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Rachel M. McLoughlin; ... et. al

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Differential Regulation of Neutrophil-Activating Chemokines by IL-6 and Its Soluble Receptor Isoforms[1]

Rachel M. McLoughlin,*‡ Suzanne M. Hurst,[2]* Mari A. Nowell,* Dean A. Harris,‡ Sankichi Horiuchi,† Llinos W. Morgan,‡ Thomas S. Wilkinson,‡ Naoki Yamamoto,† Nicholas Topley,‡ and Simon A. Jones[3]*

Interleukin-6 signaling via its soluble receptor (sIL-6R) differentially regulates inflammatory chemokine expression and leukocyte apoptosis to coordinate transition from neutrophil to mononuclear cell infiltration. sIL-6R activities may, however, be influenced in vivo by the occurrence of two sIL-6R isoforms that are released as a consequence of differential mRNA splicing (DS) or proteolytic cleavage (PC) of the cognate IL-6R (termed DS- and PC-sIL-6R). Using human peritoneal mesothelial cells and a murine model of peritoneal inflammation, studies described in this work have compared the ability of both isoforms to regulate neutrophil recruitment. In this respect, DS- and PC-sIL-6R were comparable in their activities; however, these studies emphasized that IL-6 *trans* signaling differentially controls neutrophil-activating CXC chemokine expression. In vitro, stimulation of mesothelial cells with IL-6 in combination with either DS-sIL-6R or PC-sIL-6R showed no induction of CXC chemokine ligand (CXCL)1 (GROα) and CXCL8 (IL-8), whereas both isoforms enhanced CXCL5 (ENA-78) and CXCL6 (granulocyte chemotactic protein-2) expression. Moreover, when complexed with IL-6, both isoforms specifically inhibited the IL-1β-induced secretion of CXCL8. These findings were paralleled in vivo, in which induction of peritoneal inflammation in IL-6-deficient (IL-6$^{-/-}$) mice resulted in enhanced keratinocyte-derived chemokine and macrophage-inflammatory protein-2 (the murine equivalent of CXCL1 and CXCL8) levels, but reduced LPS-induced CXC chemokine (the murine equivalent of CXCL5) expression. Reconstitution of IL-6 signaling in IL-6$^{-/-}$ mice with IL-6 and its soluble receptor isoforms corrected this chemokine imbalance and suppressed overall neutrophil infiltration. These data confirm that sIL-6R-mediated signaling primarily limits neutrophil influx; however, induction of CXCL5 and CXCL6 may regulate other neutrophil responses. ***The Journal of Immunology*****, 2004, 172: 5676–5683.**

Interleukin-6 belongs to a family of cytokines whose members act via a receptor complex containing at least one subunit of the signal-transducing protein gp130 (1). Although gp130 is expressed extensively in almost all organs of the body, cellular distribution of the cognate IL-6R is largely restricted to hepatocytes and leukocyte subsets (1, 2). In addition to the membrane-bound form of the IL-6R, there exists a soluble form of this cognate receptor (3, 4), which controls many of the biological activities assigned to IL-6. Indeed, the soluble IL-6R (sIL-6R)[4] forms a stimulatory complex with IL-6 that regulates cellular events through direct activation of gp130 (1, 2), a process referred to as *trans* signaling. Consequently, the sIL-6R/IL-6 complex is an agonist of cell types that, although express gp130, do not inherently respond to IL-6 itself.

Extensive analysis of sIL-6R production in vitro and in vivo has established that this soluble receptor is differentially released through both proteolytic (shedding) cleavage (PC) of the cognate IL-6R and differential IL-6R mRNA splicing (DS) (2, 5–8). This infers that two distinct isoforms of sIL-6R control the overall properties of this soluble receptor, which will be referred to as PC-sIL-6R and DS-sIL-6R. To date, it is unclear why two mechanisms control sIL-6R release (2). Although both forms are structurally related, DS-sIL-6R can be distinguished from its shed counterpart by a novel COOH-terminal sequence (GSRRRGSCGL), which is introduced as part of the splicing process (8, 9). Abs specific for this unique sequence have enabled the relative abundance of DS- and PC-sIL-6R to be distinguished in normal and diseased states. These studies show that release of each isoform may ultimately depend on the age of an individual, the inflammatory condition, or stage of disease progression (2, 9–11). Indeed, several in vitro studies have shown that expression of DS- and PC-sIL-6R may be individually controlled by a given cell type (12–15). Consequently, PC- and DS-sIL-6R might act at distinct stages during inflammation to orchestrate unique biological events.

During an inflammatory episode, neutrophils are rapidly recruited to inflammatory foci in response to neutrophil-activating chemokines, which are characterized by the NH_2-terminal amino acid motif ELR (termed (ELR$^+$)CXC chemokines) (16, 17). All (ELR$^+$)CXC chemokines bind the receptors CXCR1 and CXCR2 with varying affinities to elicit individual responses and are

*Cardiff School of Biosciences, Cardiff University, Cardiff, Wales, United Kingdom; †Department of Microbiology, Tokyo Medical & Dental University, Tokyo, Japan; and ‡Institute of Nephrology, University of Wales College of Medicine, Cardiff, Wales, United Kingdom

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[2] Current address: Centre for Research in Biomedicine, Faculty of Applied Sciences, University of West of England, Frenchay Campus, Coldharbour Lane, Bristol BS16 1QY, U.K.

[3] Address correspondence and reprint requests to Dr. Simon A. Jones, Molecular Cell Biology Research Group, Cardiff School of Biosciences, Biomedical Sciences Building, Cardiff University, Museum Avenue (P.O. Box 911), Cardiff CF10 3US, Wales, U.K. E-mail address: JonesSA@cardiff.ac.uk

[4] Abbreviations used in this paper: s, soluble; CCL, CC chemokine ligand; CXCL, CXC chemokine ligand; DS, differential mRNA splicing; HPMC, human peritoneal mesothelial cell; KC, keratinocyte-derived chemokine; LIX, LPS-induced CXC chemokine; MCP, monocyte chemoattractant protein; MIP, macrophage-inflammatory protein; PAF, platelet-activating factor; PC, proteolytic cleavage; RIPA, radioimmunoprecipitation; SES, *Staphylococcus epidermidis* cell-free supernatant; sgpl30, soluble gp130.

