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IL-6 Regulates Neutrophil Trafficking during Acute Inflammation via STAT3[1]

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The successful resolution of inflammation is dependent upon the coordinated transition from the initial recruitment of neutrophils to a more sustained population of mononuclear cells. IL-6, which signals via the common receptor subunit gp130, represents a crucial checkpoint regulator of neutrophil trafficking during the inflammatory response by orchestrating chemokine production and leukocyte apoptosis. However, the relative contribution of specific IL-6-dependent signaling pathways to these processes remains unresolved. To define the receptor-mediated signaling events responsible for IL-6-driven neutrophil trafficking, we used a series of gp130 knockin mutant mice displaying altered IL-6-signaling capacities in an experimental model of acute peritoneal inflammation. Hyperactivation of STAT1 and STAT3 in *gp130*$^{Y757F/Y757F}$ mice led to a more rapid clearance of neutrophils, and this coincided with a pronounced down-modulation in production of the neutrophil-attracting chemokine CXCL1/KC. By contrast, the proportion of apoptotic neutrophils in the inflammatory infiltrate remained unaffected. In *gp130*$^{Y757F/Y757F}$ mice lacking *IL-6*, neutrophil trafficking and CXCL1/KC levels were normal, and this corresponded with a reduction in the level of STAT1/3 activity. Furthermore, monoallelic ablation of *Stat3* in *gp130*$^{Y757F/Y757F}$ mice specifically reduced STAT3 activity and corrected both the rapid clearance of neutrophils and impaired CXCL1/KC production. Conversely, genetic deletion of *Stat1* in *gp130*$^{Y757F/Y757F}$ mice failed to rescue the altered responses observed in *gp130*$^{Y757F/Y757F}$ mice. Collectively, these data genetically define that IL-6-driven signaling via STAT3, but not STAT1, limits the inflammatory recruitment of neutrophils, and therefore represents a critical event for the termination of the innate immune response. ***The Journal of Immunology,*** **2008, 181: 2189–2195.**

Transition from innate to adaptive immunity is critical for the successful resolution of any inflammatory response, and represents a fundamental requirement for competent host defense. At the cellular level, this immunological switch is characterized by an initial influx of neutrophils which is subsequently replaced by inflammatory monocytes and T cells (1). Inappropriate regulation of this leukocyte trafficking can lead to impaired neutrophil clearance and increased tissue damage from the accumulation of neutrophil-secreted proteases and reactive oxygen species at the site of inflammation (2). Indeed, this situation has been implicated in the pathogenesis of many inflammatory diseases, such as chronic peritonitis, sepsis, inflammatory bowel disease, chronic obstructive pulmonary disease, and renal injury (3–7). Thus, identifying key inflammatory mediators and the pathophysiological consequences of their action in modulating neutrophil recruitment and clearance is a crucial step in understanding the molecular pathogenesis of these diseases.

Recent advances have identified certain inflammatory cytokines, TLR signaling, and complement activation as being pivotal in directing the tightly coordinated shift from innate to adaptive immunity (8). In particular, the cytokine IL-6 can dictate the profile of leukocyte recruitment during the inflammatory response via selective regulation of inflammatory chemokines and apoptotic events (1, 9, 10). The classical responsiveness to IL-6 is governed by a receptor complex consisting of two membrane-bound subunits, an 80-kDa cognate α-chain (IL-6Rα), and a ubiquitously expressed 130-kDa β-chain receptor (gp130) which acts as the universal signal-transducing element for all IL-6 family cytokines (11). Alternatively, IL-6 regulation of leukocyte trafficking relies upon signaling via its soluble IL-6Rα (termed IL-6 trans-signaling) (12). However, in either case, the receptor-mediated signaling events orchestrating this inflammatory response remain undefined.

Cytokine signaling through gp130 is triggered by receptor-associated JAKs, which phosphorylate specific tyrosine residues in the gp130 cytoplasmic domain to enable activation of STAT1 and STAT3 (13), as well as Src-homology phosphatase

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2 (SHP2)[5]-mediated MAPK and PI3K pathways (14). The phosphotyrosine residue at position 757 (pY_{757}) of murine gp130 (759 in human gp130) not only serves as a binding site for SHP2, but also plays a crucial role in the negative regulation of gp130 signaling by recruiting suppressor of cytokine signaling 3 (SOCS3) (15).

To genetically dissect the contribution of these gp130-mediated signaling events in directing neutrophil trafficking during inflammation, studies presented here have adopted two strains of gp130 knockin mice, *gp130*$^{\Delta STAT/\Delta STAT}$ and *gp130*$^{Y757F/Y757F}$, in which gp130-mediated activation of STAT1/3 and SHP2-ERK MAPK and -PI3K pathways, respectively, have been genetically abolished (16, 17). Notably, in *gp130*$^{\Delta STAT/\Delta STAT}$ mice, the absence of STAT1/3-binding motifs on gp130 results in impaired STAT3-mediated induction of SOCS3, leading to increased activation of the ERK MAPK and PI3K pathways (16, 17). Conversely, in *gp130*$^{Y757F/Y757F}$ mice, the phenylalanine substitution at Y_{757} in gp130 also prevents negative regulation of gp130-mediated STAT1/3 signaling by SOCS3, resulting in STAT1/3 hyperactivation (17). Using an established model of acute peritoneal inflammation which mimics many of the clinical features of bacterial peritonitis (9, 10), our studies conclude that the control of neutrophil clearance by IL-6 is reliant on gp130 signaling via STAT3, but not STAT1. Consequently, we propose that gp130-mediated STAT3 signaling represents a critical checkpoint regulator in the successful transition from innate to adaptive immunity, and therefore in the resolution of acute inflammation.

