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Therapeutic Targeting of IL-6 Trans Signaling Counteracts STAT3 Control of Experimental Inflammatory Arthritis

Mari A. Nowell,2* Anwen S. Williams,2† Sarah A. Carty,‡ Jürgen Scheller,§ Anthony J. Hayes,¶ Gareth W. Jones,* Peter J. Richards,‡ Simon Slinn,‡ Matthias Ernst,¶ Brendan J. Jenkins,‖ Nicholas Topley,* Stefan Rose-John,‡ and Simon A. Jones3*

Cytokine control of the synovial infiltrate is a central process in the development of inflammatory arthritis. In this study, we combine genetic approaches and intervention strategies to describe a fundamental requirement for IL-6-mediated STAT3 signaling in orchestrating the inflammatory infiltrate in monoarticular and systemic models of experimental arthritis. STAT3 activation via the common gp130 signal-transducing receptor for all IL-6-related cytokines led to increased retention of neutrophils and T cells within the inflamed synovium, which included STAT3-regulated IL-17A-secreting T cells. Control of leukocyte infiltration was reliant upon IL-6 signaling via its soluble receptor (termed IL-6 trans signaling), as evidenced by selective blockade of this alternative IL-6 signaling pathway using an engineered variant of soluble gp130 (sgp130Fc). This therapeutic intervention led to substantial clinical improvement in mice with emerging or established incidence of systemic arthritis. These data illustrate that IL-6 control of STAT3 is critical for regulating the synovial infiltrate in inflammatory arthritis, and suggest that selective inhibition of IL-6 trans signaling may provide a more refined intervention strategy for blocking IL-6-driven proarthritic activities. The Journal of Immunology, 2009, 182: 613–622.

Interleukin-6 is traditionally considered a regulator of acute-phase responses and a lymphocyte stimulatory factor (1, 2). However, recent advances have identified crucial roles for this cytokine in orchestrating aspects of both innate and acquired immunity that influence the outcome of inflammatory responses. In this respect, IL-6 has been implicated in the control of leukocyte recruitment, differentiation, activation, and survival (1, 2). A pivotal function for IL-6 in dictating disease outcome has been highlighted by the identification of IL-6 involvement in the commitment and effector properties of IL-17-secreting Th cells and CD25+ inducible regulatory T cells (3–8).

Studies in chronic models of disease collectively endorse a central role for IL-6 in disease progression and advocate the therapeutic targeting of IL-6 as a strategy for treating chronic disease (9). Dissecting the immunological regulation of these IL-6-mediated events is, however, complicated by the fact that two distinct processes control IL-6 responses in vivo. Specifically, IL-6 signals are transmitted via gp130, which serves as the universal signal-transducing receptor β-subunit for all IL-6-related cytokines. Although this classically occurs through IL-6 engagement of a membrane-bound receptor α-subunit (IL-6R), gp130 activation can also be triggered through IL-6 binding of a soluble form of IL-6R (sIL-6R). This alternative mode of cell activation is termed IL-6 trans signaling (1, 2). In either case, IL-6 engagement of its specific α-subunit leads to gp130 homodimerization and activation of several signaling cascades, in particular the JAK-STAT1/3, Src homology region 2 domain-containing phosphatase 1-MAPK, and Src homology region 2 domain-containing phosphatase 1-P3K pathways (10).

Therapeutic interventions that selectively target inflammatory cytokines have been particularly successful in the management of autoimmune conditions such as rheumatoid arthritis (RA) (11). This approach is best illustrated by the clinical application of the TNF-α blockers. However, these therapies are often associated with increased risk from infection and possible malignancies (11). The humanized blocking IL-6R Ab Tocilizumab (MRA, Actemra, Atizumab) is currently in advanced clinical trial testing for the treatment of RA, juvenile idiopathic RA, Crohn’s disease, and multiple myeloma, and has been licensed in Japan for treatment of Castleman’s disease (12–17). The potential beneficial outcome of IL-6R blockade must, however, be balanced with the negative impact on host immunity, as evidenced by the reduced ability of IL-6-deficient mice to clear bacterial infections and mount an effective T cell memory response (18–20). It is currently unclear how IL-6 brings about these contrasting detrimental and beneficial activities.

Recent studies have highlighted that a number of IL-6 activities are governed in vivo by sIL-6R (1, 2). However, it is unclear how both classical IL-6R signaling and IL-6 trans signaling coordinate
IL-6 responsiveness (1, 2). Evidence for this dual mode of IL-6 signaling has been aided by the demonstration that two soluble forms of human gp130 (sgp130 and gp130 of the rheumatoid arthritis antigenic peptide-bearing soluble form) selectively counteract IL-6 trans signaling (21–29). In this respect, in vitro and in vivo approaches advocate a role for IL-6 trans signaling in cartilage and joint destruction, whereas analysis of synovial fluids from active arthritis patients shows a close association among sIL-6R levels, disease activity, and leukocyte recruitment in the inflamed joint (27, 29–32). Furthermore, studies in murine Ag-induced arthritis (ArIA) suggest that synovial regulation of IL-6 trans signaling promotes disease activities that include CCL2-driven monocellular cell recruitment and synovial hyperplasia (27).