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produced with distinct profiles during an inflammatory episode (17–23). Particular (ELR$^+$)CXC chemokines may therefore serve distinct biological functions. Recently, the IL-6/sIL-6R complex was shown to direct transition from neutrophil to mononuclear cell recruitment during acute peritoneal inflammation (11, 24). Regulation of this event occurs through modulation of local chemokine expression and by control of leukocyte apoptosis (11, 24, 25). Given that PC- and DS-sIL-6R differ with respect to their kinetics of release during bacterial peritonitis (11), we have now examined the impact of both isoforms on various (ELR$^+$)CXC chemokines during peritoneal inflammation.

Materials and Methods

Reagents

All chemicals were from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. Recombinant human IL-1β, IL-6, and soluble gp130 (sgp130) were from R&D Systems (Minneapolis, MN). Endotoxin levels in recombinant cytokines were $<$0.1 ng/μg (1 EU/μg) protein. Cell culture reagents were from Life Technologies (Paisley, U.K.), and all cell culture vessels were obtained from Falcon (BD Biosciences, Oxford, U.K.). Lymphoprep was purchased from Nycomed (Oslo, Norway). Baculovirus-expressed PC- and DS-sIL-6R isoforms were obtained, as previously described (9, 11). The concentration of individual isoforms was determined using a commercial sIL-6R ELISA (R&D Systems), which does not distinguish between the two isoforms, and with an anti-DS-sIL-6R Ab mAb-2F3 raised against the unique COOH-terminal sequence of DS-sIL-6R (GSRRRGSCGL) (8, 9).

Isolation and culture of human peritoneal mesothelial cells (HPMC)

HPMC were isolated by tryptic digestion of greater omental tissue obtained from consenting patients undergoing abdominal surgery and characterized as previously described (26). All experiments were performed with confluent HPMC from the second passage. Cells were washed and growth was arrested for 48 h in serum-free culture medium before cytokine stimulation. Growth-arrested HPMC were stimulated at 37°C under the conditions specified in the figure legends. Culture supernatants were rendered cell free by centrifugation and assayed using ELISA.

Isolation of human leukocytes

Venous blood was collected by antecubital venipuncture from healthy nonsmoking individuals (aged 24–35) and mixed with an equal volume of 2% (w/v) dextran/0.8% (w/v) trisodium citrate in PBS (pH 7.4) to allow erythrocyte sedimentation. Plasma was collected and underlayered with Lymphoprep (2:1 (v/v) plasma:Lymphoprep) before centrifugation at 4°C for 20 min at 800 $\times$ *g*. The interface containing the mononuclear cell population was washed with 0.4% (w/v) trisodium citrate in PBS (pH 7.4) to eliminate platelet contamination and finally resuspended in serum-free RPMI 1640 containing 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The neutrophil pellet was collected, and contaminating erythrocytes were removed by hypotonic lysis. Neutrophils were subsequently resuspended in serum-free RPMI 1640 medium containing appropriate supplements. Leukocytes from the drain effluent of end-stage renal failure patients receiving peritoneal dialysis were isolated by density gradient centrifugation, as described above.

sIL-6R release from human neutrophils

Neutrophils (2×10^6 cells) were stimulated under serum-free conditions for 30 min at 37°C with the indicated concentrations of C5a, C3a, and platelet-activating factor-16 (PAF). Culture medium was rendered cell free, and sIL-6R levels were determined using matched Ab pairs from R&D Systems.

FACS analysis

Expression of IL-6R and gp130 was determined by FACS analysis, as previously described (27). Briefly, cells were labeled with PE-conjugated anti-IL-6R (551850; BD Biosciences) and anti-gp130 (FAB228P) Abs, and data were acquired from 10,000 gated events. To determine the distribution of IL-6R and gp130 on leukocyte subpopulations, analysis was performed on cells gated according to CXCR1 (neutrophils), CD14 (monocytes), and CD3 (T lymphocytes). Although high CXCR1 expression was confined to the neutrophil population as gauged by forward and side light scatter, some low CXCR1 expression was also evident within the mononuclear leukocyte field. Consequently, analysis was performed on cells gated according to high CXCR1 expression. With respect to IL-6R and gp130 expression on HPMC, FACS analysis was performed on both freshly isolated mesothelial cells before their establishment in culture, and cells cultured through to the second passage. In both instances, HPMC remained IL-6R^{-ve}, gp130^{+ve}.

Immunofluorescence

Monocytes were adhered to glass coverslips at 37°C for 1 h. The nonadherent lymphocytes were removed by washing, and the adherent population was cultured overnight at 37°C, 5% CO_2. The culture medium was replaced with RPMI 1640 containing 10 μg/ml brefeldin A (Sigma-Aldrich) and incubated for an additional 5 h. Cells were fixed for 5 min with 4% (w/v) formaldehyde and permeabilized with PBS containing 0.01% Tween 20. Nonspecific Ab binding was blocked at room temperature with 10% (v/v) goat serum, and the cells were exposed to 5 μg/ml mouse anti-human DS-sIL-6R (mAb 2F3) or a control mouse IgG for 1 h in PBS/Tween 20. Cells were subsequently incubated with a FITC-conjugated goat anti-mouse secondary Ab (DAKO, Carpenteria, CA), and counterstained with 2 μg/ml propidium iodide. Immunofluorescent labeling was visualized using a fluorescent microscope.