Materials and Methods

Mice

The generation of *gp130*$^{\Delta STAT/\Delta STAT}$, *gp130*$^{Y757F/Y757F}$, and compound *gp130*$^{Y757F/Y757F}$ mice on either a *Stat3* heterozygous (*gp130*$^{Y757F/Y757F}$:*Stat3*$^{+/-}$) or *IL-6* homozygous (*gp130*$^{Y757F/Y757F}$:*IL-6*$^{-/-}$) background has been previously described (16–19). *Stat1*$^{-/-}$ mice (20) were crossed with *gp130*$^{Y757F/Y757F}$ mice to generate *gp130*$^{Y757F/Y757F}$:*Stat1*$^{-/-}$ mice (21). All experiments were performed following approval by the Monash Medical Centre "A" Animal Ethics Committee and the U.K. Home Office, and included wild-type (*gp130*$^{+/+}$) littermate controls. All mice were maintained under specific pathogen-free conditions and were age matched for each experiment.

Staphylococcus epidermidis *cell-free supernatant (SES)-induced peritoneal inflammation*

Peritoneal inflammation was induced in vivo by i.p. injection of SES (9, 10). At the indicated times, animals were sacrificed, and their peritoneal cavities were lavaged with ice-cold PBS. Total cell counts were assessed using an automated hematology analyzer (Sysmex), and the composition of the leukocyte infiltrate was assessed by differential cell counting of Wright-Giemsa-stained slides. Lavage fluids were rendered cell-free by centrifugation for analysis of inflammatory mediators.

Cytokine and chemokine production

Murine KC, MCP-1 (R&D Systems), and IL-6 (BD Pharmingen) were quantified using commercial ELISA kits according to the manufacturers' instructions.

Preparation of nuclear extracts from peritoneal biopsies

Nuclear extracts were prepared from peritoneal biopsies using a modified version of a rapid technique for the extraction of nuclear proteins (22, 23). Protein concentrations were determined using the Bradford method.

Electrophoretic mobility shift assay

EMSAs were performed using 10 μg of nuclear extract as previously described (23, 24). Oligonucleotides containing the serum-inducible element STAT-binding element were annealed and labeled with [α-^{32}P]dTTP (Amersham Biosciences) using the Klenow fragment of DNA polymerase I.

Antibodies

ERK1/2, STAT1, and STAT3 Abs were purchased from Santa Cruz Biotechnology. Phospho-STAT1 (Tyr^{701}), phospho-STAT3 (Tyr^{705}), and phospho-ERK1/2 Abs were obtained from Cell Signaling Technology.

Western blot analysis of peritoneal biopsies

Protein extracts from frozen peritoneal biopsies were prepared using ice-cold lysis buffer, following which they were precleared of cellular debris before separation by SDS-PAGE and immunoblotting with specific Abs (18, 25, 26). Immunolabeled proteins were detected using either the ECL detection system (Amersham Biosciences) or the Odyssey Infrared Imaging System (LI-COR) with the appropriate secondary Abs as per the manufacturer's instructions.

Flow cytometric analyses

For assessment of apoptosis in vivo, at the indicated time points post-administration of SES, mice were culled and the peritoneal cavity was lavaged. Total leukocytes were recovered, and neutrophils were stained using a FITC-labeled Gr1 Ab (BD Biosciences). The extent of apoptosis in Gr1-positive neutrophils was determined by double staining with Annexin V^{PE} and 7-aminoactinomycin D (7-AAD; BD Biosciences), and subsequent analysis using a FACSCantoII flow cytometer (BD Biosciences). Quadrants were assigned according to autofluorescence controls, and 5000 events acquired for each sample. Cell surface expression levels of CXCR2 on murine peripheral blood neutrophils were determined on a FACSCalibur flow cytometer (BD Biosciences) by staining leukocytes from mouse whole blood that were subjected to red cell lysis with the appropriate fluorescence-labeled Ly6G (BD Biosciences) and CXCR2 (R&D Systems) Abs.

Calculation of the resolution index

The resolution index was calculated as previously reported (27). Briefly, the maximal number of neutrophils recruited (neutrophil ψ_{max}) following SES administration was quantified and the optimal time (T_{max}) of infiltration was recorded in each genetic strain. These values were then used to calculate the time point, which reflected 50% of the peak neutrophil infiltration (T_{50}), and the theoretical neutrophil numbers present at that time point (R_{50}). The resolution index (R_i) therefore reflects the difference between T_{50} and T_{max}.

Statistical analysis

Data are expressed as means ± SEM, and statistical analysis was performed using an independent samples *t* test with SPSS version 11 statistical software. A p value of <0.05 was considered statistically significant.

Results

Deregulated gp130-mediated signaling alters neutrophil trafficking

To define the gp130-mediated signaling events regulating the trafficking of neutrophils during acute peritoneal inflammation, *gp130*$^{+/+}$, *gp130*$^{\Delta STAT/\Delta STAT}$, and *gp130*$^{Y757F/Y757F}$ mice were administered with a defined dose of SES known to elicit a robust and reproducible inflammatory response in the peritoneal cavity (9, 10). In *gp130*$^{Y757F/Y757F}$ mice, the absolute numbers of infiltrating neutrophils peaked at 3 h ($3.1 \pm 0.9 \times 10^6$) compared with 12 h ($3.7 \pm 0.4 \times 10^6$) in *gp130*$^{+/+}$ mice, and neutrophils were more rapidly cleared and significantly reduced in numbers at 6 and 12 h ($p < 0.01$) in *gp130*$^{Y757F/Y757F}$ mice relative to *gp130*$^{+/+}$ mice (Fig. 1*A*). By contrast, the profile of neutrophil trafficking in SES-challenged *gp130*$^{\Delta STAT/\Delta STAT}$ mice was comparable to that observed in *gp130*$^{+/+}$ mice (Fig. 1*A*), although at the 3-, 6-, and 12-h time points absolute neutrophil numbers appeared consistently elevated, albeit not significantly, in *gp130*$^{\Delta STAT/\Delta STAT}$ mice (e.g., *gp130*$^{+/+}$, $2.48 \pm 0.39 \times 10^6$ vs *gp130*$^{\Delta STAT/\Delta STAT}$, $3.06 \pm 0.78 \times 10^6$ at 6 h). Further analysis of the proportion of neutrophils within the inflammatory infiltrates revealed that the trend of augmented and impaired clearance in *gp130*$^{Y757F/Y757F}$ and

[5] Abbreviations used in this paper: SHP2, Src-homology phosphatase 2; SOCS3, suppressor of cytokine signaling 3; SES, *S. epidermidis* cell-free supernatant; 7-AAD, 7-aminoactinomycin D.