In this present communication, we have identified a crucial role for IL-6/gp130 signaling through STAT3 in directing leukocyte infiltration within the inflamed joint during repeated episodes of monoaarticular arthritis. Collectively, we show that the deleterious activities of IL-6 primarily promote an aberrant control of leukocyte responses during inflammatory arthritis, and can direct the phenotype of the T cell population retained within the inflamed synovium. Successful therapeutic intervention with an engineered sgp130Fc fusion protein in a systemic arthritis model now allows us to suggest that IL-6 trans signaling represents a critical step in the pathogenesis and development of inflammatory arthritis. Consequently, this more selective management of IL-6 activities offers a refinement to current IL-6-based therapies, which may help reduce the likelihood of additional clinical complications arising from a complete blockade of IL-6 bioactivity.

Materials and Methods

Animals

Experiments were undertaken in adult (over 7 wk) male C57BL/6j and DBA-1 wild-type (WT) animals (Harlan), and inbred IL-6 knockout (KO) (C57BL/6). The gp130Y757F:Y757F mice and gp130Y757F:Y757F mice bearing a monoallelic ablation of STAT3 (gp130Y757F:Y757F:STAT3+/−) were generated, as previously described (24, 33, 34). Procedures were performed in accordance with Home Office-approved project licenses PPL-30/1820 and PPL-30/2361.

Murine ArIA

Adult C57BL/6j male mice were immunized with 100 μl of 1 mg/ml methylated BSA (mBSA; Sigma-Aldrich) in water, emulsified with an equal volume of CFA (Sigma-Aldrich) by s.c. injection. Animals also received 100 μl of heat-inactivated Bordetella pertussis toxin (Sigma-Aldrich) by i.p. injection. The immune response was boosted 1 wk later. Twenty-one days after the initial immunization, arthritis was induced by intra-articular (i.a.) administration of 10 mg/ml mBSA (10 μl vol) in the hind right joint. Sequelae of inflammatory arthritis were triggered every 14 days following administration (i.a.) of an equivalent mBSA dose. Arthritis severity was assessed through a comparison between the experimental right limb and that of the control left limb. Animals were inspected every 14 days following administration (i.a.) of an equivalent mBSA dose. Arthritis severity was assessed on day 34, when the experiment was terminated. Inflammatory parameters were assessed in up to 10 animals sacrificed at defined intervals. The experiments were repeated on two separate occasions with three animals sacrificed at each time point (i.e., n = 6 animals in total).

Histological assessment of arthritis

Joints were fixed in neutral buffered formal saline and decalcified with 10% formic acid at 4°C before embedding in paraffin. Midsagittal serial sections (of 7 μm thickness) were stained with H&E. Bone and cartilage erosions were evaluated following Safranin-O and Fast Green staining. Two independent observers blinded to the experimental groups scored sections according to subsynovial inflammation (0 = normal, to 5 = ablation of adiposity due to inflammatory infiltrate), synovial exudate (0 = normal, to 3 = substantial number of cells with large fibrin deposits), synovial hyperplasia and pannus formation (0 = normal or 1–3 layers thick, to 3 = over three layers thick with overgrowth over joint surfaces with evidence of cartilage loss), and cartilage/bone erosions (0 = normal, to 3 = destruction of a significant part of the bone). The aggregate score for all parameters is presented as an arthritis index (mean ± SEM).

Immunohistochemistry

Phenotypic markers for leukocytes, phosphorylated STAT3, and IL-17A were localized in paraffin sections using Abs to murine Ly-6G (BD Biosciences), F4/80 (Serotec), CD3e (Santa Cruz Biotechnology), pSTAT3 (Cell Signaling Technology), and IL-17A (R&D Systems). Ab unmasking using 10 mM sodium citrate buffer for CD3e, pSTAT3, and IL-17A, or 0.05% trypsin/0.53 mM EDTA (Life Technologies Invitrogen) for Ly-6G and F4/80 was necessary before staining. To eliminate endogenous peroxidase activity, sections were treated with 3% H2O2 (Sigma-Aldrich). Sections were subsequently treated with 10% normal rabbit or swine serum (DakoCytomation) and an avidin/biotin blocking reagent (DakoCytomation). Ab labeling was detected using either biotinylated rabbit anti-rat IgG or biotinylated swine anti-rabbit IgG. StreptABComplex/HRP (DakoCytomation) with diaminobenzidine chromogen (Sigma-Aldrich). Relevant fractions were concentrated, and the protein was further purified by gel filtration on a Sephacryl S-200 HR (16/60) column (GE Healthcare).

