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Differences in Wound Healing in Mice with Deficiency of IL-6 versus IL-6 Receptor

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IL-6 modulates immune responses and is essential for timely wound healing. As the functions mediated by IL-6 require binding to its specific receptor, IL-6Rα, it was expected that mice lacking IL-6Rα would have the same phenotype as IL-6-deficient mice. However, although IL-6Rα-deficient mice share many of the inflammatory deficits seen in IL-6-deficient mice, they do not display the delay in wound healing. Surprisingly, mice with a combined deficit of IL-6 and IL-6Rα, or IL-6-deficient mice treated with an IL-6Rα–blocking Ab, showed improved wound healing relative to mice with IL-6 deficiency, indicating that the absence of the receptor contributed to the restoration of timely wound healing, rather than promiscuity of IL-6 with an alternate receptor. Wounds in mice lacking IL-6 showed delays in macrophage infiltration, fibrin clearance, and wound contraction that were not seen in mice lacking IL-6 alone and were greatly reduced in mice with a combined deficit of IL-6 and IL-6Rα. MAPK activation-loop phosphorylation was elevated in wounds of IL-6Rα–deficient mice, and treatment of wounds in these mice with the MEK inhibitor U0126 resulted in a delay in wound healing suggesting that aberrant ERK activation may contribute to improved healing. These findings underscore a deeper complexity for IL-6Rα function in inflammation than has been recognized previously. The Journal of Immunology, 2010, 184: 7219–7228.

IL-6 is a pleiotropic cytokine with roles in generating acute phase responses, inflammation, and lymphocyte differentiation. Numerous cell types produce IL-6, especially at sites of inflammation. IL-6 functions are mediated by binding to its specific receptor, IL-6Rα (gp80 and CD126). IL-6Rα is the only known receptor for IL-6, and its expression is predominantly restricted to hepatocytes and immune cells. Recently, ciliary neurotrophic factor (CNTF) and IL-27, two other members of the IL-6 family, have been demonstrated to bind IL-6Rα in vitro but with lower affinity than IL-6, and thus, the in vivo relevance of these findings remains unclear (1, 2). IL-6 engagement of IL-6Rα induces homodimerization of the ubiquitously expressed, common IL-6 family signaling receptor gp130 (IL-6 signaling transducer). Mammalian IL-6, however, cannot directly engage gp130 in the absence of IL-6Rα (3).

IL-6Rα contains an extracellular Ig-like domain, a cytokine receptor homology module, a transmembrane domain, and a non-signaling intracellular domain. The cytokine receptor homology module is made up of two fibronectin type III domains and is responsible for recognition and binding of IL-6 and gp130 (reviewed in Ref. 4). Typical for a cytokine receptor, IL-6Rα does not possess kinase activity. Ligand-bound IL-6Rα induces dimerization of gp130 (5). The Janus family kinases, JAK1, JAK2, and TYK2, are constitutively associated with the cytoplasmic tail of gp130 and phosphorylate gp130 dimers (6, 7). The transcription factors Stat3 and, to a lesser extent, Stat1 dock at any of four C-terminal tyrosines on gp130 (8, 9). JAKs phosphorylate the Stat proteins inducing Stat dimerization, translocation to the nucleus, and transcription of genes with IL-6-responsive elements (10). IL-6–induced Stat3 activation is negatively regulated by suppressor of cytokine signaling 3 and Src homology region 2 domain-containing phosphatase 2 binding to a membrane-proximal gp130 phospho-tyrosine residue (Y759), resulting in Ras activation and ERK signaling (11–13). Both Stat3 and Erk activation result in cell proliferation, survival, differentiation, and inflammation, and both are frequently upregulated in tumors.

IL-6 signaling can also occur through a soluble form of the receptor that can bind IL-6 and subsequently induce gp130 dimerization (3). This process is known as trans-signaling and allows cells that do not express IL-6Rα to respond to IL-6 (14). Soluble forms of IL-6Rα occur as the result of either of two distinct processes: alternative splicing or proteolytic cleavage (15–17). The importance of each isoform in physiologic processes is not clearly understood. It is known from mice overexpressing human soluble IL-6Rα that the soluble isoform plays a role in sensitizing cells to IL-6 signaling and prolonging IL-6 half-life (18). Inhibition of the soluble form of IL-6Rα in vivo is sufficient to block many of the known functions of IL-6 signaling, such as modulation of the inflammatory response (19, 20). Neutrophil infiltration and apoptosis are early events in the inflammatory response that trigger IL-6Rα shedding and subsequent monococyte infiltration (19–22). Despite high expression of membrane-bound IL-6Rα, human osteoblasts are unable to respond to IL-6 unless the receptor is cleaved to produce the soluble form (23). It has not
been well established whether any nonredundant function exists for the membrane-bound form of IL-6Ra.