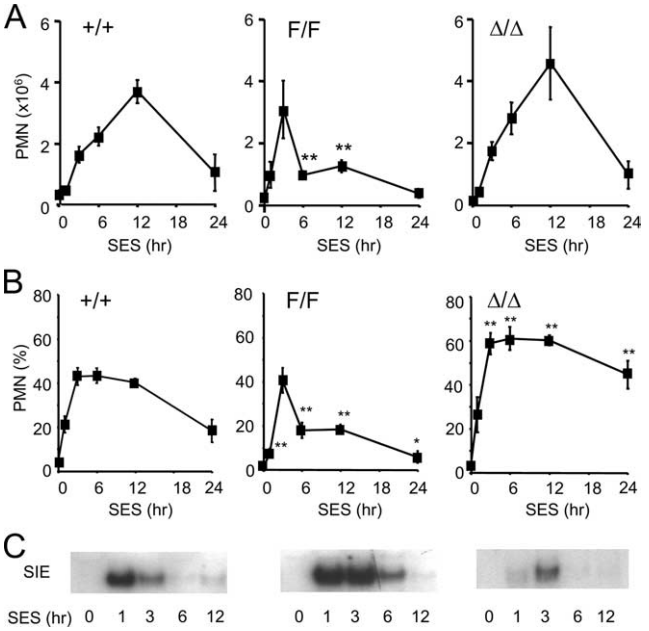

FIGURE 1. The balance between gp130-mediated STAT and SHP2/ERK signaling influences neutrophil trafficking in a model of acute peritoneal inflammation. $gp130^{+/+}$ (+/+), $gp130^{Y757F/Y757F}$ (F/F), and $gp130^{\Delta STAT/\Delta STAT}$ (Δ/Δ) mice were administered with SES, and at defined intervals during the inflammatory episode the peritoneal cavity was lavaged with PBS and a biopsy of the peritoneal membrane was taken. *A*, The absolute numbers and, *B*, percentage of peritoneal neutrophils (PMNs) were assessed by differential cell counting of leukocytes within the lavage fluid. Results are expressed as the mean ± SEM (n = at least 4 mice/time point; **, $p < 0.01$ vs +/+ at the corresponding time point). *C*, Total STAT activation within the peritoneal membrane was measured by EMSA with a radiolabeled STAT-binding oligonucleotide probe (SIE; serum-inducible element). EMSA are representative of results from at least three mice.

$gp130^{\Delta STAT/\Delta STAT}$ mice, respectively, compared with $gp130^{+/+}$ mice was even more pronounced (Fig. 1*B*). We note that the differences between absolute numbers of neutrophils and their relative proportion in the SES-induced inflammatory infiltrates within each genotype are a likely consequence of the impact that altered gp130 signaling also has on the production of other cell types within the infiltrates, namely mononuclear cells and lymphocytes (18, 25).

Compared with $gp130^{+/+}$ mice, the rapid termination of the neutrophil infiltrate in $gp130^{Y757F/Y757F}$ mice following SES administration correlated with an enhanced and prolonged nuclear STAT DNA-binding activity in nuclear extracts from the peritoneal membrane (Fig. 1*C*). Consistent with the normal neutrophil trafficking profile of $gp130^{\Delta STAT/\Delta STAT}$ mice following SES challenge, the kinetics of SES-induced STAT DNA-binding activity in the peritoneum of $gp130^{+/+}$ and $gp130^{\Delta STAT/\Delta STAT}$ mice was similar, although maximal STAT binding in $gp130^{\Delta STAT/\Delta STAT}$ mice (3 h) appeared slightly delayed compared with $gp130^{+/+}$ mice (1 h). Western blot analyses of peritoneal extracts confirmed that SES-induced STAT1 and STAT3 tyrosine phosphorylation was greatest in $gp130^{Y757F/Y757F}$ mice, while $gp130^{\Delta STAT/\Delta STAT}$ mice showed the least extent of STAT1 and STAT3 activation (Fig. 2).

Because we have previously shown that gp130-mediated activation of ERK1/2 via SHP2 is diminished in $gp130^{Y757F/Y757F}$ mice and elevated in $gp130^{\Delta STAT/\Delta STAT}$ mice (17), we next examined the phosphorylation status of ERK1/2 in response to SES

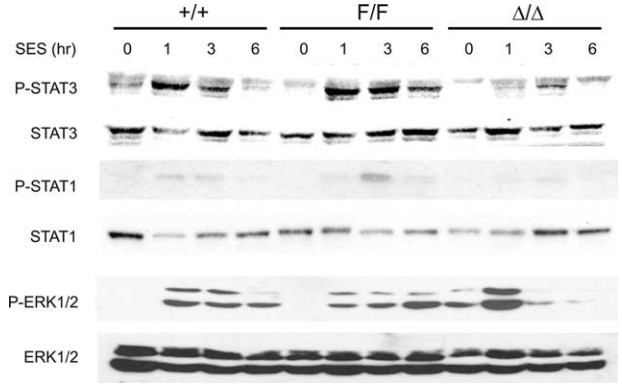

FIGURE 2. Altered activation of gp130-signaling pathways in gp130 mutant mice following SES-induced acute peritoneal inflammation. $gp130^{+/+}$ (+/+), $gp130^{Y757F/Y757F}$ (F/F), or $gp130^{\Delta STAT/\Delta STAT}$ (Δ/Δ) mice were administered with SES, and at defined intervals during the inflammatory episode a biopsy of the peritoneal membrane was taken. STAT1, STAT3, and ERK1/2 tyrosine phosphorylation in peritoneal membrane extracts was measured by immunoblotting with phospho- and pan-specific Abs. Results shown are representative of at least three mice.