Murine collagen-induced arthritis (CIA)

Adult DBA-1 male mice were immunized with 100 μl of chick collagen (2 mg/ml; Sigma-Aldrich) dissolved in 10 mM acetic acid and emulsified with an equal volume ofIFA (Sigma-Aldrich) containing 5 mg/ml Mycobacterium tuberculosis H37 Ra (BD Biosciences) by intradermal injection (day 0). Each animal received a second, identical intradermal injection on day 21. In the early intervention study, cohorts of mice were randomly assigned to treatment groups on day 21, when no arthritis was apparent in any of the mice. Animals received seven defined doses of sgp130Fc (22, 29) dissolved in vehicle (PBS) or vehicle alone, administered on alternate days by i.p. injection from day 21. Arthritis severity was scored in each paw using a scale ranging from 0 to 4, as follows: 0, no evidence of erythema and swelling; 1, erythema and mild swelling confined to the tendons or ankle joint; 2, erythema and mild swelling extending from the ankle to the toes; 3, erythema and moderate swelling extending from the ankle to metatarsal joints; 4, erythema and severe swelling encompass the ankle, foot, and digits, or ankylosis of the limb. The aggregate values for each animal gave the clinical score. A maximal score of 12 was attainable before necessitating sacrifice for ethical reasons and in strict accordance with Home Office guidelines. For the treatment of established disease, mice were divided into two groups and matched for clinical score on day 27 (sgp130Fc group, 4.28 ± 0.6; vehicle control (PBS) group, 4.47 ± 0.86; mean ± SEM) when arthritis incidence was 100% and treatment commenced. Grouped mice received a daily dose (i.p.) of either 2.5 mg/kg sgp130Fc or vehicle control (PBS; as above) for a total of 7 days. Arthritis severity was assessed on day 34, when the experiment was terminated. Inflammatory parameters were assessed in up to 10 animals sacrificed at defined intervals. The experiments were repeated on two separate occasions with three animals sacrificed at each time point (i.e., n = 6 animals in total).

Production and purification of recombinant sgp130Fc protein

Cell supernatants from Chinese hamster ovary cells stably transfected with cDNA encoding sgp130Fc were kept at −80°C and filtered (0.45 μm) before purification. Affinity chromatography was performed with protein A-Sepharose (Roche). Relevant fractions were concentrated, and the protein was further purified by gel filtration on a Sephacryl S-200 HR (16/60) column (GE Healthcare).

In vitro Th17 cell expansion

Single-cell suspensions were prepared by mechanical disruption of spleens and passage through a 70-μm nylon cell strainer. Contaminating RBC were removed by washing in lysis buffer (155 mM NH4Cl, 12 mM NaHCO3, 1
mM EDTA (pH 7.3)). Cultures were maintained in RPMI 1640 supplemented with 10% FCS, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 50 μM 2-ME (Life Technologies Invitrogen). A total of 1 × 10^5 cells was cultured in anti-CD3-coated 96-well plates (1 μg/ml; R&D Systems) containing soluble anti-CD28 (5 μg/ml; BD Biosciences) for 4 days. To promote Th17 expansion, cultures were supplemented with 10 ng/ml IL-6 and 1 ng/ml TGF-β (R&D Systems). Where indicated, IL-2 was neutralized with 10 ng/ml anti-IL-2 (clone JES6-1A12; R&D Systems).

Flow cytometry of human and murine T cells
Surface IL-6R expression was detected on murine CD3^+ and CD4^+ gated cells using anti-CD4 allophycocyanin (Caltag Invitrogen), anti-CD3 FITC, and anti-IL-6R PE (both from BD Biosciences). For intracellular cytokine staining, T cell suspensions were supplemented with 50 ng/ml PMA, 500 ng/ml ionomycin, and 3 μM monensin (Sigma Aldrich) 4 h before analysis. To reduce nonspecific Ab staining, cells were incubated with BD Fc block (BD Biosciences). CD4^+ T cells were stained with anti-CD4 PE-Cy5 (clone RM4-5; BD Biosciences) and anti-IFN-γ (clone RM4-5; BD Biosciences) before fixation and permeabilization in BD Cytofix/Cytoperm (BD Biosciences). Intracellular IL-17A and IFN-γ were detected in cells with anti-IL-17A PE (clone TC11-18H10.1; BD Biosciences) and anti-IFN-γ FITC (clone XMG1.2; Caltag Invitrogen), respectively. For the analysis of human samples, leukocyte pellets obtained from blood and synovial fluid were prepared for flow cytometry following the removal of contaminating erythrocytes by hypotonic lysis. Expression of IL-6R on CD4^+ gated T cells was determined using anti-IL-6R PE and anti-CD4 FITC (both from BD Biosciences). Data were acquired on a FACSCalibur flow cytometer (BD Biosciences) using BD CellQuest (BD Biosciences), and analyzed using FlowJo software (Tree Star).

Patient synovial fluid and serum samples
Ethical approval for the collection of synovial fluids and serum samples was obtained from the Bro Taf and Bro Morgannwg research ethics committees, and sample collection was undertaken following informed patient consent. Synovial fluid and serum samples were obtained from patients presenting with active joint effusions (as defined by the 1987 revised criteria of assessment outlined by the American College of Rheumatology (formerly the American Rheumatism Association)). Fluids were rendered cell free by centrifugation, and supernatants were stored at −80°C.