IL-6-deficient (Il6−/−) mice were generated ∼15 y ago (24, 25). Their multiple phenotypic abnormalities are not surprising, because of the pleiotropic functions ascribed to IL-6. Although Il6−/− mice have no apparent developmental abnormalities, they have an impaired capacity to mount immune responses to several pathogens and to generate acute-phase responses (24). In addition, they have reduced capacity for liver regeneration and develop age-related obesity (26, 27). Il6−/− mice display resistance to experimental autoimmune conditions, such as encephalitis, and protection against bone loss after ovariectomy (25, 28). Studies using experimental tumor models in Il6−/− mice have reported both tumor-protective and tumor-promoting effects for IL-6, depending on the tumor model used (29–31). A better understanding of the functions of IL-6 and its receptor in different contexts may provide opportunities for more effective manipulation of IL-6 signaling in the treatment of inflammation and cancer.

Il6−/− mice display significant defects in wound healing. Wound healing is a tightly regulated process in which platelets and fibrin immediately fill the wound bed with an insoluble clot, and significant neutrophil immigration occurs shortly thereafter (reviewed in Ref. 32). Re-epithelialization proceeds by adjacent keratinocyte proliferation and migration over the wound area. Neutrophils in wounds are important for microbe neutralization, but their absence from sterile wounds does not delay healing (33, 34). Monocyte/macrophages are subsequently recruited and play roles in the clearance of debris and the provision of growth and angiogenic factors. Macrophages in wounds typically express markers indicating alternate activation pathways with functions in tissue remodeling and angiogenesis, rather than classically activated macrophages that function to kill pathogens. Wounds in mice with inhibited macrophage function display excess fibrin and cellular debris and heal more slowly than those in untreated mice (35). Wound healing is delayed in Il6−/− mice as a result of impaired re-epithelialization, angiogenesis, and macrophage infiltration (36, 37).

In this paper, we report on mice with complete and conditional deficiencies of IL-6Rx, which were generated to better understand the relationship between soluble and membrane-bound receptor and to identify any phenotypic discrepancies that may exist, as compared with Il6−/− mice. Although IL-6 and IL-6Rx are believed to be exclusive and essential components of IL-6 signaling in vivo, we report both similarities and differences between the phenotypes of Il6−/− and Il6ra−/− mice. These studies indicate an unanticipated compensatory improvement in wound healing in Il6ra−/− mice but not in Il6−/− mice, which suggests a greater complexity in IL-6Rx function than was previously appreciated.

Materials and Methods

Generation of Il6ra-knockout mice

IL-6ra-deficient mice were generated by the insertion of a targeting vector into the Il6-6ra genomic locus. The targeting vector included the selection cassette loxP-flt-mPC1neoA-flt in which the neo gene is flanked by frt sites. Murine Il6-6ra exons 4, 5, and 6 were inserted between the loxP sequences. The construct was introduced into embryonic stem (ES) cells on a C57BL/6 background for homologous recombination. To prevent changes in the level of gene expression in mice carrying the floxed allele, the resulting loxP sites of the recombinated allele were located in nonconserved intronic sequences known to share no sequence conservation between mouse and human and to be devoid of transcription factor binding sites. Recombined ES cell clones were identified by PCR, and a successful single insertion was confirmed by Southern blot analysis. Blastocysts containing ES cells with the floxed allele were implanted into pregnant mice, and litters were bred with Il6ra-mice on a C57BL/6 background, resulting in the deletion of Il6-6ra exons 4, 5, and 6 (the cytokine receptor binding module) and the Neo cassette. Further breeding with C57BL/6 mice was carried out to remove the Cre construct, and mice were then bred to homozygosity for the deleted Il6-6ra allele. Genotypes were confirmed by PCR analysis and Southern blotting. Studies included in this paper have been reviewed and approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Southern blot analysis

Genomic DNA was prepared by phenol/chloroform extraction and digested overnight with restriction enzymes. Samples were loaded onto a 0.8% agarose gel in tris-acetate EDTA buffer and electrophoresed. The gel was stained with ethidium bromide for 10 min in tris-acetate EDTA buffer, imaged by UV, and then transferred overnight in 20× SSC onto a positively charged nylon membrane (Roche, Basel, Switzerland). Digoxigenin (DIG)-labeled probes were prepared using the PCR DIG Probe Synthesis Kit (Roche), according to the manufacturer’s instructions. The membrane was hybridized with 50 ng/ml DIG-labeled probe, followed by chemiluminescent detection with anti-DIG alkaline phosphatase Ab and CSPD substrate.