Coimmunoprecipitation techniques

HPMC monolayers were stimulated for 15 min, washed in ice-cold PBS (pH 7.4), and treated with radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 50 mM NaF, 3 mM sodium orthovanadate containing a mixture of protease inhibitors (supplied by Boehringer Mannheim, Indianapolis, IN), and 1% (v/v) Nonidet P-40). Lysates containing ~2 mg of protein were diluted to 1 ml with RIPA buffer and precleared for 2 h with protein A-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ). The Sepharose beads were removed and gp130 immunoprecipitated overnight at 4°C with 2.5 μg/ml polyclonal anti-gp130 Ab (C-20 from Santa Cruz Biotechnology, Santa Cruz, CA). Immune complexes were recovered by incubation with protein A-Sepharose for 2 h at 4°C. Sepharose beads were pelleted by centrifugation and washed three times with RIPA buffer. The immune complexes were then solubilized with 62.5 mM Tris-HCl (pH 6.8) containing 2% (w/v) SDS for analysis by Western blot. Proteins were separated under reducing conditions in a 7.5% gel by SDS-PAGE and electroblotted onto Hybond membrane (Amersham Pharmacia Biotech). For analysis of gp130, proteins were resolved under nonreducing conditions. Membranes were blocked with PBS containing 5% BSA and 3 mM sodium orthovanadate for 1 h at room temperature and probed with a mixture of anti-phosphotyrosine Abs (4G10 (Upstate Biotechnology, Lake Placid, NY) and PY-20 (Santa Cruz Biotechnology)) or anti-gp130 (C-20). Blots were washed and incubated with appropriate HRP-conjugated secondary Abs, and protein bands were visualized using ECL (SuperSignal; Pierce, Rockford, IL). Washing steps were performed with PBS containing 5% BSA and 3 mM sodium orthovanadate.

Analysis of nuclear extracts

Nuclear extracts were prepared from HPMC using a rapid technique for the extraction of DNA-binding proteins. Briefly, cells were harvested in ice-cold PBS (pH 7.4) and pelleted by centrifugation. Cells were resuspended in cold buffer A (10 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF) and incubated on ice for 10 min. The cell pellet was collected by centrifugation, resuspended in buffer B (20 mM HEPES-KOH (pH 7.9), 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.3 mM DTT, 0.2 mM PMSF), and incubated on ice for 20 min. Cellular debris was removed by centrifugation (1200 $\times$ *g* for 10 s, 4°C), and supernatants (nuclear extract) were collected. Protein concentrations were determined using the Bradford method. EMSAs were performed, as described previously (28). Oligonucleotides containing a STAT-binding consensus sequence (SIE-m67) were annealed for use in EMSA (24, 27). These double-stranded fragments were labeled with [α-^{32}P]dTTP (Amersham Pharmacia Biotech) using the Klenow fragment of DNA polymerase I. The composition of protein/DNA complexes was determined by supershift assays using 2 μg/ml anti-STAT1 (M-22) and anti-STAT3 (C-20) Abs (27, 28).

Animals

Experiments were performed in weight-matched 7- to 12-wk-old inbred C57BL/6J wild-type (IL-6$^{+/+}$) and IL-6$^{-/-}$ mice (29). Procedures were performed in accordance with Home Office-approved project license PPL-40/2131.

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

Peritoneal inflammation was established in IL-$6^{+/+}$ and IL-$6^{-/-}$ mice through i.p. administration of a cell-free supernatant (SES) prepared from *S. epidermidis* (11, 24). At defined intervals, animals were sacrificed, and their peritoneal cavity was lavaged with 2 ml of ice-cold PBS. Composition of the leukocyte infiltrate was assessed using a Coulter counter (Coulter Z2; Beckman Coulter, High Wycombe, U.K.) and by differential cell staining. Lavage fluids were rendered cell free by centrifugation for analysis of inflammatory mediators. To assess the in vivo properties of the sIL-6R isoforms, signaling was modulated in IL-$6^{-/-}$ mice by administration (i.p.) of DS- or PC-sIL-6R (25 ng/mouse) in combination with IL-6 (20 ng/mouse). SES and sgp130 (150 ng/mouse) were included, as indicated in the figure legends. In Ab inhibition experiments, IL-$6^{+/+}$ mice were injected with 50 μg of a rabbit polyclonal anti-keratinocyte-derived chemokine (KC) Ab (PeproTech, Rocky Hill, NJ) or isotype control IgG for 90 min before SES challenge.

Analysis of chemokine expression

Human CC chemokine ligand (CCL) (monocyte chemoattractant protein-1 (MCP-1)) was detected using a matched Ab pair OptEIA kit from BD PharMingen (San Diego, CA). Human CXC chemokine ligand (CXCL)5 (ENA-78), CXCL6 (granulocyte chemotactic protein-2), CXCL9 (monokine induced by IFN-γ), CCL11 (eotaxin), and murine KC and macrophage-inflammatory protein-2 (MIP-2) were quantified using commercially available ELISA kits (R&D Systems). Human CXCL8 (IL-8) and murine LPS-induced CXC chemokine (LIX) levels were measured using matched Ab pairs from R&D Systems.

Statistical analysis

Data are expressed as mean ± SEM, and statistical analysis was performed using Student's unpaired *t* test (Statview SE + Graphics (version 1.03) software). Value of $p < 0.05$ was considered significantly different.

Results

Leukocyte subpopulations as a source of the sIL-6R isoforms

Analysis of effluent from peritoneal dialysis patients with overt peritonitis revealed that increases in IL-6R shedding directly correlate with an initial infiltration of neutrophils, whereas increases in DS-sIL-6R coincide with the influx of mononuclear leukocytes (11). To identify the potential cellular source of PC- and DS-sIL-6R during peritonitis episodes, IL-6R and gp130 expression was first defined on HPMC and leukocytes obtained from whole blood and peritoneal exudates. Although similar levels of gp130 were detected on all cell types, IL-6R expression was strictly confined to leukocyte subpopulations (Fig. 1*A*). Appropriate activation of leukocytes may therefore contribute to sIL-6R levels in vivo.