ELISA
Concentrations of human IL-6, sIL-6R, and sgp130 were quantified using commercial ELISA (R&D Systems).

Graphical representation and data analysis
XY scatter plots (mean ± SEM) and one-grouping variable vertical scatter plots were plotted using GraphPad Prism v.4.00 (GraphPad). Statistical analyses were conducted in Prism using one-tailed nonparametric Mann-Whitney U test.

Results
Synovial infiltration and arthritis severity are governed by IL-6
Previous studies have shown that when compared with WT littermates, IL-6KO mice are resistant to disease induction in models of experimental arthritis (27, 35, 36). However, the molecular and cellular mechanisms underlying their resistance to disease remain largely unresolved. Recurrent episodes of inflammatory activity are an underlying feature of RA and invariably contribute to an exacerbation of inflammation and joint destruction (37). To examine how these processes might influence IL-6 involvement in arthritis progression, we have used a monoarticular model of AIA, which has been modified to examine changes in the inflammatory response as a consequence of recurrent inflammatory activation. This model therefore provided an opportunity to examine the role of IL-6 during relapsing/remitting inflammatory arthritis, and a genetic appraisal of cytokine involvement through the use of transgenic mice. WT and IL-6KO mice were immunized with mBSA and inflammatory arthritis triggered sequentially on days 0, 14, and 28 by i.a. administration of WT and IL-6KO mice with mBSA (Fig. 1). Ab (IgG) responses to mBSA and inflammatory arthritis triggered sequentially on days 0, 14, and 28 by i.a. administration of WT and IL-6KO mice with mBSA (Fig. 1). Ab (IgG) responses to mBSA were measured in WT and IL-6KO and shown to be similar at disease onset (\(n = 3\), WT OD 450 nM; 1.61 ± 0.49, vs IL-6KO OD 450 nM; 1.03 ± 0.27, mean ± SEM). In agreement with previous studies, inflammation of the knee following Ag challenge (as measured by differences in knee diameters between i.a. injected knee and noninjected knee) in IL-6KO mice was significantly impaired compared with WT mice (Fig. 1A). In addition, WT showed enhanced inflammation following repeated challenge, which was noticeably absent in IL-6KO (Fig. 1A).

Histological sections were prepared during each round of inflammatory activation and arthritis parameters scored. Although arthritis severity progressively worsened following each round of mBSA activation in WT mice, joints from IL-6KO mice showed limited pathological changes, and showed no signs of disease progression as a consequence of recurrent inflammatory activation (Fig. 1, A and B). This is illustrated in Fig. 1B, in which histological sections prepared at the experimental end point (day 49) following three rounds of activation show increased evidence of leukocyte infiltration, together with gross alterations in joint morphology. Specifically, the tissue underlining the synovial membrane of WT mice was awash with inflammatory cells, and...
evidence of a pronounced inflammatory infiltrate or exudate (tions in IL-6KO mice, IL-6KO joint sections showed little or no C). In contrast, despite repeated mBSA injec-
tion (Fig. 1, B), representative arthritis index scores observed during the course of the ex-
periment (Fig. 1, C). These changes in the joint mor-
phology of WT mice were accompanied by a gross alteration in leukocyte infiltration of the inflamed synovial tissue (infiltrate), which was sustained with no evidence of clearance from the inflamed joint (Fig. 1, A and B). This was reflected by the consistently high arthritis index scores observed during the course of the experiment (Fig. 1, C). In contrast, despite repeated mBSA injec-
tions in IL-6KO mice, IL-6KO joint sections showed little or no evidence of a pronounced inflammatory infiltrate or exudate (p < 0.05; Fig. 1, B and C). The involvement of IL-6 in arthritis therefore appears to be closely associated with an ability of IL-6 to dysregulate leukocyte trafficking.

To define the nature of the inflammatory cells influenced by IL-6, sections taken from WT and IL-6KO mice during each round of the AIA model were probed using Abs against pan leukocyte markers for neutrophils (Ly6G), monocytes (F4/80), and T cells (CD3). As shown in Fig. 2A, IL-6 deficiency was associated with an impaired influx of neutrophils and T cells. No substantive difference in F4/80 monocyte cell recruitment was observed between the two strains (Fig. 2). Significantly, both neutrophil and mononuclear cell infiltration resolved by day 49 in WT and IL-6KO mice, suggesting that IL-6 is not required for their effective clearance (Fig. 2). Conversely, the presence of IL-6 appears to promote maintenance of CD3+ T cells in lymphoid aggregates within the inflamed joint (Fig. 2A). This is consistent with lymphoid aggregations observed in human RA synovial tissue (38) and was observed only in WT animals.