Generation and maintenance of mice with conditional expression of IL-6Ra

Mice with a floxed allele of IL-6ra (Il6rafl/−) were first crossed with FLPe+/- mice (38) to delete the FRT-flanked Neo cassette in the targeting vector. Progeny with successful excision of Neo, as determined by PCR, were crossed with mice expressing Cre recombinase under the control of the albumin (Alb) or the lymphocyte-specific (LysM) or the collagenase (Col1a1 or Cre) (40). Progeny lacking the FLPe allele were selected for further breeding. Il6rafl/−/AbCre−/− mice were maintained as homozygotes for both alleles. Il6rafl/−/LysMCre−/− mice used in experiments were bred from colonies of Il6rafl/− and Il6rafl/−/LysMCre−/− mice. All mouse strains were on a C57BL/6 background.

Genotyping

DNA from ear clippings was isolated by using the SYBR Green Extract-N-Amp Tissue PCR kit (Sigma-Aldrich, St. Louis, MO). Primers for the wild-type (WT) IL-6-6ra allele (5′-CTGGACACGGTAGTTACTC-3′ and 5′-CAGCAACCCGTGAACCTCTTT-3′), the floxed Il6-6ra allele (5′-GGCTGGTGGAGGGCTTTTTT-3′ and 5′-CCCCATGGACTCCA-CAATCAA-3′), and the excised Il6-6ra allele (5′-CTGGACAGGAGACGCTTTT-3′ and 5′-CCCCATGGACTCCACATCAAA-3′) were used in real-time PCR analysis. AlbCre, LysMCre, and FLPe mice were genotyped as per protocols from their originating laboratories (JAX Mouse Database, The Jackson Laboratory, Bar Harbor, ME).

Harvesting of normal and inflammatory-challenged tissues

Spleen, liver, and lung tissues were collected from euthanized mice for analysis. Plasma was prepared from EDTA-treated blood collected from the retro-orbital plexus. Hepatocytes were prepared from anesthetized mice by in situ liver perfusion and Percoll gradient separation, as described previously (41). Thioglycolate-elicited macrophages and granulocytes were collected by peritoneal lavage 24 or 48 h after i.p. injection of 3% thioglycolate broth. Cells were incubated on plastic for 1 h to enrich for adherent macrophages. Acute-phase responses were assessed in plasma and livers collected from mice given a s.c. injection of 100 μl turpentine at various intervals prior to sacrifice.

Air-pouch experiments were performed as described previously (20). Briefly, mice were injected s.c. in the center of the back with 6 and 4 ml sterile air at days −6 and −3, respectively, to create an air pouch. Inflammation was elicited at day 0 by injecting 1 ml 1% carrageenan (Sigma-Aldrich) in PBS into the air pouch. Lavage was performed at days 3 and 7 according to Papanicolaou staining. Lavage fluid was spun at 10,000 × g for 10 min to remove the cytospin (Thermo Shandon, Pittsburgh, PA) and stained with Kwik-Diff (Thermo Shandon). Differential cell counts were performed by a hematologist (C.A.M.) on three to four microscope fields per sample.

Full-thickness excisional wounds were prepared on shaved dorsal skin by 4-mm-punch biopsy (small wounds; Acuderm, Fort Lauderdale, FL) or with a fine-scissor cutting around a 1-cm2 template (large wounds). Mice were housed individually, and wounds were left undressed. Wounds were measured by guest on April 14, 2017 http://www.jimmunol.org/ Downloaded from on April 14, 2017
Real-time PCR analysis

RNA from WT and Il6ra−/− mice was isolated using the Nucleospin II Kit (Macherey-Nagel, Bethlehem, PA). One microgram of mRNA was reverse transcribed with the Thermoscript II Kit (Invitrogen, Carlsbad, CA). Twenty nanograms of cDNA was used for subsequent PCR analysis. Quantitative PCR was performed with the following primers: F3 (5′-GCCAATATTACCTCTCCTGTAAGGAA-3′) and R3 (5′-GAAGGATCTGACCATAGTCTGTTTCC-3′). Each analysis was repeated at least twice to ensure reproducibility. To ensure specificity, real-time melt curve analyses were performed, and band sizes were checked on agarose gels. Values were normalized against murine L32, housekeeping gene.