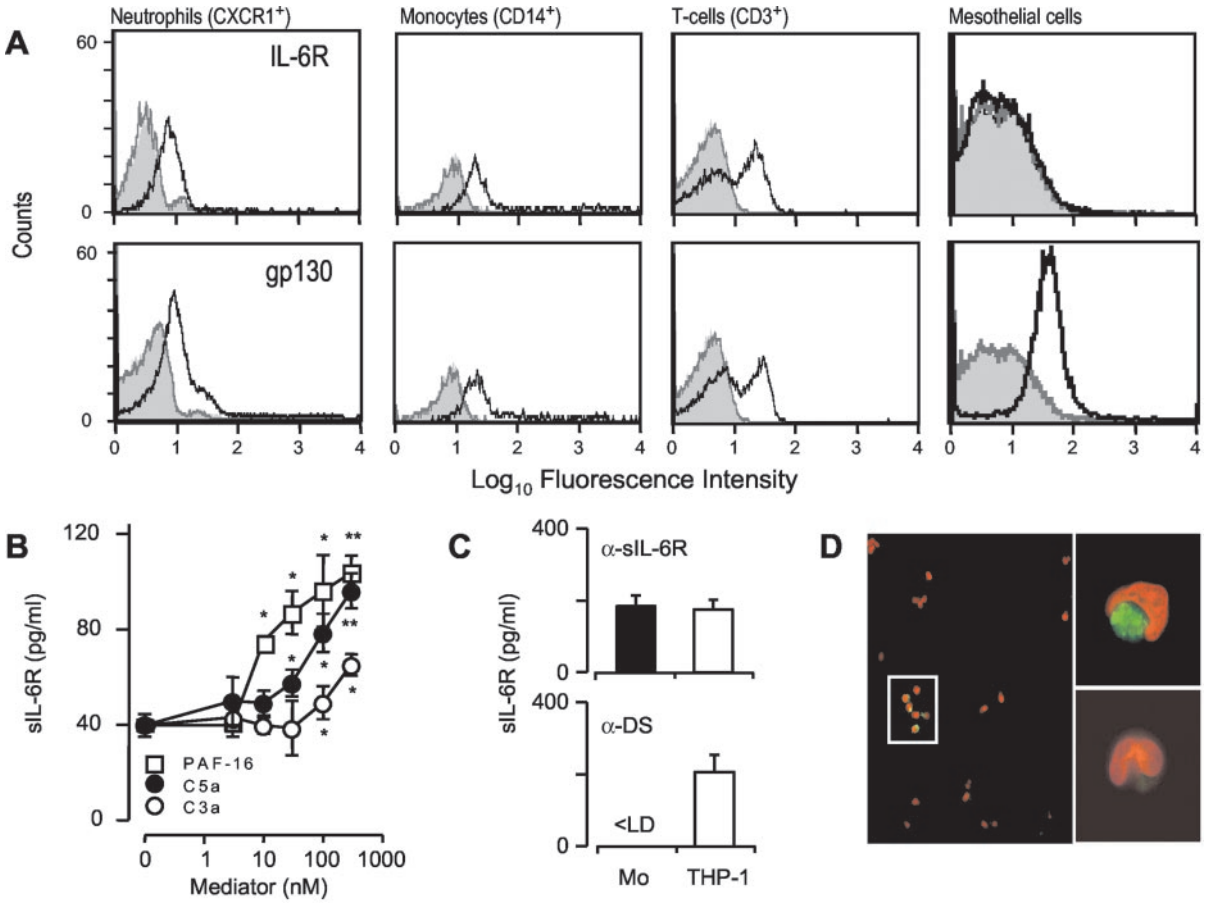

FIGURE 1. Cellular source of the sIL-6R isoforms. *A*, Cognate IL-6R and gp130 on leukocytes and freshly isolated HPMC. Leukocytes were gated according to CXCR1, CD14, and CD3 staining and histograms for IL-6R and gp130 labeling acquired from 10,000 gated events. Data are presented for circulating leukocytes. Similar patterns were obtained for leukocytes isolated from peritoneal exudates (data not shown). Shaded histograms refer to labeling obtained from isotype control Abs. *B*, IL-6R shedding by human neutrophils stimulated for 30 min with increasing concentrations of C5a, C3a, and PAF-16. sIL-6R levels were quantified using ELISA. Mean ± SEM from three donors (*, $p < 0.05$; **, $p < 0.01$) is shown. *C*, Culture supernatants were collected from overnight cultures of human mononuclear phagocytes (Mo) and monocytic THP-1 cells. Total sIL-6R and DS-sIL-6R were quantified using ELISA. Values represent the mean ± SEM from 12 determinations. *D*, Monocytes were stained with mAb-2F3 (green) and counterstained with propidium iodide (red). *Left-hand panel*, Shows a field of cells (×20 magnification), with a cluster of DS-sIL-6R-secreting cells highlighted by the box. *Top right-hand panel*, Shows a single cell expressing DS-sIL-6R. *Lower right-hand panel*, Shows staining with an isotype control (both ×100 magnification). Data are representative of three experiments.