Activation of STAT3 promotes T cell responses and arthritis progression

To define the molecular and biochemical mechanisms governing the IL-6 control of arthritis progression, we next examined changes in IL-6 signaling events within the inflamed synovium following repeated challenge with mBSA. Synovial joint sections were examined by immunohistochemistry for changes in pY-STAT1 and pY-STAT3. As previously described, small, but transient increases in pY-STAT1 were observed during the initiation of AIA (data not shown) (29, 38). In contrast, activated STAT3 was rapidly enhanced following arthritis induction (Fig. 3). Comparative analysis of pY-STAT3 staining in joint sections from both WT and IL-6KO mice showed that the activation of STAT3 was significantly impaired in IL-6KO mice (Fig. 3A). Histological inspection of the pY-STAT3 staining pattern in WT sections showed that STAT3 activity was closely associated with that of the CD3+ infiltrate (Fig. 3B, inset). Because STAT3 has recently been linked with the expansion of IL-17-secreting Th cells (4), we next examined the potential relationship between STAT3 activity and the T cell infiltrate by immunohistochemistry using an anti-IL-17A Ab (Fig. 3). IL-17A expression colocalized with the CD3+ T cell infiltrate found within distinct lymphoid aggregates in the inflamed tissue.

FIGURE 2. IL-6 governs neutrophil and lymphocyte infiltration of the joint. A, Immunohistochemical staining of leukocyte markers in joint section from WT and IL-6KO mice during recurrent monoarticular AIA. Staining with Abs for Ly6G (neutrophil), F4/80 (monocy-
cytic cells), and CD3 (T cells) was analyzed. Results are presented as the mean ± SEM (n = 3–4; *, p < 0.05; **, p < 0.01; ***, p < 0.005). Representative staining for each Ag in serial joint sections from WT (B) and IL-6KO (C) is shown. Sections were derived from joints taken at day 29 (i.e., 1 day following the third mBSA challenge). Insets. Show high-power images of the dia-
minobenzidine staining of Ags (brown). Scale bar, 100 μM.

FIGURE 3. IL-6 enhances STAT3 activation in the arthritic synovium. A, Ab staining for pY-STAT3 in joint sections from WT and IL-6KO mice taken during recurrent mBSA-triggered arthritis. Results presented as the mean scores ± SEM (n = 3–4 sections per point; *, p < 0.05; **, p < 0.01; ***, p < 0.005). B, Representative immunohistochemical staining at day 29 (i.e., 1 day post-third mBSA challenge) from WT and IL-6KO of pY-STAT3. Inset, Shows CD3 staining corresponding to the area marked on the figure. C, Representative immunohistochemical staining of CD3 and IL-17A in serial joint sections taken from WT and IL-6KO mice on day 49. Scale bar, 50 μM.
FIGURE 4. Hallmarks of arthritis are linked to gp130-mediated STAT3 activation. A, Histological analysis of AIA joint sections for WT, gp130Y757F:Y757F (FF), and gp130Y757F:Y757F:STAT31/−/− (FFS3) mice, following a single challenge with mBSA. Results presented are the mean scores ± SEM, n = 6 (histopathology) and n = 3 (immunohistochemistry). B, Representative histology at experimental end point (day 35) for WT, gp130Y757F:Y757F, and gp130Y757F:Y757F:STAT31/−/− mice. Contralateral nonarthritic knee joint sections were taken from gp130Y757F:Y757F:STAT31/−/− as control. Evidence of inflammatory infiltrate is shown with an asterisk. Scale bar, 100 μM. C, Immunohistochemical staining for CD3 and IL-17A in representative serial joint sections (day 35) from gp130Y757F:Y757F and gp130Y757F:Y757F:STAT31/−/−, were cultured in the presence of anti-CD3 and anti-CD28 stimulatory Abs (n = 3). Data show TGF-β (1 ng/ml)- and IL-6 (10 ng/ml)-mediated expansion of Th17 cells. Ab blockade of IL-2 (aIL-2) is known to augment Th17 commitment and was used in WT cultures to emphasize the degree of Th17 expansion observed by selective manipulation of gp130 signaling (WT aIL2).

In contrast, negligible IL-17A staining was observed in joint sections taken from IL-6KO mice following arthritis induction (Fig. 3C). The absence in IL-17A detection is therefore consistent with the lack of CD3 staining seen in sections from IL-6KO (Fig. 3C).

Arthritis progression is associated with enhanced gp130-mediated STAT3 control of the inflammatory infiltrate

The data presented to date collectively infer that IL-6 may influence the recruitment and activation status of the inflammatory infiltrate in the progression of AIA. To genetically dissect whether the T cell-associated STAT3 activity illustrated in Fig. 3 is linked to arthritis severity, AIA was triggered in a series of gp130 knockin strains, gp130Y757F:Y757F and gp130Y757F:Y757F:STAT31/−/−, were cultured in the presence of anti-CD3 and anti-CD28 stimulatory Abs (n = 3). Data show TGF-β (1 ng/ml)- and IL-6 (10 ng/ml)-mediated expansion of Th17 cells. Ab blockade of IL-2 (aIL-2) is known to augment Th17 commitment and was used in WT cultures to emphasize the degree of Th17 expansion observed by selective manipulation of gp130 signaling (WT aIL2).