Western blotting and ELISA

Protein lysates were prepared from liver and spleen tissue and quantified by protein assay (Bio-Rad DC; Bio-Rad, Hercules, CA) for Western blotting. Reduction samples were treated with 8% polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked in 5% milk for 1 h. Primary Ab dilutions were applied at 4°C overnight at the following dilutions: rabbit anti-mouse IL-6R IgG (1/400; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti–β-actin (1/1000; Cell Signaling Technology, Beverly, MA), phospho-Stat3 (1/800, Ser237/2377, Cell Signaling Technology), Stat3 (1/1000, C-20, Santa Cruz Biotechnology), phospho-p44/42 MAPK (1/1000, and numbers 4370 and 4695; Cell Signaling Technology). Primary Abs were detected with donkey anti-rabbit IgG-HRP (1/10,000; Novus Biologicals, Littleton, CO) and luminol (ECL Plus; PerkinElmer, Wellesley, MA). ELISAs were used to detect murine soluble IL-6Rα (R&D Systems, Minneapolis, MN) and SAA (Invitrogen) in EDTA-treated plasma.

Histology and immunohistochemistry

Wound tissues were collected into Glyofixx fixative (Thermo Shandon) at necropsy and processed into paraffin. A separate group of wounds was fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and in thioglycolate-elicited macrophages of LysMCre+/- mice. Given that IL-6Rα is believed to be the exclusive binding partner required to facilitate the interaction with the common IL-6 family receptor gp130, it was not surprising to find that deficiencies of either IL-6 or IL-6R are not detected in null mice while detecting abundant product in WT mice. Il6ra−/− mice were normal in appearance and were born in the expected Mendelian ratio.

Il6ra−/− mice have a defective response to IL-6

IL-6 is critical for the acute-phase response to s.c. injected turpentine (43, 44). To assess the response of Il6ra−/− mice, groups of mice were s.c. injected with turpentine. Hepatic production of the acute-phase reactants fibrinogen, haptoglobin, and SAA was markedly diminished or absent in Il6ra−/− mice, as assessed by real-time PCR (Fig. 2A) and by ELISA for SAA (Fig. 2B). Similar results were obtained in Il6−/− mice (Fig. 2A, 2B), as reported previously (43). Thus, IL-6 signaling function is abrogated in Il6ra−/− mice in the context of generating an acute-phase response. IL-6 has been shown to be important in the progression of inflammatory responses from a neutrophil-dominant phase to a macrophage-dominant phase (21). To determine whether this is also the case in Il6ra−/− mice, we created s.c. air pouches in mice and injected carrageenan as described previously (20). Although the proportion of neutrophils and macrophages in the pouch fluid was not different between the groups (data not shown), it was not surprising to find that deficiencies of either IL-6 or IL-6Rα induced the same phenotypic abnormalities compared with WT mice.

Hepatocyte production of IL-6Ra is critical for the acute-phase response but myeloid cells secrete most of the circulating IL-6Ra

IL-6Ra expression has been reported to be predominantly restricted to hepatocytes and immune cells. To elucidate the importance of each compartment in IL-6Ra-dependent processes, we generated mice with conditional deficiency of IL-6Ra in either hepatocytes or lysozyme M-expressing cells (macrophages and granulocytes) by crossing Il6rafl/fl mice with mice expressing Cre recombinase under the control of the albumin (AlbCre) or lysozyme M (Ly5McCre) promoters, respectively. Significant reduction in IL-6Ra expression was observed in hepatocytes of AlbCre+/Il6rafl/fl mice (Fig. 3A) and in thioglycolate-elicited macrophages of Ly5McCre+/Il6rafl/fl mice (Fig. 3B). Interestingly, expression of soluble IL-6Ra in plasma was more dependent on immune cell secretion than on hepatic production, because Ly5McCre+/Il6rafl/fl mice had lower levels of IL-6Ra levels (39.95% of WT) than AlbCre+/Il6rafl/fl mice (67.95%) (Fig. 3C). No differences were seen between WT and Il6rafl/fl mice lacking Cre (data not shown). It is also noteworthy that macrophages, granulocytes, and hepatocytes together apparently account for almost all of the circulating IL-6Ra in unchallenged mice, because the sum of the proportional

