Defective gp130-mediated Signal Transducer and Activator of Transcription (STAT) Signaling Results in Degenerative Joint Disease, Gastrointestinal Ulceration, and Failure of Uterine Implantation

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Abstract

The receptor subunit gp130 transduces multiple cell type–specific activities of the leukemia inhibitory factor (LIF)/interleukin (IL)-6 family of cytokines through the signal transducer and activator of transcription (STAT) and src homology 2 domain–bearing protein tyrosine phosphatase (SHP)-2/ras/Erk pathways. To define STAT-dependent physiological responses, we generated mice with a COOH-terminal gp130ΔSTAT “knock-in” mutation which deleted all STAT-binding sites. gp130ΔSTAT mice phenocopied mice deficient for IL-6 (impaired humoral and mucosal immune and hepatic acute phase responses) and LIF (failure of blastocyst implantation). However, unlike mice with null mutations in any of the components in the gp130 signaling pathway, gp130ΔSTAT mice also displayed gastrointestinal ulceration and a severe joint disease with features of chronic synovitis, cartilaginous metaplasia, and degradation of the articular cartilage. Mitogenic hyperresponsiveness of synovial cells to the LIF/IL-6 family of cytokines was caused by sustained gp130-mediated SHP-2/ras/Erk activation due to impaired STAT-mediated induction of suppressor of cytokine signaling (SOCS) proteins which normally limits gp130 signaling. Therefore, the joint pathology in gp130ΔSTAT mice is likely to arise from the disturbance of the otherwise balanced activation of the SHP-2/ras/Erk and STAT signaling cascades emanating from gp130.

Key words: infertility • signal transduction • STAT • interleukin • gene targeting

Introduction

The activities of a family of cytokines that comprises IL-6, IL-11, leukemia inhibitory factor (LIF),* oncostatin M (OsM), ciliary neurotrophic factor (CNTF), and cardiotrophin-1 are mediated by the common signal transducing receptor β chain gp130 (1, 2). LIF/IL-6 cytokines exert multiple activities on target organs such as liver, heart, immune, hematopoietic, and nervous systems that are pivotal during embryogenesis, morphogenesis, and inflammation. Aberrant expression of LIF/IL-6 family cytokines has been associated with autoimmune disease, septic shock, and neoplasia (2, 3). Somewhat surprisingly, mice with engineered null mutations for individual LIF/IL-6 family ligands or receptor α subunits develop normally and exhibit rather mild phenotypes. IL-6–deficient (IL-6−/−) mice, for instance, showed impaired acute phase protein production and decreased high affinity immune responses...
(4, 5), while mice deficient for LIF or the IL-11 receptor α subunit (IL-11Rα1) were unable to support pregnancies due to defective blastocyst implantation (6, 7) and decidua formation (8, 9), respectively. This finding has been attributed to partial functional redundancy between family members due to overlapping expression patterns and the sharing of common receptor β chains. Predictably, receptor β chain deficiency resulted in more severe and global effects. Depending on the genetic background, gp130<sup>−/−</sup> mice died in utero or shortly after birth due to myocardial hypoplasia and reduced hemopoiesis in the fetal liver (11, 12), while the LIF receptor β chain (LIF-Rβ)<sup>−/−</sup> mice died perinatally due to degeneration of motor neurons and astrocytes (10).

The LIF/IL-6 family of cytokines recruits gp130 according to two principal mechanisms. In the case of IL-6 and IL-11, signaling is initiated by binding to ligand-specific receptors followed by recruitment and homodimerization of two gp130 receptor β chains. In contrast, binding of LIF, OSM, CNTF, or cardiotrophin-1 induces the formation of β chain heterodimers consisting of gp130 and one of the structurally and functionally related β chains, either the LIF-Rβ or OSM receptor (1, 2). Thus, IL-6 and IL-11 are totally dependent on gp130 for signal transduction, while signals emanating from LIF, OSM, CNTF, and cardiotrophin-1 may be transduced, at least in part, by LIF-Rβ or OSM receptor. The cytoplasmic domains of all three β chains are characterized by conserved modular structures with each module thought to connect to a particular signal transduction pathway. First, the membrane-proximal homology region associates with cytoplasmic Jak kinases, which, upon receptor dimerization, phosphorylate tyrosine (Y) residues within the β chains (1). Second, a YxxV-phosphotyrosine motif (where Y is followed by any two amino acids and valine (V)) at positions Y<sub>57</sub> and Y<sub>65</sub> in mouse gp130 (13) and LIF-Rβ (14), respectively, is required for recruitment of the phosphatase src homology 2 domain-bearing protein tyrosine phosphatase (SHP)-2, subsequent activation of the Erk mitogen-activated protein kinase (MAPK) and transduction of mitogenic signals (15). Third, the membrane distal portion of the β chains, which contain several YxxQ-phosphotyrosine motifs, with a glutamine (Q) in position +3 relative to Y<sub>765/812/904/914</sub> of mouse gp130 and Y<sub>976/996/1,023</sub> of mouse LIF-Rβ, mediates binding and activation of the latent transcription factors signal transducer and activator of transcription (STAT)1 and STAT3 (16). This region, and in particular the YxxQ motifs, is necessary and sufficient to control differentiation and apoptosis of cells in vitro and to induce acute phase proteins in cultured hepatocytes (17–20). Moreover, the duration and intensity of the signals emanating from gp130 and LIF-Rβ appear to be limited by transcriptional induction of negative regulatory proteins of the SOCS family which impair Jak-mediated substrate phosphorylation (21, 22).

To identify the biological responses to LIF/IL-6 cytokines specifically mediated by STATs in vivo, we generated a gp130 “knock-in” mouse containing a COOH-terminal truncation mutation in gp130 (gp130<sub>ΔSTAT</sub>) which deleted all STAT binding sites in a manner likely to be functionally equivalent to phenylalanine substitutions (Y→F) in all YxxQ motifs (17–19).