STAT3 signaling via gp130 promotes T cell commitment and synovial infiltration

IL-6 activation of STAT3 has been widely acknowledged as being a transcriptional regulator of the TGF-β-mediated expansion of murine IL-17-secreting T cells (Th17 cells) (7, 8). By monitoring IL-17A expression in joint sections from each of the gp130 knockin strains following a single round of AIA, it was evident that the proportion of IL-17-secreting T cells was more pronounced in gp130Y757F:Y757F:Stat31/−/− mice as a consequence of enhanced gp130-mediated STAT3 signaling (Fig. 4C). This enhanced accumulation of IL-17-secreting T cells in the inflamed joints of gp130Y757F:Y757F:Stat31/−/− mice closely reflected the increased Th17 expansion observed in splenocyte cultures from gp130Y757F:Y757F:Stat31/−/− mice (WT vs gp130Y757F:Y757F:Stat31/−/− mice; p < 0.05) that had been made under IL-6/TGF-β-polarizing conditions (Fig. 4D). Collectively, these data infer that IL-6 not only coordinates T cell differentiation events, but also directs their trafficking and retention within the inflamed joint.

IL-6 trans signaling directs arthritis progression

Structural cells of the joint have previously been shown to lack cognate IL-6R expression, and consequently may only respond to IL-6 via its soluble receptor (27, 30). To evaluate the potential clinical significance of IL-6 trans signaling, synovial concentrations of IL-6, sIL-6R, and the trans signaling antagonist sgp130
FIGURE 5. IL-6 trans signaling promotes arthritis onset. A, ELISA quantification of IL-6, sIL-6R, and sgp130 in synovial fluids from patients with RA (n = 24–26) or OA (n = 14–16). Mean values for each patient cohort are indicated with a thick line (***, p < 0.005). B, T cell IL-6R expression was examined by flow cytometry in peripheral blood (PB) and synovial fluids (SF) from seven RA patients with active arthritic flares (*, p < 0.05). C, Activated T cells lose IL-6R expression. Splenocytes were cultured for 4 days with stimulatory anti-CD3 and anti-CD28 Abs, in the presence of IL-6 and TGF-β, as indicated. Representative flow cytometry plots from a total of three independent experiments are shown highlighting changes in mean fluorescence intensity for IL-6R and the isotype control. D, Early intervention of CIA with sgp130Fc. Mice (n = 6 per group) were treated with either sgp130Fc (0.1, 0.5, or 2.5 mg/kg) or vehicle control (PBS). Each group received seven doses administered (i.p.) on alternate days commencing 21 days after the first immunization with collagen II (*, p < 0.05; **, p < 0.01; ***, p < 0.005). E, Representative H&E-stained midsagittal sections (scale bar, 500 μM) taken from the hind paws of mice treated with vehicle control (PBS) and 2.5 mg/kg sgp130Fc at experimental end point (day 35). F, Histological scores for mice treated with 2.5 mg/kg sgp130Fc (n = 12 joints per group) (*, p < 0.05; ***, p < 0.005).
were recorded in synovial fluids from patients with RA and osteoarthritis (OA). When compared with effusions from OA patients, IL-6 and sIL-6R levels were found to be significantly elevated in synovial fluids from RA patients during active disease (Fig. 5A). In contrast, sgp130 concentrations were remarkably comparable in both patient groups, suggesting that this natural antagonist of IL-6 trans signaling does not appear to be regulated as a consequence of inflammatory disease, and that its levels may be insufficient to counteract the raised IL-6 and sIL-6R levels recorded in individuals with active RA (Fig. 5A). Although IL-6 trans signaling has primarily been associated with the activation of stromal tissue cells (1), T cell from synovial effusions taken from individuals with active arthritic flares showed that the proportion of CD4+ IL-6R+ T cells is markedly lower than those identified in peripheral blood (Fig. 5B). In this respect, we noted that activation of human CD4+ T cells with stimulatory anti-CD3 and anti-CD28 Abs triggered a rapid loss of membrane-bound IL-6R (Fig. 5C). Consequently, these activation-induced processes may considerably influence the regulation of IL-6 responses in RA, and suggests that classical IL-6R signaling may be less prominent than that of IL-6 trans signaling within the inflamed joint.

To directly examine the involvement of IL-6 trans signaling in arthritis progression, we tested the efficacy and potency of a sgp130Fc fusion protein delivered systemically in a collagen-induced model of arthritis (Fig. 5). This model provides a distinct advantage over AIA because it affects multiple joints and more closely resembles the systemic situation seen in human disease. Induction of CIA led to increased clinical scores, which included histological signs of joint destruction and leukocyte infiltration, including the presence of IL-17A-secreting CD3+ T cells (data not shown). Early intervention experiments were initially performed to assess the impact of selective IL-6 trans signaling antagonism on arthritis development. The sgp130Fc was administered (i.p.) every second day before the onset of CIA, and disease severity was monitored by a daily inspection of visible joint inflammation. As shown in Fig. 5D, arthritis severity was substantially impaired by sgp130Fc intervention with the optimal 2.5 mg/kg dose significantly reducing arthritis incidence. Histological assessment of ankle joint sections showed that systemic delivery of sgp130Fc (2.5 mg/kg) reduced synovial hyperplasia, inflammatory infiltrate/exudate, and joint erosion, and was reflected by an overall improvement in arthritis index (p < 0.005; Fig. 5, E and F).