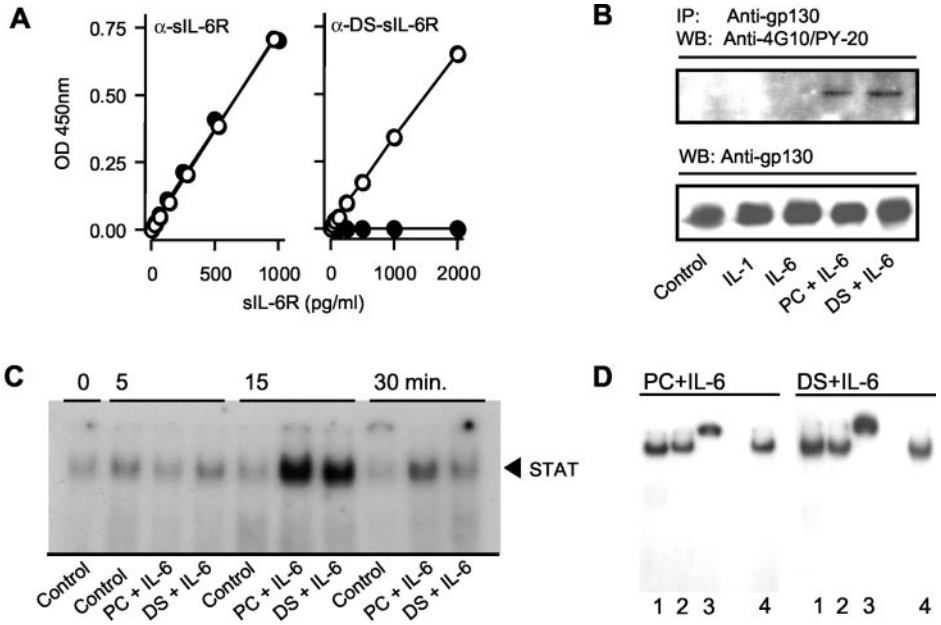

FIGURE 2. Characterization of the sIL-6R isoforms and their signaling activities. *A*, Baculovirus forms of PC-sIL-6R and DS-sIL-6R were assayed using total sIL-6R (α-sIL-6R) and DS-sIL-6R (α-DS-sIL-6R) ELISA systems. ●, Indicate total sIL-6R; ○, indicate DS-sIL-6R. *B*, Growth-arrested HPMC were stimulated with IL-1β (100 pg/ml), IL-6 (10 ng/ml) alone, and in combination with either PC- or DS-sIL-6R (30 ng/ml) for 24 h. gp130 was immunoprecipitated with mAb C-20 and Western blotted with anti-phosphotyrosine Abs (4G10/PY-20 combination). *C*, HPMC were incubated with either medium alone (control) or IL-6 (10 ng/ml) in combination with PC (PC + IL-6)-, or DS-sIL-6R (DS + IL-6) (30 ng/ml). At defined intervals, nuclear extracts were prepared and STAT activation was monitored by EMSA. *D*, Supershift analysis of the STAT complex using anti-STAT1 (*lane 2*) and anti-STAT3 (*lane 3*) Abs. No Ab was included in *lane 1*. An irrelevant control Ab (SC-8432; Santa Cruz Biotechnology) was included in *lane 4*.

Consequently, sIL-6R isoform production by these cells was monitored in vitro. Consistent with reports that chemotactic agents induce IL-6R shedding (11, 30, 31), we observed that stimulation of human neutrophils with complement components C5a and C3a and the lipid mediator PAF increased sIL-6R production (Fig. 1*B*). These increases in sIL-6R were most likely the product of enhanced IL-6R shedding, because no detectable levels of DS-sIL-6R were recorded using a specific anti-DS-sIL-6R (mAb 2F3) Ab (data not shown) (14). Monocytes derived from whole blood and drain effluent were also found to actively secrete sIL-6R during overnight cultures; however, DS-sIL-6R levels remained below the detection sensitivity (15 pg/ml) of this ELISA method (Fig. 1*C*). Given that monocytic cell lines such as THP-1 constitutively release DS-sIL-6R (Fig. 1*C*; see also Ref. 13), production of this isoform by human monocytes was assessed by immunofluorescence. As shown in Fig. 1*D*, DS-sIL-6R production was confined to 10–15% of the total monocyte population. Thus, monocytes produce PC-sIL-6R, while DS-sIL-6R production is confined to a defined subset of cells.

Signaling characteristics of the sIL-6R isoforms

To examine the individual biological characteristics of both receptor isoforms, baculovirus-expressed forms of PC- and DS-sIL-6R were obtained and characterized using ELISA that distinguish between the isoforms (Fig. 2*A*). Because HPMC lack expression of the cognate IL-6R and therefore require sIL-6R to be responsive to IL-6, we next tested whether both recombinant forms activate the Janus kinase-STAT pathway through gp130. Immunoprecipitation of gp130, followed by Western blot analysis with anti-phosphotyrosine Abs (PY-20 and 4G10), showed that both sIL-6R isoforms induced phosphorylation of a single 130-kDa protein, suggesting activation of a gp130 homodimerization event (Fig. 2*B*). In accordance with these findings, both PC- and DS-sIL-6R regulated the IL-6 activation of the Janus kinase-STAT pathway, with peak STAT activation occurring some 15 min after stimulation (Fig. 2*C*). Supershift assays using anti-STAT1 and anti-STAT3 Abs confirmed that both isoforms activate STAT3 (Fig. 2*D*).

Regulation of CCL2/MCP-1 expression by PC- and DS-sIL-6R

It is widely accepted that sIL-6R-mediated signaling promotes secretion of the CC chemokine CCL2 (MCP-1) in a variety of cell types (1). Consistent with these findings, we now confirm that both PC- and DS-sIL-6R are comparable in their ability to regulate CCL2 in HPMC (Fig. 3). In contrast, neither isoform effected expression of CCL11 (eotaxin), CXCL1 (GROα), CXCL8 (IL-8), or CXCL9 (monokine induced by IFN-γ) (Fig. 3*A*).

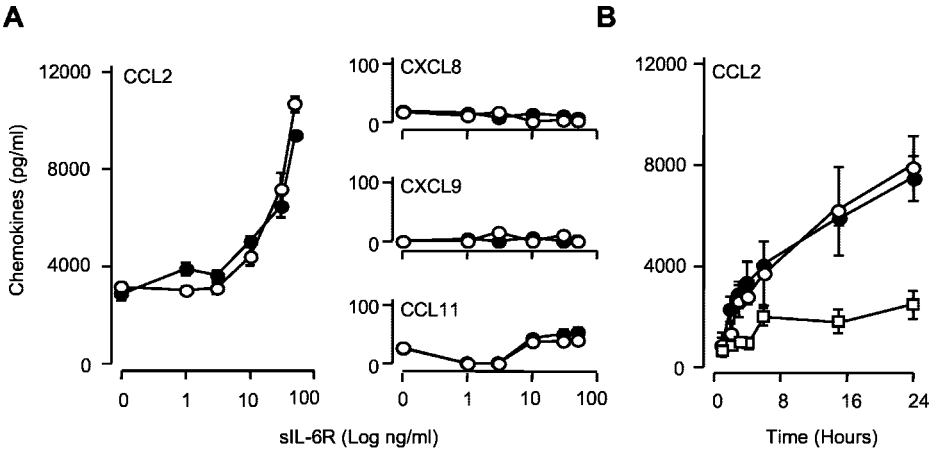

FIGURE 3. Chemokine expression in response to the sIL-6R isoforms. *A*, HPMC were stimulated for 24 h with IL-6 alone (10 ng/ml) or in combination with 0–50 ng/ml PC-sIL-6R (○) or DS-sIL-6R (●). *B*, HPMC were stimulated for defined times with IL-6 (10 ng/ml) in combination with 20 ng/ml PC-sIL-6R (○) or DS-sIL-6R (●). CCL2 was quantified in supernatants using ELISA. □, Show CCL2 release by nonstimulated HPMC. Mean ± SEM from five HPMC isolates is shown (*, $p < 0.05$; **, $p < 0.01$).