Materials and Methods

Targeting Construct and Generation of Mice. A mouse genomic 129/Sv phage library was screened with a cDNA fragment encoding the cytosolic domain of gp130, yielding a 14-kb genomic clone that contained the final two coding exons encompassing amino acids 645–671 and 672–917 (13) and a 5.4 kb of 3′ flanking sequence. PCR-mediated mutagenesis was used to introduce the gp130<sub>ΔSTAT</sub> truncation mutation by introducing the Y<sub>765</sub>→F (TAC to TTC), Q<sub>769</sub>→A (CAG to GCC), and V<sub>776</sub>→A (GTC to TAC) substitutions which were encoded by the oligonucleotide 5′-TTCCAG.GAC.GCC.TAG.GAC-3′. The newly introduced stop codon was part of an AvrII site (underlined) and was fused to the fourth nucleotide (bold A) of the 3′ untranslated region of the endogenous g130 gene. A cDNA encoding neomycin resistance preceded by an internal ribosomal entry site (IRES) was introduced into the AvrII site as a Xbal/NheI fragment, thereby generating gp130-neo dicistronic RNA. The targeting vector also contained 0.8 kb and 4.3 kb of sequence homologous to the final intron and the 3′ flanking sequence, respectively.

From 25 × 10<sup>6</sup> W9.5 embryonic stem (ES) cells (129/Sv) electroporated with the Sall linearized targeting construct, 12/120 neomycin resistant colonies were correctly targeted as judged by Southern analysis. Allele-specific amplification with PCR primers P1 and P2 was used to confirm the nucleotide sequence of the mutated exon in the targeted allele (see Fig. I). Two recombinant ES cell lines (129/Sv) were injected into C57BL/6 blastocysts and the germline chimeras were mated with C57BL/6 females. Colonies homozygous for the mutation (gp130<sub>ΔSTAT/ΔSTAT</sub>) were established and subsequently maintained by breeding gp130<sub>ΔSTAT/ΔSTAT</sub> males with gp130<sub>ΔSTAT/ΔSTAT</sub> females, and their offspring were routinely genotyped at weaning age by PCR analysis using primers P1 and P2 of DNA prepared from tail biopsies.

Synovial Cell Assays. Primary cultures of synovial fibroblasts were established by sequential collagenase digestion (collagenase type II CLS-4, Worthington; 165 U/ml in DMEM) of clinically normal knee joints taken from gp130<sub>ΔSTAT/ΔSTAT</sub> mice and their gp130<sub>ΔSTAT/ΔSTAT</sub> littermate controls as well as compound homozygous SOCS<sup>−/−</sup>, INF-γ<sup>−/−</sup> mice (23), and SOCS<sup>−/−</sup>; INF-γ<sup>−/−</sup> (24) controls were established as described previously (25). Cell populations with the highest intensity of vascular cell adhesion molecule 1 staining, a marker for synovial fibroblasts (26), were maintained in DMEM supplemented with 20% FCS and assayed at passage 5.

The mitogenic activity of mouse LIF (ESGRO; AMRAD) or recombinant human IL-6 on synovial cells was assessed by culturing 2 × 10<sup>4</sup> cells per well in 96-well plates. After serum starvation (0.5% FCS) for 24 h, quadruplicate cultures were stimulated for 24 h with the indicated concentration of LIF or IL-6 and pulsed for the last 4 h with 0.5 µCi per well methyl-[<sup>3</sup>H]thymidine (Du Pont NEN) before harvesting and measuring incorporated radioactivity.

For transient transfection assays, 5 × 10<sup>6</sup> cells were electroporated (500 µF; 270V) with 20 µg of a luciferase reporter construct containing the MAPK responsive region (−404 to
+41) of the human c-fos promoter (27), 20 μg of the Sreα-β-galatosidase plasmid and increasing concentrations of a Flag epitope-tagged SOCS-1 expression construct (pSOCS-1; reference 28). Cells in 24-multiwell plates were maintained in 0.5% FCS for 48 h in the presence of IL-6 plus sIL-6R (500 ng/ml each), saline, or FCS (10%) before lysis with 40 μl of reporter lysis buffer (Promega). Lysates were assayed for luciferase activity which was normalized according to the β-galactosidase activity. Expression levels from the transfected SOCS-1 plasmid were monitored by anti-Flag immunoblotting (28).

Immunoblotting and In Vitro Phosphorylation Assay. Serum-starved cultures of synovial fibroblasts were stimulated with IL-6 plus sIL-6R (both at 500 ng/ml) for the indicated period of time. 1 mg of cell lysate was immunoprecipitated with an antiserum against Jak-2 (UBI) and used for in vitro phosphorylation experiments in the presence of γ-[32P]ATP as described previously (19). SHP-2 immunoprecipitates were resolved by SDS-PAGE and blots were probed with antiphosphotyrosine (UBI) and SHP-2 (Santa Cruz Biotechnology, Inc.) antibodies to assess protein loading (19). For detection of active Erk MAPK, 50 μg of total cell lysate were resolved by SDS-PAGE and transferred proteins were reacted with an antibody that recognizes phosphorylated Erk1/2 (New England Biolabs, Inc.). The level of Erk protein was subsequently visualized by reaction with anti-Erk1/2 antibodies (Santa Cruz Biotechnology, Inc.). STAT3 phosphorylation in tissues was monitored after STAT3 immunoprecipitation (Santa Cruz Biotechnology, Inc.) of 2 mg of tissue lysate obtained from mice 20 min after a single intravenous injection of either 5 μg IL-6, LIF, IL-11, or saline and detection with antiphosphotyrosine and anti-STAT3 (Transduction Laboratories) antibodies, respectively (19).

Northern Blot Analysis. Poly(A)+ RNA was prepared from livers of mice 40 min (for SOCS induction) or 4 h (for acute phase protein) after a single intravenous injection of 5 μg of LIF, IL-6, or saline according to standard protocols. Poly(A)+ RNA was also isolated from cultures of primary synovial cells stimulated with IL-6 plus sIL-6R (both at 500 ng/ml) for 45 and 180 min, respectively. 3 μg of poly(A)+ RNA were hybridized with cDNAs encoding either cytokine-inducible src homology 2 protein (CIS), SOCS-1, SOCS-2, or SOCS-3, gp130 or neomycin, and serum amyloid A or haptoglobin. All RNA blots were finally hybridized for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to assess the amounts of RNA analyzed.