(Fig. 2). In $gp130^{+/+}$ mice, SES induced maximal ERK1/2 phosphorylation between 1 and 3 h before subsiding at 6 h. Although the magnitude of ERK1/2 phosphorylation in $gp130^{Y757F/Y757F}$ mice resembled $gp130^{+/+}$ mice, ERK1/2 phosphorylation was sustained and remained maximal at 6 h post-SES challenge. By contrast, in $gp130^{\Delta STAT/\Delta STAT}$ mice, the high basal ERK1/2 activity (0 h) was comparable to that induced by SES in $gp130^{+/+}$ and $gp130^{Y757F/Y757F}$ mice at 1 and 3 h, and was even further induced by SES, peaking at 1 h. Collectively, these data suggest that deregulated SES-induced STAT (increased magnitude and duration) and/or ERK1/2 (increased duration) signaling in $gp130^{Y757F/Y757F}$ mice has a major influence on the rate of neutrophil trafficking during acute peritoneal inflammation.

Deregulation of STAT signaling via IL-6 alters neutrophil trafficking

We have previously used mice deficient in IL-6 to demonstrate the significance of IL-6 signaling in coordinating neutrophil clearance during acute peritoneal inflammation (9). As shown in Fig. 3*A*, genetic ablation of IL-6 in $gp130^{Y757F/Y757F}$ mice restored the profile of neutrophil infiltration and clearance in the resultant $gp130^{Y757F/Y757F}$:$IL\text{-}6^{-/-}$ mice back to a similar pattern seen in $gp130^{+/+}$ mice upon SES administration, thus confirming that IL-6 is a primary gp130-acting cytokine responsible for the altered neutrophil trafficking in $gp130^{Y757F/Y757F}$ mice. Furthermore, SES-induced STAT1 and STAT3 activation in $gp130^{Y757F/Y757F}$:$IL\text{-}6^{-/-}$ mice were reduced back to wild-type levels (Fig. 3*B*). Because IL-6-induced ERK1/2 activation via gp130 is abolished in $gp130^{Y757F/Y757F}$ mice (17), these data suggest a causal role for exaggerated IL-6-induced STAT signaling in altered neutrophil trafficking.

STAT3 signaling via gp130 regulates neutrophil trafficking

Although the data presented above infer that the threshold of IL-6-mediated STAT signaling is critical for governing normal neutrophil trafficking during acute peritoneal inflammation, the relative involvement of STAT1 and STAT3 in this process is unclear. We therefore genetically assessed the contribution of exaggerated STAT3 activation to the altered SES-induced neutrophil trafficking in $gp130^{Y757F/Y757F}$ mice by using $gp130^{Y757F/Y757F}$:$Stat3^{+/-}$ mice in which the level of gp130-dependent STAT3 activation has

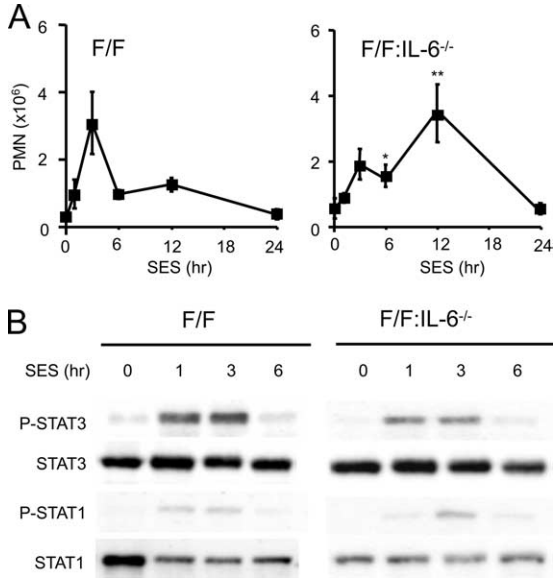

FIGURE 3. IL-6 drives altered neutrophil trafficking and deregulated STAT activation in *gp130*$^{Y757F/Y757F}$ mice. *gp130*$^{Y757F/Y757F}$ (F/F) and *gp130*$^{Y757F/Y757F}$:*IL-6*$^{-/-}$ (F/F:IL-6$^{-/-}$) mice were administered with SES, following which the peritoneal cavity was lavaged and peritoneal membrane biopsies were collected at various time points during the inflammatory episode. *A*, Absolute numbers of neutrophils (PMN) within peritoneal lavage fluid. Results are expressed as the mean ± SEM (n = at least 4 mice/time point; **, $p < 0.01$ and *, $p < 0.05$ vs +/+ mice at the corresponding time point). *B*, Immunoblot analyses of STAT1 and STAT3 tyrosine phosphorylation in peritoneal membrane extracts. Results shown are representative of at least three mice.