Regulation of (ELR$^+$)CXC chemokine expression by sIL-6R isoforms

sIL-6R-mediated signaling has been shown to suppress proinflammatory cytokine (IL-1β and TNF-α)-induced expression of certain adhesion molecules and chemokines (CXCL1, CXCL8, and CX3CL1/fractalkine) (11, 32, 33). To compare the effect of each sIL-6R isoform on (ELR$^+$)CXC chemokine release, HPMC were stimulated with IL-1β (10 pg/ml), and CXCL8 secretion in response to IL-6 was monitored in the presence of each sIL-6R isoform. Both isoforms specifically blocked CXCL8 secretion (Fig. 4*A*). The ability of sIL-6R to suppress (ELR$^+$)CXC chemokine expression was not, however, universal, because IL-6 *trans* signaling promoted CXCL5 (ENA-78) and CXCL6 (granulocyte chemotactic protein-2) secretion to levels comparable to those elicited by IL-1β (Fig. 4*B*), a known inducer of these chemokines (34). However, IL-1β stimulation when combined with IL-6 and the sIL-6R isoforms was not synergistic and only resulted in an additive induction of CXCL5 (1509 $\pm$ 486 pg/ml: predicted additive value 1548 pg/ml) and CXCL6 (1201 $\pm$ 150 pg/ml: predicted additive value 1559 pg/ml).

(ELR$^+$)CXC chemokine expression in IL-6-deficient mice

Previous studies using experimental models of inflammation have illustrated that IL-6 can suppress neutrophil recruitment in vivo (35, 36) (T. Wilkinson and N. Topley, unpublished observation). This process is, however, coordinated via sIL-6R, because selective blockade of IL-6 *trans* signaling by sgp130 disrupts this regulatory event (11, 24, 25). Experiments were therefore performed to substantiate the differential regulation of individual (ELR$^+$)CXC chemokines by IL-6 and its soluble receptor isoforms in vivo. First, KC, MIP-2, and LIX (murine counterparts of CXCL1, CXCL8, and CXCL5, respectively) expression was monitored over a 12-h period in IL-6$^{+/+}$ and IL-6$^{-/-}$ mice following induction of peritoneal inflammation with SES. Although the profile of each chemokine in IL-6$^{+/+}$ mice showed a transient expression, KC and MIP-2 secretion preceded that of LIX (Fig. 5*A*). Consistent with previous reports using this model (11, 24), IL-6 deficiency was associated with enhanced KC and MIP-2 expression (Fig. 5*A*; see also Ref. 11). However, the augmented induction of KC and MIP-2 levels in the absence of IL-6 does not appear to be common to all (ELR$^+$)CXC chemokine, because IL-6 deficiency was accompanied by a significantly reduced secretion of LIX (Fig. 5*A*). This differential control of (ELR$^+$)CXC chemokines appears to be regulated via IL-6 signaling through its soluble receptor, because i.p. reconstitution of IL-6 signaling through sIL-6R in IL-6$^{-/-}$ mice suppressed the expression of MIP-2 and KC, while concomitantly promoting the release of LIX. This was true irrespective of the sIL-6R isoform tested. To substantiate these findings, sIL-6R-mediated signaling was specifically blocked by i.p. administration of its natural antagonist sgp130 (11, 24, 37). Addition of sgp130 prevented the regulation of KC, MIP-2, and LIX, thus supporting an active role for sIL-6R in this process (Fig. 5*B*).

Control of neutrophil recruitment in IL-6-deficient mice by sIL-6R

Having established that the sIL-6R isoforms differentially regulate the pattern of (ELR$^+$)CXC chemokine expression, we next tested the overall capacity of both isoforms to regulate leukocyte infiltration. As shown in Fig. 6*A*, IL-6 deficiency was associated with heightened neutrophil infiltration following SES administration. Reconstitution of IL-6 signaling via the coadministration of IL-6 in combination with PC- or DS-sIL-6R significantly blocked neutrophil influx, while inhibition of sIL-6R activity with sgp130 substantiated that regulation of neutrophil recruitment by IL-6 was mediated through its soluble receptor and not via IL-6 activation of its cognate receptor (Fig. 6*B*). In terms of leukocyte infiltration, these data infer that suppression of KC and MIP-2 expression by IL-6 may override its ability to induce LIX. To exemplify this point, KC activity was blocked in IL-6$^{+/+}$ mice by administration of an anti-KC Ab before the induction of inflammation. Blockade of KC significantly reduced (65–70% inhibition over controls) the number of neutrophils recruited to the peritoneal cavity (Fig. 6*C*). Although these data do not exclude the likelihood that other chemotactic agents, such as MIP-2, also contribute to the overall recruitment of neutrophils, these studies, coupled with the different time course of LIX expression and the induction of LIX by IL-6 and sIL-6R, indicate that the primary function of KC, and presumably MIP-2, is distinct from that of LIX.

Discussion

In this study, we have characterized the ability of the sIL-6R isoforms to coordinate neutrophil recruitment during acute inflammation. Although both isoforms behaved similarly, these studies emphasize that individual (ELR$^+$)CXC chemokines may elicit unique functions during an inflammatory episode.

IL-6 responses are mediated through interaction with either a membrane-bound or a soluble form of its cognate receptor. However, only a limited number of cell types express IL-6R, suggesting that sIL-6R-mediated signaling may represent the most prominent mechanism of IL-6 signaling (1). Surprisingly, sIL-6R is generated through proteolytic cleavage of the cognate IL-6R, and as the product of differential mRNA splicing (2). Consequently, two distinct isoforms of the sIL-6R contribute to the properties of sIL-6R in vivo. To date, it is unclear why two release mechanisms are used to control sIL-6R activities, while the processes that contribute to their regulation are not fully understood (2). Abs raised against the unique COOH-terminal sequence of DS-sIL-6R have enabled the relative abundance of each isoform to be established in normal and diseased states. These studies reveal that sIL-6R expression depends on the age of an individual and the disease condition (2, 9,