Clinical Assessment and Histology. For each limb, clinical scores were allocated for erythema, swelling, and flexibility of joints twice per week for up to 25 wk as described previously (29). The scoring system for each limb was as follows: 0, normal; 1, moderately affected; and 2, severely affected. Soft tissues were fixed in formalin for 24 h, while joints were fixed in 10% formalin for 48 h, decalcified, and embedded in paraffin (29). Sections were stained with hematoxylin and eosin. Histopathological features associated with the large appendicular joints (shoulder, elbow, wrist, hip, knee, ankle) were scored as 0, normal; 1, minor synovial changes without degenerative alterations or cartilage metaplasia; 2, mild degenerative joint disease with cartilage deposits with or without synovial hyperplasia; and 3, severe degenerative joint disease with marked cartilage metaplasia with or

Figure 1. Generation of gp130STAT−/−STAT mice. (A) Targeting strategy for the introduction of the gp130STAT truncation. The cytoplasmic domain of mouse gp130 with its homology motifs Box1, Box2, and Box3 (black) are schematically depicted alongside the corresponding genomic structure with exons numbered according to Betz et al. (reference 31). The targeting vector contains a Y765RHQ768 to FRHA substitution and a translational stop codon at position 769 preceding a ribosomal reentry site and the sequence encoding neomycin resistance (IRES-neo). A diagnostic digest with BglII (Bg) yields a 3.8 kb fragment from the wt allele and a 5.6-kb fragment from the targeted allele when hybridized with a probe external to the targeting vector (>). P1 and P2 refer to primers used for an allele-specific PCR reaction. S, SalI. (B) Southern blot analysis of BglII digests of DNA prepared from a targeted ES cell line and tail biopsies obtained from offspring of gp130 STAT-neo ES cell line and tail biopsies obtained from offspring of gp130 STAT/wt intercrosses. The immobilized DNA was hybridized with a probe (STAT) to assess the amounts of RNA analyzed.
without synovial hyperplasia. All clinical and histological assessments were performed blind.

Immunization, ELISA, and Immunohistochemistry. Mice were immunized by injecting ~1 μl of OVA, (1 mg/ml in PBS, pH 7.4, emulsified with Freund’s Complete Adjuvant) into each Peyer’s patch (30). Mice were boosted intraduodenally 14 d later with OVA and euthanized 5 d after the secondary challenge. The proteinacious fraction of fecal pellets, obtained from intestinal washes, was prepared as described previously (30) and the small intestine was collected, fixed in cold ethanol, embedded in paraffin, and sectioned (5 μm) for immunohistochemistry of OVA-containing cells (5). OVA-specific ELISA assays were carried out for IgA, IgG, and IgM using Ig-specific biotin conjugated goat anti–mouse antibodies (Zymed Laboratories). Total isotype-specific Ig levels in sera of unchallenged mice were determined by ELISA using commercially available kits according to the manufacturer’s recommendations (Bethyl Laboratories, Inc.).

Embryo Cross-Fostering. Blastocysts were flushed from uteri on day 4 of gestation (day 1 is day of plug) and transferred to day 3 pseudopregnant recipients as described previously (6). All offspring were genotyped by Southern blotting of DNA from tail biopsies.

Results

To inactivate the four YxxQ STAT-binding sites in gp130 at positions Y765/812/904/914, we replaced exon 18, encoding amino acids 672–917, with a version truncated at the COOH-terminal side of amino acid 768 and containing the Y765F and Q766A substitutions. After homologous recombination in ES cells, the presence of the expected mutations was confirmed by sequencing allele-specific PCR amplicons generated from the targeted gp130 allele with primers P1 and P2 (Fig. 1 A). Transcription of the mutated gp130 allele generated a dicistronic gp130-neo mRNA species ~1.4 kb larger than wild-type (wt) gp130 mRNA which encoded a truncated gp130ΔSTAT protein (Fig. 1 C). Interbreeding of gp130ΔSTAT/wt mice yielded homozygous offspring close to the expected Mendelian ratio (Fig. 1 B and Table I). However, subsequent matings of gp130ΔSTAT/wt females with gp130ΔSTAT/ΔSTAT males yielded significantly fewer gp130ΔSTAT/ΔSTAT than gp130ΔSTAT/wt offspring at weaning age (Table I), although mice of the two genotypes were recovered at equal ratios during embryonic development (E18, and data not shown). At birth, gp130ΔSTAT/ΔSTAT mice appeared superficially healthy, but in comparison to gp130wt/wt and gp130ΔSTAT/wt littermates, they showed a 20–30% reduction in body weight (1.93 g ± 0.38 g vs. 2.93 g ± 0.12 g; P < 0.05, n = 20) and trunk length, which persisted throughout adulthood. The average life span of gp130ΔSTAT/ΔSTAT mice was reduced (6 ± 1 mo for males, 8 ± 2 mo for females) compared with gp130wt/wt littermates (15 ± 2 mo for both sexes) which were indistinguishable from gp130wt/wt mice in all aspects analyzed.

Table I. Reproductive Frequency in gp130 Mutant Mice

| Male   | Female | Live pups |
|--------|--------|-----------|
| wt/Δ (17) | wt/Δ (17) | wt/wt (66) |
|        |        | wt/Δ (156) |
|        |        | Δ/Δ (57)   |
| Δ/Δ (47) | wt/Δ (47) | wt/Δ (328)b |
|        |        | Δ/Δ (179)c |
| wt/Δ (15) | Δ/Δ (6) | Δ/Δ (0) |
| Δ/Δ (8)  | Δ/Δ (3) | Δ/Δ (0) |

| Male | Female | Numbers of blastocysts transferred | Foster mother | Live pups |
|------|--------|-----------------------------------|--------------|----------|
| wt/Δ | Δ/Δ (2) | 28 | wt/wt (2) | wt/Δ (4) |
| wt/Δ | wt/wt (5) | 28 | Δ/Δ (3) | wt/wt (0) |
| wt/Δ | wt/wt (5) | 29 | wt/wt (3) | wt/Δ (3) |

*a Test breeding of gp130 mice of the indicated genotypes. All parents and offspring were genotyped by PCR analysis of DNA from tail biopsies. Each gp130ΔSTAT/ΔSTAT female was test mated at least twice with a male of proven fertility. Number of animals is indicated in parentheses.

*b 67 pups in total were cannibalized by their mothers before genotyping.