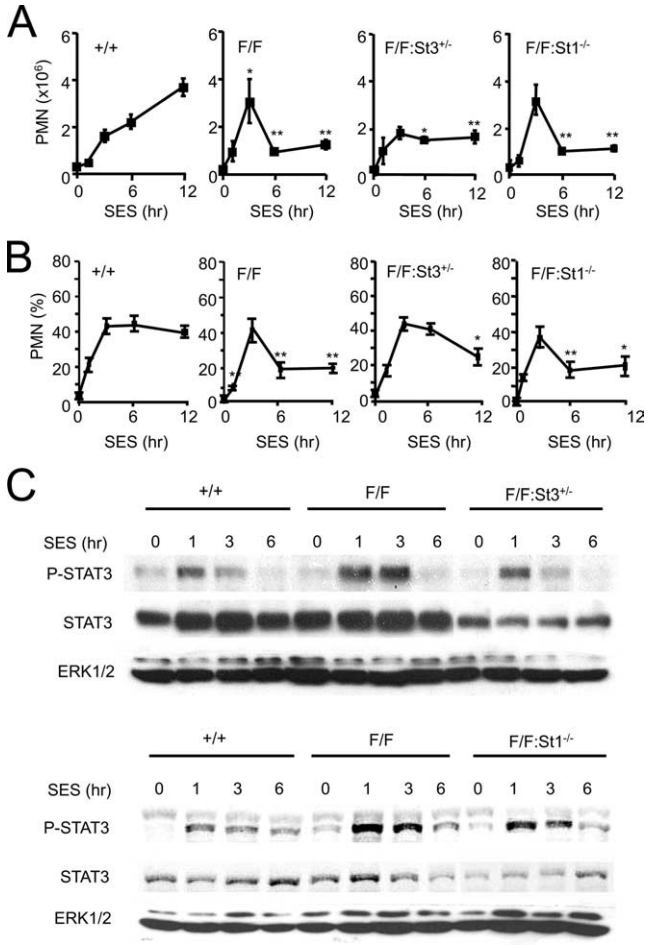

FIGURE 4. STAT3, but not STAT1, activation via gp130 regulates neutrophil clearance. *gp130*$^{+/+}$ (+/+), *gp130*$^{Y757F/Y757F}$ (F/F), *gp130*$^{Y757F/Y757F}$:*Stat3*$^{+/-}$ (F/F:St3$^{+/-}$), and *gp130*$^{Y757F/Y757F}$:*Stat1*$^{-/-}$ (F/F:St1$^{-/-}$) mice were administered with SES, and at defined intervals during the inflammatory episode the peritoneal cavity was lavaged and a biopsy of the peritoneal membrane taken. *A*, Absolute numbers and, *B*, percentage of neutrophils (PMN) within the peritoneal lavage fluid are shown. Results are expressed as the mean ± SEM (n = at least 4 mice/time point, except for F/F:St1$^{-/-}$ 1- and 3-h time points, n = 3; **, $p < 0.01$ and *, $p < 0.05$ vs +/+ mice at the corresponding time point). *C*, Immunoblot analysis of STAT3 tyrosine phosphorylation in peritoneal membrane extracts. Results shown are representative of at least three mice.

been normalized (18, 26). The profile of neutrophil trafficking in *gp130*$^{Y757F/Y757F}$:*Stat3*$^{+/-}$ mice appeared intermediate between *gp130*$^{+/+}$ and *gp130*$^{Y757F/Y757F}$ mice, with the absolute numbers of infiltrating neutrophils peaking within 3 h and remaining at these levels up to 12 h. Interestingly, the magnitude of this peak neutrophil influx in *gp130*$^{Y757F/Y757F}$:*Stat3*$^{+/-}$ mice at 3 h was significantly reduced ($1.8 \pm 0.2 \times 10^6$, $p > 0.05$) compared with the corresponding peak neutrophil numbers at 3 and 12 h in *gp130*$^{Y757F/Y757F}$ and *gp130*$^{+/+}$ mice, respectively (Fig. 4*A*). Despite these observations, it is worth noting the similar proportion of neutrophils within the initial inflammatory infiltrate and early clearance phase of trafficking in *gp130*$^{+/+}$ and *gp130*$^{Y757F/Y757F}$:*Stat3*$^{+/-}$ mice compared with *gp130*$^{Y757F:Y757F}$ mice (Fig. 4*B*), the latter of which is significantly ($p > 0.01$) reduced at 6 h (*gp130*$^{+/+}$, 43 ± 3% vs *gp130*$^{Y757F/Y757F}$, 18 ± 4% vs *gp130*$^{Y757F/Y757F}$:*Stat3*$^{+/-}$, 38 ± 2%). A similar trend in absolute numbers (*gp130*$^{+/+}$, $0.78 \pm 0.24 \times 10^6$, and *gp130*$^{Y757F/Y757F}$:*Stat3*$^{+/-}$, $0.62 \pm 0.14 \times 10^6$ vs *gp130*$^{Y757F/Y757F}$, $0.36 \pm 0.09 \times 10^6$; $p > 0.01$) and proportions (*gp130*$^{+/+}$, 18.2 ± 5.2%, and *gp130*$^{Y757F/Y757F}$:*Stat3*$^{+/-}$ 12.5 ± 2.25% vs *gp130*$^{Y757F/Y757F}$, 5.8 ± 2.7%; $p > 0.05$) of neutrophils in *gp130*$^{+/+}$ and *gp130*$^{Y757F/Y757F}$:*Stat3*$^{+/-}$ mice compared with *gp130*$^{Y757F:Y757F}$ mice was also apparent at the later 24-h time point. This rescue in the pattern of neutrophil recruitment in *gp130*$^{Y757F/Y757F}$:*Stat3*$^{+/-}$ mice was characterized by a reduction in the level of SES-induced STAT3 tyrosine phosphorylation which was similar to that seen in *gp130*$^{+/+}$ mice (Fig. 4*C*).

We have previously shown that STAT1 hyperactivity remained unchanged in *gp130*$^{Y757F/Y757F}$:*Stat3*$^{+/-}$ mice (25), which is consistent with the notion that the specific reduction of STAT3 in these mice was responsible for alleviating the neutrophil trafficking phenotype. However, to assess whether exaggerated STAT1 activation also contributed to the aberrant neutrophil trafficking phenotype observed in *gp130*$^{Y757F/Y757F}$ mice, we next analyzed SES-induced neutrophil recruitment and clearance in *gp130*$^{Y757F/Y757F}$ mice crossed onto a *Stat1*-deficient background (*gp130*$^{Y757F/Y757F}$:*Stat1*$^{-/-}$). As expected, STAT3 activity remained elevated in *gp130*$^{Y757F/Y757F}$:*Stat1*$^{-/-}$ mice (Fig. 4*C*), and the neutrophil trafficking profile in these mice resembled that observed in *gp130*$^{Y757F/Y757F}$ mice (Fig. 4, *A* and *B*).