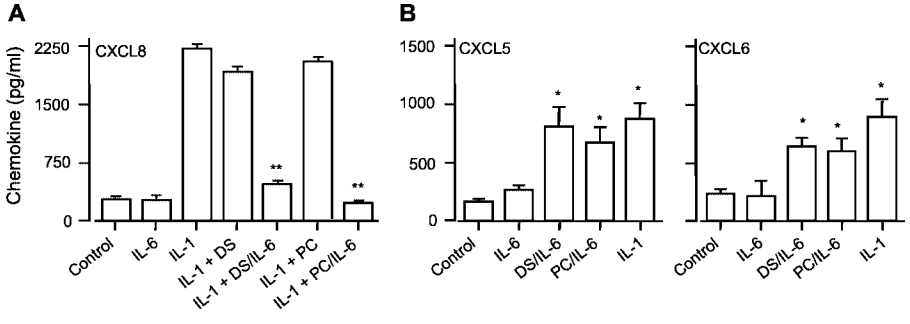

FIGURE 4. Regulation of CXCL5, CXCL6, and CXCL8 by the sIL-6R isoforms. HPMC were stimulated (24 h) with IL-1β (10 pg/ml), IL-6 (10 ng/ml), and IL-6 in combination with PC- or DS-sIL-6R (50 ng/ml), as indicated. Chemokine levels were quantified using ELISA for *A*, CXCL8, and *B*, CXCL5 and CXCL6. Mean $\pm$ SEM from five HPMC isolates (*, $p < 0.05$; **, $p < 0.01$) is shown.

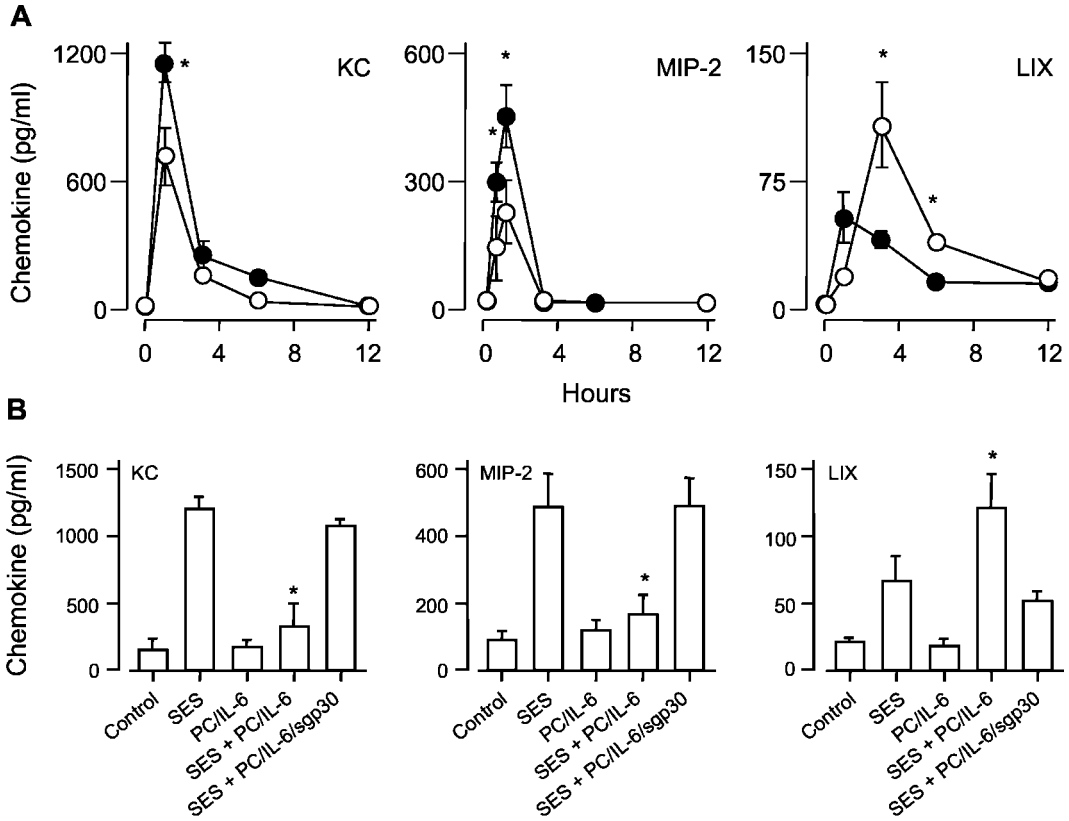

FIGURE 5. Differential regulation of (ELR$^+$)CXC chemokines in IL-6$^{-/-}$ mice. *A*, SES-induced inflammation was established in IL-6$^{+/+}$ (○) and IL-6$^{-/-}$ (●) mice (∗, $p < 0.05$). Chemokine levels were quantified using ELISA in lavage fluid obtained at defined intervals. *B*, Modulation of chemokine expression in IL-6$^{-/-}$ mice by administration (i.p.) of IL-6 (10 ng/mouse) with sIL-6R (25 ng/mouse) for 1 h. sgp130 (150 ng/mouse) was added, as indicated. Mean ± SEM (n = 5–6 mice/condition; ∗, $p < 0.05$ SES vs SES + sIL-6R/IL-6 complex) is shown.

10, 11, 38). Specifically, during progression of acute inflammation, DS-sIL-6R levels increase at a later stage in the inflammatory process than PC-sIL-6R, and coincide with the onset of mononuclear leukocyte infiltration (11). In this study, we show that chemotactic agents promote IL-6R shedding from human neutrophils, while only a limited proportion of human monocytic cells actively secretes DS-sIL-6R. Although it remains to be established whether DS-sIL-6R expression is confined to one of the recently defined monocytic subsets (39), detection of sIL-6R in culture supernatants suggests that human monocytes release both PC- and DS-sIL-6R. Such a differential regulation has also been observed in a series of transformed cell lines (8, 12, 13, 15). Taken together, these in vitro and ex vivo studies imply that DS- and PC-sIL-6R possess unique characteristics.