*c Transfer of blastocysts into pseudopregnant recipient mice. Blastocysts were flushed from the uteri on day 4 of gestation and transferred to day 3 pseudopregnant recipients. Numbers of animals in indicated in parentheses. Δ/Δ, Δ/Δ, and wt/wt refers to gp130ΔSTAT/ΔSTAT, gp130ΔSTAT/wt, and gp130wt/wt mice, respectively.

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Failure of Uterine Implantation and Hepatic Acute Phase Response. While gp130ΔSTAT/ΔSTAT male mice sired litters of equal size to those of gp130ΔSTAT/wt or wt males, female gp130ΔSTAT/ΔSTAT mice mated with males of any genotype showed no external signs of pregnancy (Table I). Histological analysis of uteri from gp130ΔSTAT/ΔSTAT mice 5.5 d after coitum revealed the presence of blastocysts that showed no overt signs of implantation at a time when embryos in uteri of gp130ΔSTAT/wt and gp130wt/wt mice had implanted and were surrounded by the secondary decidua (Fig. 2 A). However, blastocysts of all genotypes recovered from gp130ΔSTAT/ΔSTAT mice developed to term when surgically transferred to day 3 pseudopregnant wt recipient mice, while again no signs of pregnancy were observed when transferred to gp130ΔSTAT/ΔSTAT recipient mice (Table I). These results unequivocally show a maternal defect at the time of embryo implantation that is strikingly similar to the defect described in mice deficient for LIF (6, 7).

To determine whether impaired STAT signaling in the uteri of gp130ΔSTAT/ΔSTAT mice was the likely reason for the failure of blastocyst implantation and subsequent decidua formation, we monitored STAT3 phosphorylation in the liver and uterus after single bolus injections of IL-6, IL-11, or LIF. In both tissues, STAT3 phosphorylation in gp130ΔSTAT/ΔSTAT mice was markedly reduced in response to LIF and completely abrogated in response to IL-6 or IL-11, compared with identically treated heterozygous control mice (Fig. 2, B and D). This observation was further pursued in liver by measuring the induction of hepatic class I acute phase genes which are regarded as classical STAT-mediated responses (20). Induction of serum amyloid A and haptoglobin mRNAs in gp130ΔSTAT/ΔSTAT mice was
clearly diminished in response to LIF and absent in response to IL-6 (Fig. 2 C). Collectively, these results suggest that the mutant receptor β chain heterodimers between gp130ΔSTAT and LIF-Rβ (which retains the 3 YxxQ motifs in LIF-Rβ) induced by LIF, while less efficient than a wt gp130/LIF-Rβ complex containing 7 YxxQ motifs, retains some STAT-mediated signaling capacity. Meanwhile homodimers formed between two truncated gp130ΔSTAT proteins in response to IL-6 and IL-11 failed to promote STAT-mediated signaling.

Gastrointestinal Ulceration and Impaired Humoral and Mucosal Immunity. Under specific pathogen–free conditions, approximately half of all gp130ΔSTAT/ΔSTAT mice of 4 mo of age showed occult traces of fecal blood and ulceration of the gastric pylorus (Fig. 3 a). We also observed ulceration of the anorectal region which was invariably associated with rectal prolapse. Notably, the ulceration at both sites was confined to the vicinity of the sphincters suggesting defective epithelial repair in response to sustained mucosal injury. Mutant mice kept under conventional conditions also developed caecal ulcers (41%; Fig. 3 b) and acute inflammation at additional mucosal sites including the conjunctivae (41%) and the nasolacrimal ducts (29%). Furthermore, in comparison to gp130ΔSTAT/wt mice, gp130ΔSTAT/ΔSTAT mice showed a much higher load of Giardia muris which is an endemic parasite in the conventional facility in which the mice were housed.

Figure 3. Gastrointestinal ulceration and impaired immunity in gp130ΔSTAT/ΔSTAT mice. Mucosal ulcers (arrowheads) in the gastric pylorus (a) and caecum (b). High magnification (insert) shows surface ulcer debris, neutrophil-rich inflammatory exudates, and regenerative changes in adjacent epithelial cells. S, stomach; D, duodenum. (c and d) Appearance of OVA-containing plasma cells in the lamina propria of small intestinal villi in gp130ΔSTAT/wt control (c) and gp130ΔSTAT/ΔSTAT mutant (d) mice detected by FITC-mediated immunofluorescence of anti-OVA antibodies. (e) Isotype-specific anti-OVA antibody response in extracts of fecal pellets and serum of gp130ΔSTAT/ΔSTAT mutant and gp130ΔSTAT/wt control mice. Mice were immunized by injection of OVA directly into Peyer’s patches and boosted with OVA 14 d later. OVA-specific Ig levels were determined after a further 5 d as described in Materials and Methods. Mean ± SEM, n = 7. (f) Number of OVA-specific plasma cells in the small intestine, spleen, and mesenteric lymph nodes (MLN) of mice used for the experiment in Fig. 2 E. Data were obtained by immunofluorescent histology with FITC-conjugated goat–anti mouse heavy chain reagent and represent the mean ± SEM in 30–50 randomly selected high power fields per mouse with four mice per group.
Since IL-6 is required for differentiation of B cells to high affinity IgA- and IgG-producing plasma cells (4, 5, 31), we sought to determine mucosal immune responses of gp130STAT/STAT mice. In the absence of deliberate immunization and compared with heterozygous controls, mutant mice showed reduced levels of circulating IgA (gp130STAT/STAT, 0.62 ± 0.05 mg/ml, n = 7 vs. gp130STAT/wt mice, 0.85 ± 0.10 mg/ml, n = 6), IgG1 (0.17 ± 0.07 mg/ml vs. 0.35 ± 0.11 mg/ml), IgG2a (0.18 ± 0.13 mg/ml vs. 0.53 ± 0.12 mg/ml), and IgG2b (0.38 ± 0.15 mg/ml vs. 0.87 ± 0.17 mg/ml). In contrast, the levels of circulating IgM were similar for mice of both genotypes. The capacity of gp130STAT/STAT mice to mount a specific intestinal IgA response after local immunization of Peyer’s patches with OVA was grossly impaired when assessed by OVA-specific Ig isotype level in fecal pellets and serum (Fig. 3 e). Similarly, the OVA-specific IgG response in the intestine was also reduced and there were significantly less anti-OVA-containing cells in the small intestine (Fig. 3 c and d), mesenteric lymph nodes, and spleen of gp130STAT/STAT mice (Fig. 3 f) reminiscent of the findings reported previously in IL-6–deficient mice (30). Collectively, these data imply that gp130 transduces the IL-6–mediated final maturation of B cells to high affinity Ig-secreting plasma cells via its STAT-binding sites in the COOH-terminal domain.

