–22. https://doi.org/10.1189/jlb.3HI0517-198R