To further characterize changes in the kinetics of neutrophil clearance following SES stimulation in each of the genetically modified strains, we next quantified the neutrophil "resolution index" (R_i) (27). The data from these calculations (outlined in *Materials and Methods*) are presented in Table I. The R_i calculated for *gp130*$^{+/+}$ mice (8 h) was consistent with previously published results (27). Enhanced gp130-STAT signaling in the *gp130*$^{Y757F/Y757F}$ mice reduced the R_i (2 h), whereas the R_i values for the *gp130*$^{\Delta STAT/\Delta STAT}$ and *gp130*$^{Y757F/Y757F}$:

Table I. *Resolution intervals calculated for the different gp130 genotypes*[a]

Genotype	PMN ψ_{max} ($\times 10^6$)	PMN T_{max} (h)	PMN R_{50} ($\times 10^6$)	PMN T_{50} (h)	R_i (h)
+/+	3.7	12	1.85	20	8
F/F	3.1	3	1.55	5	2
Δ/Δ	4.6	12	2.3	20	8
F/F:IL-6$^{-/-}$	3.5	12	1.75	19	7
F/F:St3$^{+/-}$	1.8	3	0.9	21	18
F/F:St1$^{-/-}$	3.2	3	1.6	5	2

[a] Resolution intervals (27) were calculated on the basis of polymorphonuclear neutrophil (PMN) levels during SES-induced peritoneal inflammation obtained experimentally. The maximum levels of PMN (PMN ψ_{max}) were used to calculate the 50% PMN levels (PMN R_{50}). The time, to the nearest hour, which coincided with the 50% levels (T_{50}), was determined from the time course of PMN levels. The resolution interval (R_i) was calculated as (PMN T_{50}) − (PMN T_{max}).

IL-6$^{-/-}$ mice (8 and 7 h, respectively) were similar to *gp130*$^{+/+}$ mice. By contrast, a reduction in the activation of STAT3 in *gp130*$^{Y757F/Y757F}$:*Stat3*$^{+/-}$ mice dramatically increased the R_i to 18 h, whereas the absence of STAT1 in *gp130*$^{Y757F/Y757F}$:*Stat1*$^{-/-}$ had no effect on the R_i. Collectively, these data therefore support a more prominent role for the IL-6/STAT3-signaling axis in controlling SES-induced neutrophil infiltration during acute peritoneal inflammation.

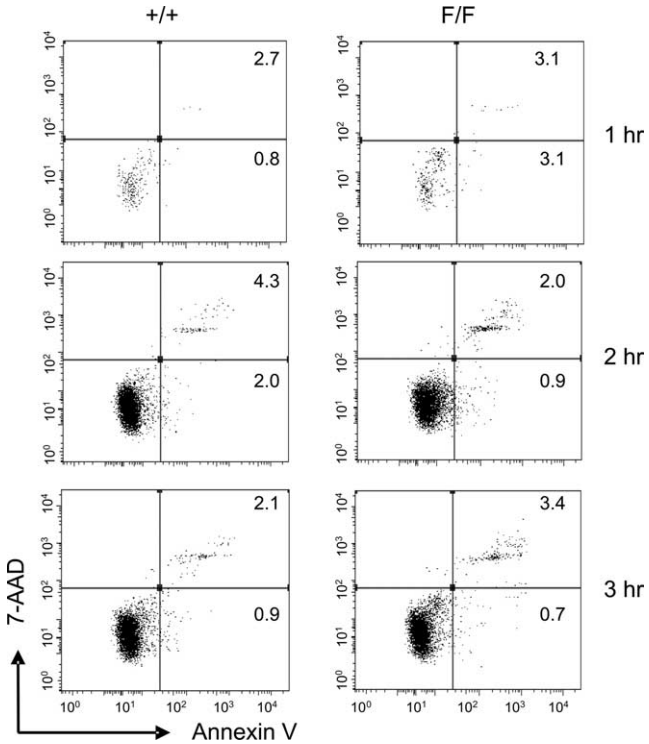

FIGURE 5. Normal neutrophil apoptosis in *gp130*$^{Y757F/Y757F}$ mice displaying rapid neutrophil clearance during acute peritoneal inflammation. *gp130*$^{+/+}$ (+/+) and *gp130*$^{Y757F/Y757F}$ (F/F) mice were administered with SES, and at various time points during the inflammatory episode the peritoneal cavity was lavaged. Shown are representative scatter plots (n = 2–3 mice/genotype/time point) of Gr1-positive (+) peritoneal neutrophils that were stained with Annexin-V-PE/7-AAD and analyzed by flow cytometry. Apoptotic cells were identified according to Annexin-V+/7-AAD− (*lower right quadrant*, early apoptosis) and Annexin-V+/7-AAD+ (*upper right quadrant*, late apoptosis/necrosis) staining. The proportion of cells residing in each quadrant is expressed as a percentage.