Severe Degenerative Joint Disease in gp130STAT/STAT Mice. Unexpectedly, swelling of one or more of the large weight-bearing joints was observed in 60% of adult gp130STAT/STAT mice (n = 49, mean age 89 d ranging from 56–412 d), which resulted in flexion deformities of the hind limbs and reluctance of the mice to stand (Fig. 4, a and b). Joints of gp130STAT/STAT mice were often affected bilaterally but only rarely showed signs of erythema. Systematic clinical assessment showed that all gp130STAT/STAT mice showed stiffness of one or more limbs with restricted flexion and extension of the ankles as well as dorsiflexion at the wrist joints (Fig. 4 b to e). Notably, some degree of joint restriction was already evident in one or more limbs of P7–neonatal gp130STAT/STAT mice but did not increase significantly with age and only rarely affected previously healthy joints (Table II). By contrast, none of the gp130STAT/wt control mice showed any clinical evidence
of swelling or restricted joint movement at any age. Abnormal joints were also observed in gp130ΔSTAT/ΔSTAT mice maintained under specific pathogen–free conditions, indicating that this pathology was unlikely to be secondary to infections at mucosal sites.

The joints of 49 clinically affected mice were examined histologically and graded as described in Materials and Methods. Of the 292 large appendicular joints examined (16 hips, 75 knees, 67 ankles, 40 shoulders, 50 elbows, 44 wrists), 76% were scored as 0 or 1 while 24% were graded as 2 or 3. Curiously, joints showing degenerative changes (grade 2 or 3) were twice as common in male (27.8%, n = 65) than female (15.1%, n = 8) gp130ΔSTAT/ΔSTAT mice. Histologically, these joints showed cartilaginous nodules along the joint capsule extending from the edge of the articular cartilage. In the knee joints, these nodules invariably replaced the menisci and supporting connective tissue (Fig. 5, a and b) and occasionally the joint spaces contained dislodged and rounded cartilaginous nodules (Fig. 5 c). These changes were often accompanied by enlargement and deformation of the joints which occasionally affected the growth plate (Fig. 5 b). In addition, joints of older gp130ΔSTAT/ΔSTAT mice often showed erosion of the articular surface and mild synovial hyperplasia associated with the tendon sheaths and nonswollen joints. Since histological examination of clinically affected joints of newborns revealed considerable structural variability, we were unable to unambiguously ascribe histological abnormalities to these joints. However, no primary irregularities were detected in the growth plate of young adult gp130ΔSTAT/ΔSTAT mice. Occasionally, marked synovial hyperplasia was observed in the recesses of the joint cavities with expansion of the subsynovial connective tissue adjacent to the articular surfaces (Fig. 5, c and d). However, foci of metaplastic cartilage were rarely observed within this de novo–formed connective tissue suggesting that the cartilaginous nodules were composed of newly formed cartilage and not disordered menisci or articular cartilage. Inflammatory cells, primarily lymphocytes and macrophages, were present in low numbers in the synovium and subsynovial tissues of pathological joints; however, neutrophils were usually absent. Collectively, these observations suggest that the joint pathology in gp130ΔSTAT/ΔSTAT mice was primarily a result of synovial hyperplasia with progressive chronic inflammation and secondary cartilaginous metaplasia restricting joint movement and culminating in the degeneration of the articular cartilage of the major weight-bearing joints in older mice (Fig. 5, f and g).

**Enhanced Mitogenic Response of Synovial Fibroblasts to IL-6.** Since the LIF/IL-6 family cytokines have been implicated in promoting synovial hyperplasia (32, 33), we investigated whether altered signaling through gp130ΔSTAT receptors affected the mitogenic response of mutant synovial cells.

### Table II. Clinical Assessment of Joint Stiffness in gp130ΔSTAT/ΔSTAT Mice

| Age (d) | 30 | 50 | 71 | 98 | 141 | 176 |
|---------|----|----|----|----|-----|-----|
| **Limb** |    |    |    |    |     |     |
| Mouse 1 |    |    |    |    |     |     |
| Rear left | 1  | 1  | 1  | 1  | 2   | 2   |
| Rear right | 1  | 1  | 1  | 1  | 1   | 1   |
| Front left | 0  | 0  | 0  | 0  | 0   | 0   |
| Front right | 2  | 2  | 2  | 2  | 2   | 2   |
| Mouse 2 |    |    |    |    |     |     |
| Rear left | 1  | 1  | 1  | 1  | 2   | 2   |
| Rear right | 1  | 1  | 1  | 1  | 1   | 1   |
| Front left | 0  | 0  | 0  | 1  | 1   | 1   |
| Front right | 0  | 0  | 0  | 1  | 1   | 1   |
| Mouse 3 |    |    |    |    |     |     |
| Rear left | 1  | 1  | 0  | 1  | 1   | 2   |
| Rear right | 1  | 1  | 1  | 2  | 1   | 1   |
| Front left | 0  | 0  | 0  | 0  | 0   | 0   |
| Front right | 0  | 0  | 0  | 0  | 0   | 0   |

Average score per gp130ΔSTAT/ΔSTAT mouse (n = 14) | 3.3 ± 1.2 | 2.9 ± 1.7 | 3.0 ± 1.2 | 3.3 ± 1.2 | 3.5 ± 1.1 | 3.5 ± 1.1 |

Average score per gp130ΔSTAT/wt mouse (n = 21) | 0  | 0  | 0  | 0  | 0   | 0   |

Mice were clinically assessed and scores for joint movement were allocated as described in Materials and Methods. Each limb of mice in cohorts of gp130ΔSTAT/ΔSTAT and gp130ΔSTAT/wt animals was assessed twice per week for restriction in wrist and ankle movement as illustrated in Fig. 4 c to f. Scores were allocated for: 0, no restriction; 1, moderate restriction; and 2, severe restriction, with 8 being the maximal score per mouse. None of the gp130ΔSTAT/wt control mice scored positive at any time point. The table shows the results for selected time points of three representative gp130ΔSTAT/ΔSTAT mice and the average score per mouse in the mutant cohort (mean ± SD, n = 14).