Based on these outcomes, we next tested whether sgp130Fc intervention could arrest progression of established disease (Fig. 6). CIA was triggered, and on day 27, when 100% of mice had active disease, animals were treated with a daily administration (i.p.) of either 2.5 mg/kg sgp130Fc (mean clinical score at commencement of treatment: 4.28 ± 0.6) or vehicle control (PBS) (mean clinical score at commencement of treatment: 4.47 ± 0.86). All mice treated with vehicle (PBS) alone showed worsening histological signs of disease (see Fig. 6B). Conversely, administration of sgp130Fc arrested disease development, and animals receiving sgp130Fc showed no advancement in arthritis above that observed at the start of treatment. In this respect, the sgp130Fc regime led to a significant improvement in arthritis index (p < 0.05), and markedly reduced the degree of synovial infiltration observed histologically (p < 0.05; Fig. 6). Further evaluation of joint and cartilage erosion showed that sgp130Fc protected the joint from additional damage, and prevented disease progression (Fig. 6B).

Discussion

A newfound interest in IL-6 signaling has recently emerged due to the ability of this inflammatory cytokine to modulate TGF-β polarization of murine IL-17-secreting Th cells and inducible FoxP3 regulatory T cells (3–8). However, IL-6 activity has also been linked with a capacity to direct chemokine-mediated T cell recruitment, and can prevent their apoptotic clearance from sites of inflammation (21, 26). In the models adopted in this study, arthritis severity strongly correlated with an increased accumulation of CD3+ inflammatory cells within the inflamed joint. Specifically, IL-6 acting through its soluble receptor (IL-6 trans signaling) was shown to promote synovial hyperplasia, and fundamentally affected neutrophil and lymphocyte trafficking (Fig. 7). These processes appear to require STAT3 with enhanced pY-STAT3 staining within the synovium predominantly associated with the CD3+ infiltrate. The enhanced pY-STAT3 staining displayed within these pockets of inflammatory T cells also colocalized with IL-17A expression. IL-17 activity has been linked with increased joint erosion, metalloprotease expression, and receptor activator of NF-kB ligand (RANKL)-induced osteoclastogenesis, with T cells from IL-6KO displaying altered production of IL-17, RANKL, and the RANKL decoy receptor osteoprotegerin (36). However, it is unclear whether the reduced joint destruction observed in IL-6KO mice during AIA is attributable to an overall reduction in joint...
mokine receptors CCR2 and CCR6 (40–42). Significantly, Ab blockade of CCL20 (chemokine ligand for CCR6) has been shown to reduce arthritis severity and prevent CCR6+ T cell trafficking to the joint, whereas a lack of arthritis pathology in IL-6KO mice following AIA is associated with the impaired synovial infiltration of CCR2+ mononuclear cells (27, 41). Collectively, these data suggest that IL-6 regulates the chemokine-directed trafficking of T cells, including Th17 cells.

The principal protagonist underpinning these IL-6-driven responses is the latent transcription factor STAT3. In models of chronic disease, STAT3 signaling exacerbates pathology and in many instances correlates with lesion severity (43). By tracking development of AIA in gp130 knockin strains, studies outlined in this work show that heightened gp130-mediated STAT3 signaling exaggerates the inflammatory process and leads to a worsening of disease. Similarly, genetic overexpression of the STAT-inducible inhibitor of STAT3 signaling suppressor of cytokine signaling 3 in experimental models of disease has shown that negative regulation of STAT3 activity leads to substantive improvements in inflammatory conditions, including arthritis (44–46). Consequently, blockade of gp130-mediated STAT3 signaling might represent the main target of IL-6-directed intervention therapies.

Based on the dual regulation of IL-6 responses by classical IL-6R signaling and IL-6 trans signaling (Fig. 7B), it is conceivable that leukocyte trafficking and activation may be independently governed by these two signaling mechanisms. Based on our current understanding of IL-6 trans signaling in vivo, it is evident that this mode of IL-6 signaling orchestrates chemokine-mediated leukocyte trafficking and T cell-homing events steered by adhesion molecules (1, 24–27, 47). Generation of sIL-6R may therefore represent a rate-limiting event in the regulation of these processes. In the context of inflammatory arthritis, increased synovial levels of sIL-6R correlate with both enhanced joint destruction and leukocyte recruitment, and is typically associated with more advanced forms of human disease (30–32). Structural cells of the joint typically lack IL-6R expression, and cells including synoviocytes may only respond to IL-6 through trans signaling (27, 30). More recent advances, however, suggest that IL-6 trans signaling may directly affect T cell activation and apoptotic clearance (21, 47–50). In this respect, T cell IL-6R expression is closely associated with surface markers typically associated with naive or central memory T cell subsets, suggesting that effector and effector memory T cells at sites of inflammation lack IL-6R expression (47, 51, 52).