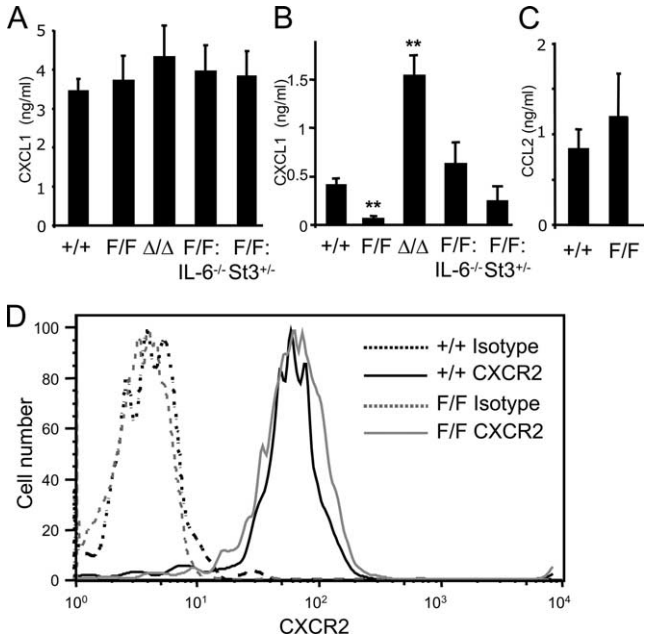

FIGURE 6. Regulation of CXCL1/KC chemokine levels by gp130-mediated STAT3 signaling during acute peritoneal inflammation. Peritoneal inflammation was induced in *gp130*$^{+/+}$ (+/+), *gp130*$^{\Delta STAT/\Delta STAT}$ (Δ/Δ), *gp130*$^{Y757F/Y757F}$ (F/F), and the indicated *gp130*$^{Y757F/Y757F}$ compound mutant mice by administration with SES. ELISAs were performed on peritoneal lavage fluid to determine levels of CXCL1/KC at 1 h (*A*) and 3 h (*B*), and CCL2 at 3 h. Results are expressed as the mean ± SEM (n = at least 3 mice/time point; **, $p < 0.05$ vs +/+ mice at the corresponding time point). *D*, Shown are representative histograms (n = 2–3 mice/genotype) of peripheral blood Ly6G$^+$ neutrophils that were stained with an Ab against CXCR2 and were detected by dual-color flow cytometric analysis. The *x*-axis represents PE intensity (for either CXCR2 Ab or appropriate isotype control Ab), and the *y*-axis represents relative cell number.

Regulation of chemokine production, but not apoptosis, by gp130 signaling influences neutrophil clearance

Neutrophil trafficking following inflammatory activation is governed by the initial production of neutrophil-activating chemokines, and the eventual clearance of neutrophils occurs via both reduced local chemotactic activity and apoptotic mechanisms. In this respect, IL-6 has been shown to inhibit the inflammatory induction of neutrophil-activating chemokines and to facilitate their apoptotic clearance (1, 10). However, the contribution of specific IL-6-signaling pathways to these processes remains to be fully elucidated. We therefore initially assessed whether the rapid clearance of neutrophils during acute peritoneal inflammation in *gp130*$^{Y757F/Y757F}$ mice was attributable to increased neutrophil apoptosis by performing Annexin-V/7-AAD staining on the Gr1$^+$ neutrophil population following SES administration. However, as shown in Fig. 5, a comparable profile of neutrophil apoptosis was recorded in *gp130*$^{+/+}$ and *gp130*$^{Y757F/Y757F}$ mice at 1, 2, and 3 h post-SES administration. This result also suggests that the low proportion of neutrophils in the peritoneal lavage of *gp130*$^{Y757F/Y757F}$ mice at the early 1 h time point (e.g., Fig. 4*B*) is unlikely to result from elevated apoptosis.

We next examined whether gp130-signaling alterations influenced chemokine-directed neutrophil recruitment by measuring the protein levels of the key neutrophil-activating chemokine CXCL1/KC in the peritoneal lavage fluid of SES-challenged mice (9, 10). No significant changes were observed after 1 h (optimal time point for CXCL1/KC induction in this model (9)) among the various genotypes (Fig. 6*A*), suggesting that, consistent with our

earlier data (Fig. 4*B*), deregulated gp130 signaling does not affect the initial CXCL1/KC-driven induction of neutrophil recruitment. However, CXCL1/KC levels were significantly reduced in *gp130*$^{Y757F/Y757F}$ mice compared with *gp130*$^{+/+}$ mice at 3 h (Fig. 6*B*). Importantly, this was in contrast to the elevated levels of CXCL1/KC in *gp130*$^{\Delta STAT/\Delta STAT}$ mice at 3 h (Fig. 6*B*). As predicted, this response was highly dependent on IL-6 as evidenced by the increased levels of CXCL1/KC detected in lavage fluid from *gp130*$^{Y757F/Y757F}$*:IL-6*$^{-/-}$ mice (Fig. 6*B*). Notably, the monoallelic deletion of *Stat3* in *gp130*$^{Y757F/Y757F}$*:Stat3*$^{+/-}$ mice restored CXCL1/KC levels similar to those observed in *gp130*$^{+/+}$ mice, suggesting an exclusive role for STAT3 via gp130 in attenuating SES-induced CXCL1/KC production, and therefore in promoting the clearance of neutrophils. In support of this notion, the reduced CXCL1/KC levels in *gp130*$^{Y757F/Y757F}$*:Stat1*$^{-/-}$ mice (68 $\pm$ 22 pg/ml) were similar to those in *gp130*$^{Y757F:Y757F}$ mice (45 $\pm$ 27 pg/ml) at 3 h postactivation. Furthermore, the specificity of the IL-6/STAT3-mediated effect on neutrophil-activating chemokines was supported by the normal expression level of the monocyte chemoattractant CCL2/MCP1 in *gp130*$^{Y757F/Y757F}$ peritoneal fluid at 3 h post-SES (Fig. 6*C*). Similarly, flow cytometric analyses confirmed that the surface expression of CXCR2, the common chemokine receptor for KC/CXCL1 and other CXC chemokines containing the ELR (Glu-Leu-Arg) motif, was comparable between *gp130*$^{+/+}$ and *gp130*$^{Y757F/Y757F}$ mice (Fig. 6*D*). Thus, the above data collectively demonstrate that STAT3-mediated down-regulation of neutrophil-activating chemokine expression represents a primary mechanism by which IL-6 controls neutrophil trafficking.

Discussion

Regulation of neutrophil trafficking during inflammation, which involves the initial recruitment of neutrophils and their subsequent controlled clearance, is critical for the successful resolution of acute inflammation. Impaired clearance of activated neutrophils can result in damage to the inflamed tissue and resulting immunopathology. To provide new molecular insights into the mechanisms governing this process, in this study we have used a unique series of gp130 knockin mutant mice displaying differential activation of specific gp130-signaling pathways in an established model of SES-induced acute peritonitis. We report here that alterations to specific IL-6/gp130-signaling pathways dramatically influence the rate of neutrophil trafficking (Table I) (27) and, in particular, that neutrophil clearance is positively regulated by gp130-mediated STAT3 signaling.