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in vitro. Primary cultures of synovial fibroblasts, derived from clinically unaffected knees of gp130\(\Delta^{STAT/\Delta^{STAT}}\) mice, showed an enhanced rate of \(^{3}H\)thymidine incorporation in response to IL-6 or LIF when compared with cells obtained from gp130\(^{wt/wt}\) mice (Fig. 6 A). Furthermore, mutant cells responded to 100- to 1,000-fold lower concentrations of cytokines than wt cells and showed sustained IL-6–dependent activation of gp130-mediated signaling, as demonstrated by autophosphorylation of Jak-2, tyrosine phosphorylation of SHP-2, and phosphorylation of MAPK isoforms Erk1/2 (Fig. 6 B). These in vitro data indicate increased sensitivity and proliferative response of mutant synovial fibroblasts to IL-6 associated with sustained gp130 signaling through the SHP-2/ras/Erk pathway.

**Sustained Activation of the SHP-2/ras/Erk Pathway Results from Impaired Induction of SOCS-1.** One possible mechanism accounting for sustained activation of the SHP-2/ras/Erk pathway in response to IL-6 could be that impaired STAT activation retarded the termination of gp130-mediated signaling. We hypothesized that this might result from impaired induction of SOCS/CIS family gene expression (21, 22). As depicted in Fig. 7 A, the expression of CIS, SOCS-1, SOCS-2, and SOCS-3 mRNA were readily induced in the livers of wt mice after a single injection of either IL-6 or LIF. IL-6 treatment also induced CIS and SOCS-2 mRNA in the livers of gp130\(^{\Delta^{STAT/\Delta^{STAT}}}\) mice, however, we observed almost complete absence of SOCS-1 mRNA induction and re-

Figure 5. Histological assessment of joint disease in gp130\(^{\Delta^{STAT/\Delta^{STAT}}}\) mice. Sagittal sections of knees of age-matched 71-d-old gp130\(^{\Delta^{STAT/\Delta^{STAT}}/wt}\) control (a) and gp130\(^{\Delta^{STAT/\Delta^{STAT}}/mutant}\) mice (b). The section of the mutant knee shows enlargement of the synovial space (*), synovial hyperplasia and pannus formation (black arrowhead), cartilaginous overgrowth in the menisci (white arrowhead), and destruction and deformation of the articular surfaces. Note that the cartilaginous metaplasia on the head of the femur (arrow) is associated with alterations of the growth plate. The shoulder (c) joint of gp130\(^{\Delta^{STAT/\Delta^{STAT}}/mutant}\) mouse illustrates the early stage manifested as enlargement of the synovial space (*), prominent synovial hyperplasia (arrowheads), exuberant pannus formation with early cartilaginous metaplasia (arrows) but little destruction of the articular surface. A high magnification (d) of the area boxed in c shows synovial hyperplasia in the recess of the cavity of the shoulder joint. The metaplastic cartilage deposits within the synovial cavity (e) contain disorganized chondrocyte clusters that occupy lacunae within the matrix and contrast the organized appearance of smaller chondrocytes in “columns” in normal articular cartilage (arrow). Safranin-O and Alcian Blue stain proteoglycan red and mineralized matrix blue, respectively. Frontal sections through knees from 180-d-old gp130\(^{\Delta^{STAT/\Delta^{STAT}}/wt}\) (f) and gp130\(^{\Delta^{STAT/\Delta^{STAT}}/mutant}\) mice (g) document the expanded synovial space (*), destruction, and erosion of the articular cartilage (arrowhead). aC, articular cartilage; F, femur; H, humerus; M, meniscus; S, scapula; T, tibia.
duced SOCS-3 mRNA induction. Notably, the induction of SOCS-1 mRNA was also markedly reduced in synovial cells from gp130/STAT/H9004/STAT mice compared with cells from wt mice (Fig. 7 B). These data support previous observations suggesting that IL-6–dependent activation of SOCS-1 and SOCS-3 genes is mediated, at least in part, by STATs (34). The reduced induction of SOCS-1 mRNA observed in the livers of LIF-treated gp130/STAT/H9004/STAT mice (Fig. 7 A) most likely resulted from diminished STAT signaling through the less effective gp130/STAT/LIF-R/R heterodimers.

To explore the concept of enhanced gp130-mediated SHP-2/ras/Erk signaling in the absence of SOCS-1, we analyzed IL-6–signaling in SOCS-1–deficient synovial cells. These cells were derived from adult SOCS-1−/− mice established on an IFN-γ-null background to rescue the perinatal lethality associated with SOCS-1 deficiency (23). As revealed in Fig. 8 A, compound mutant SOCS-1−/−, IFN-γ−/− cells, like gp130/STAT/STAT cells, showed enhanced [3H]thymidine incorporation in response to nanomolar concentrations of IL-6, compared with cells prepared from control mice of the corresponding genetic background (SOCS-1+/+, IFN-γ−/−, and gp130/STAT/wt, respectively). Compared with SOCS-1+/+ IFN-γ−/− control cells, we also observed sustained IL-6–mediated Erk1/2 phosphorylation in SOCS-1−/− IFN-γ−/− cells (Fig. 8 B). Thus, the enhanced proliferative responsiveness to IL-6 was also a feature of SOCS-1−/− IFN-γ−/− cells, however, this was less marked than in gp130/STAT/STAT cells.