Using a series of in vitro and in vivo approaches, it was found that sIL-6R activity preferentially blocks neutrophil infiltration by suppressing secretion of the (ELR$^+$)CXC chemokines, CXCL1 and CXCL8 (the functional homologues of murine KC and MIP-2) from activated resident tissue cells (data presented in this work) (11). However, this response was not common to all (ELR$^+$)CXC chemokines, because the IL-6/sIL-6R complex promoted CXCL5 and CXCL6 expression. This differential ability to regulate

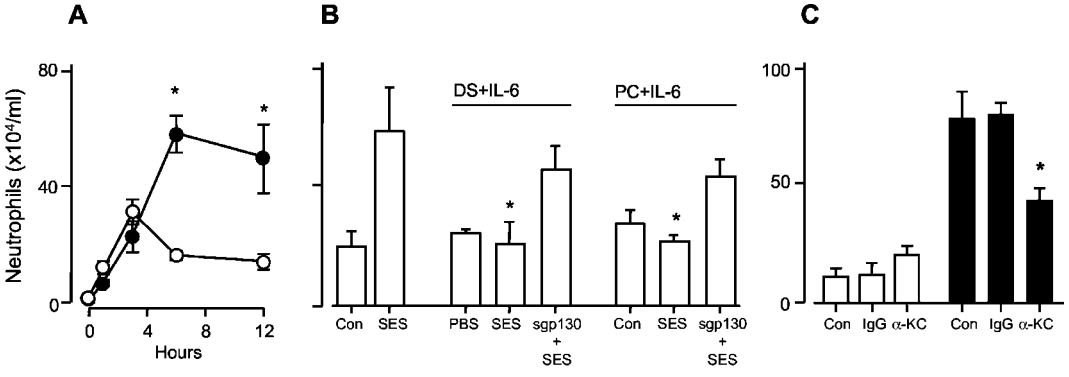

FIGURE 6. Impact of the sIL-6R isoforms on neutrophil recruitment in vivo. *A*, Neutrophil recruitment was quantified by differential cell counting in IL-6$^{+/+}$ (○) and IL-6$^{-/-}$ (●) mice. *B*, Reconstitution of sIL-6R activity in IL-6$^{-/-}$ mice by administration (i.p.) of IL-6 (10 ng/mouse) with sIL-6R (25 ng/mouse; DS + IL-6 and PC + IL-6). sgp130 (150 ng/mouse) was added, as indicated. *C*, IL-6$^{+/+}$ mice were treated with anti-KC Ab (50 μg/mouse) 90 min before challenge with PBS (□) or SES (■). For *B* and *C*, differential cell counts were derived after 3-h stimulation. Results expressed as mean ± SEM (n = 6–9 mice). ∗, $p < 0.05$ between *B*, SES and SES/PC + IL-6 or SES/DS + IL-6; and *C*, IgG- and anti-KC-treated mice.

(ELR$^+$)CXC chemokines is not restricted to IL-6, because the IL-6-related cytokine oncostatin-M governs CXCL8 and CXCL5 expression in an identical manner (27, 40, 41). Oncostatin-M and sIL-6R are liberated from the initial neutrophil influx during acute peritoneal inflammation and serve to direct transition between neutrophil and mononuclear leukocyte recruitment (11, 24, 27). The regulation of IL-6R shedding by CXCL1 and CXCL8 (11, 30) also provides an elegant mechanism for controlling the initial neutrophil influx and facilitating their subsequent clearance (1, 25). However, the importance of CXCL5 and CXCL6 in this sIL-6R-mediated process is unclear. Significantly, KC and MIP-2 induction precedes neutrophil influx, while LIX expression coincides with neutrophil infiltration in SES-challenged IL-6$^{+/+}$ mice. Thus, the overall ability of sIL-6R signaling to limit the neutrophil infiltrate suggests that CXCL5 and CXCL6 may not predominantly act as frontline chemoattractants, but might alternatively govern neutrophil responses local to the inflammatory insult. However, in the absence of a suitable blocking LIX Ab or a LIX-deficient mouse, this idea may be difficult to address.

In humans, (ELR$^+$)CXC chemokines activate neutrophils through interaction with the chemokine receptors CXCR1 and CXCR2 (17). Although both receptors elicit chemotactic responses, they show marked differences in their ability to regulate the NADPH oxidase, phospholipase D, receptor internalization, and cross-sensitization (20, 21, 42, 43). Indeed, CXCL8-mediated degranulation events can be fully elicited under conditions in which CXCR2 signaling is blocked (20, 21). These studies have led to speculation regarding the potential roles of CXCR1 and CXCR2 in regulating chemotaxis and activating cellular events at sites of inflammation (42, 44). CXCL5 and CXCL6 primarily signal through CXCR2; however, CXCL6 shares an intermediary affinity for both receptors (19). In addition, comparison of CXCL5 and CXCL8 expression by LPS-stimulated monocytes demonstrated different kinetics of production, which suggests not only different mechanisms of induction, but potentially unique functional properties (23). In line with this notion, a recent study of endothelial cells proposed that LIX could regulate NF-κB activity, and control production of inflammatory cytokines (45). The differential regulation of (ELR$^+$)CXC chemokines by IL-6 and its soluble receptor also supports this theory. However, the overall ability of sIL-6R-mediated events to suppress neutrophil infiltration suggests that the induction of CXCL5 and CXCL6 might somehow contribute to the demise of the neutrophil influx. Recently, we reported that IL-6/sIL-6R signaling controls neutrophil recruitment by suppressing local CXCL1 and CXCL8 expression, and by directing neutrophil apoptosis (11, 24). Accordingly, it is tempting to speculate whether CXCL5 and CXCL6 perform roles in the clearance of senescent neutrophils. In this respect, various chemokines have been shown to regulate apoptotic events in leukocytes (46–48).

It is firmly established that IL-6 signaling through its soluble receptor is instrumental in controlling leukocyte recruitment during inflammation and regulates transition from neutrophil to mononuclear leukocyte recruitment (1, 11, 25). This checkpoint represents an essential step in the successful resolution of an inflammatory process. The identification of two sIL-6R isoforms may ultimately have a significant bearing on the regulation of leukocyte recruitment during inflammation. Both isoforms elicit common cellular events through gp130, which include STAT activation, and the regulation of neutrophil recruitment via (ELR$^+$)CXC chemokines. Similarly, DS- and PC-sIL-6R were equally capable of regulating CCL2 expression. However, the observation that increases in DS-sIL-6R coincide with the initiation of mononuclear cell influx suggests that the activities of this isoform may be more associated with the recruitment or activation of monocytes and lymphocytes.

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