Apoptosis has been implicated in the clearance of neutrophils from sites of inflammation and is important for the resolution of the inflammatory process (10, 28). Although several lines of evidence suggest that the ERK MAPK and PI3K pathways promote the survival of neutrophils (29–32), neutrophil apoptosis during the inflammatory response in *gp130*$^{Y757F/Y757F}$ mice was comparable to *gp130*$^{+/+}$ mice. Similarly, the extent of apoptosis over 24 h was normal within the total leukocyte population of both *gp130*$^{Y757F/Y757F}$ and *gp130*$^{Y757F/Y757F}$*:IL-6*$^{-/-}$ mice genetically modified to lack gp130-mediated SHP2-ERK MAPK and -PI3K pathways (data not shown). Thus, these observations suggest that the accelerated removal of neutrophils in the peritoneal cavity of *gp130*$^{Y757F/Y757F}$ mice is not due to increased apoptosis as a consequence of impaired ERK/PI3K signaling. We speculate that one possible explanation for the removal of these neutrophils from the peritoneal cavity which is currently under investigation in our laboratory is the increased (3- to 4-fold) numbers of monocytes/macrophages in the peritoneal inflammatory infiltrates of only *gp130*$^{Y757F/Y757F}$ mice, which occurs in a STAT3-dependent manner (B. J. Jenkins, unpublished observations) and may lead to enhanced phagocytosis of dying neutrophils by macrophages as previously demonstrated (33).

A key and definitive finding of this current study was our genetic evidence that hyperactivation of STAT3, rather than STAT1, via gp130 was primarily responsible for the aberrant regulation of neutrophil trafficking in *gp130*$^{Y757F/Y757F}$ mice. Specifically, the accelerated clearance of neutrophils and impaired production of the critical neutrophil-attracting chemokine CXCL1/KC in the peritoneal cavity of *gp130*$^{Y757F/Y757F}$ mice were rescued in *gp130*$^{Y757F/Y757F}$:*Stat3*$^{+/-}$ mice displaying reduced gp130-dependent STAT3 activity. This genetic observation identified that gp130-dependent STAT3 signaling influences neutrophil clearance by negatively regulating CXCL1/KC production. Because the SES-induced neutrophil trafficking phenotype of *gp130*$^{Y757F/Y757F}$ mice was not completely corrected in *gp130*$^{Y757F/Y757F}$:*Stat3*$^{+/-}$ mice, we interpret this finding as a further indication that the STAT3-signaling threshold for specific pathophysiological responses varies in a cell/organ-type specific manner (34). For instance, we have previously reported that genetically reducing the level of STAT3 activity in *gp130*$^{Y757F/Y757F}$:*Stat3*$^{+/-}$ mice completely rescues the STAT3-driven splenomegaly and thrombocytosis observed in *gp130*$^{Y757F/Y757F}$ mice, whereas lymphadenopathy was only partially ameliorated (18, 26). Accordingly, certain cell types/organs are more sensitive to the reduction in the net STAT3 signal output in *gp130*$^{Y757F/Y757F}$:*Stat3*$^{+/-}$ mice than others. We therefore propose that this scenario also applies to the STAT3-driven molecular processes (e.g., CXCL1/KC regulation) facilitating neutrophil trafficking. Nonetheless, our data presented here are supported by other studies demonstrating that STAT3 activation negatively regulates production of a number of inflammatory genes, including chemokines (35, 36). It is possible that this is a selective effect on a specific subset of chemokines and may not be a global suppression of all chemokines. In support of this notion, inflammation-induced expression levels of the monocyte-attracting chemokine CCL2 (MCP-1) remained unchanged in *gp130*$^{Y757F/Y757F}$ mice. Although the precise molecular mechanism for STAT3-negative regulation of proinflammatory gene expression remains controversial, a number of studies have reported that STAT3 negatively regulates NF-κB signaling or DNA binding, either through a direct interaction or via an intermediate protein (37–40).

Although our data presented here suggested that IL-6 signaling has a major role in regulating the neutrophil trafficking component of peritonitis, it is still possible that other gp130-acting cytokines may augment this process either independently or in combination with IL-6. Oncostatin M, for example, displays the same capability as IL-6 to down-regulate neutrophil-activating chemokines in vitro (41), and a number of other IL-6 family cytokines (e.g., IL-11, IL-27 and IL-31) are produced during inflammation which may have distinct or overlapping roles in regulating acute inflammatory responses. Thus, the role of such cytokines in the aberrant neutrophil trafficking observed in *gp130*$^{Y757F/Y757F}$ mice during acute peritoneal inflammation warrants further investigation.

In summary, this study has demonstrated a clear and major role for gp130-mediated STAT3 signaling in regulating neutrophil clearance during acute peritoneal inflammation. These effects were driven by IL-6, were independent of other gp130-signaling pathways (e.g., STAT1, ERK1/2), and involved specific down-regulation of neutrophil-attracting chemokine production. Importantly, these current findings expand upon our earlier observations identifying the gp130-STAT3 signaling axis as a key promoter of chemokine-directed T cell recruitment during acute inflammatory responses (25), and therefore further highlight the critical role

gp130/STAT3 signaling plays in controlling both innate and adaptive arms of the immune system. Although the studies presented here are based on an acute model of peritoneal inflammation, the involvement of deregulated IL-6/STAT3 signaling in numerous chronic inflammatory conditions (e.g., inflammatory bowel disease and arthritis) (42–44) suggests a broader implication for the role of gp130/STAT3-mediated chemokine regulation during inflammatory episodes.

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Disclosures

The authors have no financial conflict of interest.

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