If impaired or reduced induction of SOCS-1 (and SOCS-3) expression accounted for sustained activation of the Jak/SHP-2/ras/Erk cascade in response to IL-6 in gp130/STAT/STAT synovial cells, then overexpression of SOCS-1 should revert the hyperresponsive phenotype of...
these cells. To explore this notion, we exploited the fact that the minimal \( c\)-\( fos \) promoter is activated through the ras/Erk pathway (27). As expected, IL-6 stimulation of \( \text{gp130}^{\Delta\text{STAT}/\Delta\text{STAT}} \) cells, transiently transfected with a \( c\)-\( fos \)-luciferase reporter construct, yielded higher luciferase activity than IL-6 stimulation of wt cells (Fig. 8 C). Furthermore, increasing the cellular concentration of SOCS-1 protein, after cotransfection with an epitope tagged SOCS-1\(^{\text{Flag}} \) expression plasmid (pSOCS-1), reduced IL-6-responsiveness in \( \text{gp130}^{\Delta\text{STAT}/\Delta\text{STAT}} \) cells to levels observed in wt cells. Finally, a complete block of IL-6–mediated luciferase activity in \( \text{gp130}^{\Delta\text{STAT}/\Delta\text{STAT}} \) cells required greater amounts of transfected pSOCS-1 protein than in wt cells. Collectively, these observations are consistent with the notion that elevated levels of recombinant SOCS-1\(^{\text{Flag}} \) protein were required to terminate IL-6 signaling in \( \text{gp130}^{\Delta\text{STAT}/\Delta\text{STAT}} \) mutant cells to compensate for impaired induction of endogenous SOCS-1 and SOCS-3 expression. Since recombinant SOCS-1\(^{\text{Flag}} \) expression did not suppress \( c\)-\( fos \)-luciferase activity induced by serum, overexpression of SOCS-1\(^{\text{Flag}} \) was not directly interfering with activity of the \( c\)-\( fos \)-luciferase reporter plasmid.

Taken together, these data strongly suggest that the hyperresponsiveness of synovial cells from \( \text{gp130}^{\Delta\text{STAT}/\Delta\text{STAT}} \) mice arose from defective or impaired STAT-mediated SOCS induction which resulted in sustained activation of the SHP-2/ras/Erk pathway, leading to synovial hyperproliferation and cartilaginous metaplasia and ultimately the degeneration of the joint.

Discussion

At the molecular level, the multiple and tissue-specific activities of cytokines are explained, in part, by activation of multiple signaling cascades emanating from discrete intracellular receptor domains which regulate distinct (sets of) target genes. While complete inactivation of multidomain receptors will result in phenotypes reflecting inactivation of all associated signaling pathways, the selective deletion of one particular domain provides the opportunity to associate a specific signaling cascade with a particular physiological or pathological process. Here we used a knock-in strategy to mimic the effect of a nonsense mutation that specifically deletes the COOH-terminal domain of gp130, along with its associated STAT activation sites, but still permits activation of the SHP-2/ras/Erk pathway (15, 19). Consequently, the phenotype in \( \text{gp130}^{\Delta\text{STAT}/\Delta\text{STAT}} \) mice is largely a direct consequence of the absence (in the case of \( \text{gp130}^{\Delta\text{STAT}} \) homodimerization) or diminution (in the case of \( \text{LIF-R}\beta \)/\( \text{gp130}^{\Delta\text{STAT}} \) heterodimerization) of STAT-mediated cellular responses. Furthermore, we also identified the importance of STAT signals in promoting inhibitory feedback on gp130 and the associated SHP-2/ras/Erk pathway, thereby reinforcing a concept that the two pathways are under reciprocal negative control (35).

The phenotypes of mice with \( \text{gp130}^{\Delta\text{STAT}/\Delta\text{STAT}} \) impairment mutations affecting either the STAT (\( \text{gp130}^{\Delta\text{STAT}/\Delta\text{STAT}} \) mice reported here) or the SHP-2/ras/Erk signaling cascade (\( \text{gp130}^{757F/757F} \) mice; reference 35) are dramatically different from the myocardial hypoplasia and associated embry-
chronic lethality observed in gp130−/− mice (11). Similarly, no profound effects on embryonic development were reported in gp130FxxQ/FxxQ mice, where STAT signaling was abolished after replacement of the cytoplasmic domain of gp130 with a human cDNA encoding phenylalanine substitutions in all YxxQ motifs (36). However, the latter mutation resulted in perinatal death associated with the apparent failure of milk uptake and perturbed CTNF-mediated astrocyte differentiation (35). Since the phenotype of gp130FxxQ/FxxQ mice is somewhat reminiscent of that observed in LIF-Rβ−/− (10) and CTNF-Rα−−/− mice (36), but entirely different to that associated with IL-6−/− (4, 5) or IL-11Rα1−/− mice (8, 9), signaling through the heterodimeric LIF-Rβ/gp130 rather than through homodimeric gp130 appears critical after birth. Therefore, it is likely that STAT signaling through LIF-Rβ/gp130FxxQ receptor heterodimers is rate limiting in gp130FxxQ/FxxQ mice, while signaling through LIF-Rβ/gp130ΔSTAT heterodimers, although decreased when compared with wt LIF-Rβ/gp130 dimers, reaches the threshold that enables postnatal survival of most of the gp130ΔSTAT/ΔSTAT pups. The “magnitude” of the STAT signal may be influenced by the rate of internalization of ligand occupied LIF-Rβ/gp130 complexes, which in turn is determined by the collective activity of individual dileucine internalization signals (L1064I in LIF-Rβ and L986L in gp130; references 37 and 38). Thus, one could speculate that LIF-Rβ/gp130FxxQ dimers are more efficiently internalized than LIF-Rβ/gp130ΔSTAT dimers, and thus signal from the LIF-Rβ/gp130ΔSTAT dimers may be sustained for longer.

Degenerative Joint Disease in gp130ΔSTAT/ΔSTAT Mice. The most striking observation in gp130ΔSTAT/ΔSTAT mice was restricted joint movement which was apparent from an early age and consistently affected older mice albeit with variable severity. While early stages of the joint disease bear hallmarks of inflammation, the intermediate stages were reminiscent of rheumatoid arthritis with the overgrowing pannus-like tissue at the edge of the articular surface. Likewise, the formation of cartilage in these pannus-like tissues resembled osteoarthritis in which cartilaginous and osseous osteophytes formed along the rim of the eroded cartilage plate. The advanced stage with its overgrowth of cartilage along the synovial lining, however, resembled neither pathology but rather the rare human condition synovial chondromatosis (39), although the characteristic multiple “loose” bodies of hyaline cartilage within the joint space were only infrequently observed in gp130ΔSTAT/ΔSTAT mice.