In this study, we noted that pan T cell activation led to a rapid loss in IL-6R expression, whereas the proportion of T cells expressing IL-6R in synovial effusions of RA patients was significantly reduced as compared with corresponding T cells from their whole blood.

Although the biological significance of IL-6 trans signaling was originally considered an in vitro oddity of IL-6 biology, this mechanism of cellular activation may now represent the predominant mode for IL-6 signaling in vivo (1, 2). Roles for IL-6 trans signaling have been linked with the control of infection, inflammation, vascular tone, and immunological responses associated with tumor progression (18, 21, 22, 27, 47–51, 53). The assignment of these activities to IL-6 trans signaling has been best illustrated in vivo when sIL-6R-mediated signaling via membrane-bound gp130 has been selectively antagonized with sgp130. In this respect, we have noted that localized delivery of sgp130 (i.a.) counteracts development of AIA (data not shown; see also Refs. 27 and 29). High levels of sgp130 have been identified in circulation; however, synovial sgp130 levels are not elevated in RA patients, suggesting inflammation or a local reduction in key disease-modifying cytokines produced by effector T cells, including IL-17 (39).

A prominent role for gp130-mediated STAT3 signaling is emphasized by the increased Th17 commitment observed in splenic T cell cultures from gp130(−/−) mice that have been maintained under appropriate polarizing conditions. Regulation of Th17 expansion may have a considerable bearing on disease progression because the increased arthritis severity observed within these mice during AIA is related to retention of inflammatory cells within the joint, and a specific accumulation of IL-17-secreting T cells. IL-17A was not, however, expressed by all synovial CD3+ T cells. In this regard, synovial tissue from AIA joints of IL-6KO and gp130(−/−);Stat3+/− mice showed little evidence of an inflammatory T cell infiltrate. Consequently, IL-6/gp130 signaling does not appear to selectively govern Th17 trafficking. Previously, we have shown in acute peritoneal inflammation that gp130-mediated STAT3 signaling promotes T cell migration and that IL-6 deficiency is associated with impaired T cell chemokine (CCL4, CCL5, CCL11, CCL17, CXCL10) and chemokine receptor (CCR3, CCR4, CCR5, CXCR3) expression (25). Recent reports have predicted that IL-6 may also regulate the Th17 signature chemokine receptor expression (26). Importantly, the inflammatory infiltrate outlined in A and retention of neutrophils and effector T cells, including IL-17A-secretory T cells, suggests that effector T cell recruitment and retention of neutrophils and effector T cells, including IL-17A-secretory T cells, is typically lack IL-6R expression, and cells including synoviocytes may only respond to IL-6 through trans signaling (27, 30). More recent advances, however, suggest that IL-6 trans signaling may directly affect T cell activation and apoptotic clearance (21, 47–50). In this respect, T cell IL-6R expression is closely associated with surface markers typically associated with naive or central memory T cell subsets, suggesting that effector and effector memory T cells at sites of inflammation lack IL-6R expression (47, 51, 52). In this study, we noted that pan T cell activation led to a rapid loss in IL-6R expression, whereas the proportion of T cells expressing IL-6R in synovial effusions of RA patients was significantly reduced as compared with corresponding T cells from their whole blood.

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that disease-associated increases in IL-6 and sIL-6R concentrations may be inadequately governed by this natural cytokine antagonist (29).

Early clinical intervention remains the most effective therapeutic strategy for suppressing inflammatory arthritis (11). In line with this clinical appraisal, systemic administration of mice with sgp130Fc during the onset of CIA led to a substantial improvement in disease activity, whereas treatment of established disease prevented worsening of the condition. Consequently, the involvement of IL-6 in arthritis progression is closely related to the presence of its soluble receptor. This represents a major translational advance, and builds on our emerging knowledge of IL-6 signaling, and the clinical benefit shown by the neutralizing IL-6R mAb Tocilizumab as a treatment for Crohn’s disease and inflammatory arthritis (12–17). The question remains whether targeted inhibition of IL-6 trans signaling is more effective than the global blockade of IL-6 signaling provided by an anti-IL-6R-based strategy. In this respect, it is important to note that IL-6 trans signaling and classical IL-6R signaling direct specific aspects of T cell effector characteristics and homeostatic control of vascular function, suggesting that regulation of both modes of IL-6 activation may be mutually distinct (25, 49, 53). This highlights the sophisticated nature of IL-6 regulation, and emphasizes the necessity to fully define the biological relationship between classical IL-6R signaling and IL-6 trans signaling. In this regard, the application of sgp130Fc illustrates that IL-6 trans signaling is the prevailing mode of IL-6 signaling in arthritis pathology. Consequently, selective antagonism of IL-6 trans signaling by sgp130Fc represents a promising alternative IL-6 intervention strategy.

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Disclosures

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References


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