Many studies have implicated the LIF/IL-6 family cytokines and their soluble receptors as proinflammatory mediators for synovial fibroblasts in inflammatory joint diseases including rheumatoid arthritis (32, 33). Our model, in which signaling through gp130ΔSTAT resulted in impaired or reduced induction of SOCS-1 and SOCS-3, implicates activation of the SHP-2/ras/Erk pathway as the likely molecular mechanism promoting hyperproliferation of synovial cells by increasing their sensitivity to physiological levels of LIF/IL-6 cytokines. In turn, the hypersensitivity of these cells may trigger a sequence of changes including chronic synovitis that results in cartilaginous metaplasia and culminates in a degenerative process affecting the major weight-bearing joints. However, we cannot categorically exclude the possibility that synovial hyperplasia and the formation of exogenous cartilage occurred independently of each other.

Since our data provide strong genetic evidence for a critical involvement of gp130, STAT, and SOCS proteins in maintaining the integrity of joints of the appendicular skeleton, why do other mouse strains with mutations in either the LIF/IL-6 cytokine signaling cascade or in SOCS genes lack overt joint pathology? This apparent anomaly can be explained by extrapolating from observations by Ohtani et al. (35) who showed that the gp130-dependent STAT and SHP-2/ras/Erk pathways play reciprocal roles in the immune response by providing a balance between positive and negative signals. This delicate balance between the two pathways at the level of gp130 is further emphasized through their reciprocal negative regulation (35, 40, 41).

Hence, balanced alterations of the signaling output from gp130-dependent STAT and SHP-2/ras/Erk pathways, either by attenuation (for instance in ligand and receptor null mutants) or augmentation (for instance in mice transgenic for the ligands), resulted in a number of phenotypes which were not observed in mice with an imbalance between the two pathways (for instance in gp130ΔSTAT/ΔSTAT or gp130FxxQ/FxxQ mice; reference 35). Similarly, the simultaneous and sustained activation of the SHP-2/ras/Erk and STAT pathways in response to IL-6 in synovial cells from SOCS-1−/− IFN-γ−/− compound mice did not result in cartilaginous overgrowth or destruction of the large weight-bearing joints. However, SOCS-1−/− IFN-γ−/− mice developed an acute synovitis of the tendons and small joints of distal limbs (unpublished data), presumably resulting from the activity of dysregulated T cells (42).

The gp130ΔSTAT Mutation Impairs Biological Responses to IL-6 and IL-11. Since IL-6 and IL-11 are the only cytokines that signal exclusively through gp130, we predicted that these cytokines would fail to elicit STAT-mediated responses in gp130ΔSTAT/ΔSTAT mice. This was clearly demonstrated by failure to induce type I acute phase proteins in the liver which is a STAT-mediated response in cultured hepatocytes (20). Furthermore, gp130ΔSTAT/ΔSTAT mice, like mice either deficient for IL-6 (4, 5, 30), or containing a conditional gp130-null allele (31), or mice transgenic for a truncated and dominant interfering gp130703 protein (43), displayed impairment of T cell–dependent high affinity immune and mucosal IgA responses. By contrast, constitutive activation of the STAT and the SHP-2/ras/Erk signaling cascades, as observed in IL-6 transgenic mice, resulted in IgG1 plasmacytosis (44), a phenotype that was never observed as a result of sustained activation of the SHP-2/ras/Erk pathway in gp130ΔSTAT/ΔSTAT mice. Collectively, these observations implicate STAT signaling, rather than activation of the SHP-2/ras/Erk pathway, as the driving force for IL-6–mediated plasma cell maturation.

Intriguingly, spontaneously occurring ulcerative lesions have not been previously observed as a consequence of the...
genetic manipulation of the IL-6/LIF cytokine axis. Notably, no intestinal phenotype was observed in IL-6−/− or IL-11Rα−/− mice, although a role for STAT3 has been proposed during cell migration in epidermal wound healing (45). Thus, it is conceivable that intestinal ulceration in gp130STAT−/− mice may result from a combination of STAT-dependent reduction in IL-6–mediated first line defense against pathogens at mucosal surfaces and impairment of epithelial homeostasis, which is maintained in the intestine, at least in part, through IL-11–mediated signaling (46).

The gp130STAT Mutation Reduces STAT Signaling from gp130/LIF-Rβ Complexes. The failure of blastocyst implantation in gp130STAT−/− mice resembled in all aspects the defect observed in LIF−/− mice (6, 7) and suggests a pivotal role for the COOH-terminal region of gp130 and associated STAT signaling during blastocyst implantation and possibly decidua formation. Since STAT signaling from the mutant gp130STAT−/−/LIF-Rβ receptor heterodimer was compromised in all tissues examined, the implantation defect is the likely consequence of a failure to reach a threshold level of LIF–induced STAT signaling in the uterus of gp130STAT−/−STAT− mice. However, we cannot exclude the possibility that the gp130STAT−/− mutation may also affect the implantation capacity of the uterine epithelium in response to simultaneous stimulation by IL-6 and IL-11. Although blastocyst implantation in mice individually deficient for either IL-6 or IL-11 signaling was not affected (8, 9), increased IL-6 expression is observed in the epithelial cells of the endometrium during the “implantation window” (47). The generation of IL-6−/− IL-11Rα−/− mice will establish whether the simultaneous action of these two cytokines may play a hitherto unknown indispensable role during the process of uterine implantation.

This study genetically implicates gp130–mediated STAT signaling in a number of physiological host responses affecting uterine implantation, type I acute phase response, humoral and mucosal immune response, and epithelial homeostasis in the gastrointestinal tract. Surprisingly, blunting of STAT signaling in the joint compartment resulted in the mitogenic hyperresponsiveness of the synovial cells thereby triggering a series of pathological responses which ultimately culminated in the degeneration of the joints. This observation is consistent with a requirement for the balanced activation of the SHP-2/ras/Erk and STAT pathways to maintain synovial homeostasis in response to gp130 signaling and highlights the crucial role for the LIF/IL-6 cytokine family in maintaining the integrity of joint architecture and function. To date, no disorder has been associated with impaired gp130 signaling in humans, however, germline mutations yielding a premature stop codon have been found in the related receptor for granulocyte colony stimulating factor in a subset of patients with severe congenital neutropenia (48). On the assumption that gp130 truncation mutations in humans would phenocopy at least some of the abnormalities observed in gp130STAT mice, one might speculate that a subset of patients with degenerative joint diseases, female infertility, or impaired mucosal immunity may result from nonsense mutations in gp130 or other genetic lesions that impair gp130-mediated STAT signaling.

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