predicted 9-kb excised allele in mice homozygous for the floxed allele (Fig. 1B). Subsequent breeding to remove the Cre-recombinase gene was performed. The absence of mRNA for Il-6ra was determined by real-time PCR on liver and lung tissue (Fig. 1C). IL-6Rα protein was absent from liver and spleen lysates prepared from Il6ra−/− mice and present in WT (Il6ra+/+) and heterozygous (Il6ra+/−) mice (Fig. 1D). Similarly, soluble IL-6Ra was abundant in Il6ra+/+ and Il6ra−/− mice and undetectable in Il6ra−/− mice (Fig. 1E). Although the presence of a truncated IL-6Ra protein in Il6ra−/− mice cannot be formally excluded, such a product is unlikely because of 1) the presence of an incomplete domain, 2) unpaired cysteine residues because of the deletion of the cytokine homology domain, and 3) the inability of a polyclonal Ab raised against the entire extracellular domain of IL-6Rα to recognize any product in null mice while detecting abundant product in WT mice. Il6ra−/− mice were normal in appearance and were born in the expected Mendelian ratio.
deficits in circulating IL-6Rα in AlbCre+/+Il6rafl/fl (30.6% deficit) and LysMCre+/2Il6rafl/fl (62.7% deficit) mice equaled 93.4% of the levels in WT mice. To determine the contributions of hepatocytes and immune cells to soluble IL-6Rα during inflammatory challenge, plasma and livers were collected from mice subjected to turpentine injection or cutaneous wounding. Despite increased expression of mRNA for IL-6ra in livers of WT mice subjected to acute-phase protein induction (Ref. 45 and our own unpublished data), no increase in the expression of soluble IL-6Rα was seen in mice challenged with turpentine or cutaneous wounding (Fig. 3C, 3D).

We next compared acute-phase reactants in mice with conditional expression of IL-6Rα. As expected, generation of an acute-phase response was completely muted in AlbCre+/+Il6rafl/fl mice compared with WT or Il6rafl/fl mice (Fig. 3E, 3F). This is consistent with hepatocytes being the predominant cell type responsible for acute-phase responses. No significant difference was seen between LysMCre+/2Il6rafl/fl mice and WT (Fig. 3E, 3F) or LysMCre+/2Il6rafl/fl mice (data not shown). Despite in vitro reports that soluble IL-6Rα can enhance acute-phase protein expression in HepG2 cells (46), no hepatic acute-phase response was detected in AlbCre+/+Il6rafl/fl mice despite their having two-thirds of the WT expression of soluble IL-6Rα (Fig. 3C, 3D). Apparently, membrane-bound IL-6Rα is essential for the hepatic acute-phase response to IL-6.

The deficit in wound healing is not as severe in Il6ra2/2 mice as in Il62/2 mice. It has previously been reported that wound healing is markedly delayed in Il62/2 mice (36, 37). We generated full-thickness small and large excision wounds to determine whether IL-6Rα2/2 mice shared a similar phenotype. To our surprise, we found that Il6ra2/2 mice did not share the same dramatic delay in wound healing seen between LysMCre+/2Il6rafl/fl mice and WT (Fig. 3E, 3F) or LysMCre+/2Il6rafl/fl mice (data not shown). Despite in vitro reports that soluble IL-6Rα can enhance acute-phase protein expression in HepG2 cells (46), no hepatic acute-phase response was detected in AlbCre+/+Il6rafl/fl mice despite their having two-thirds of the WT expression of soluble IL-6Rα (Fig. 3C, 3D). Apparently, membrane-bound IL-6Rα is essential for the hepatic acute-phase response to IL-6.

**FIGURE 1.** Generation of IL-6ra–deficient mice. A, Schematic depicting exons 2–6 of the genomic IL-6ra gene, the targeted construct containing LoxP sites (△) around exons 4–6 and a neo cassette, and the recombinant allele after Cre recombinase-mediated excision. B, Southern blot analysis of tail DNA from WT, homozygous, and heterozygous IL-6ra–targeted alleles, indicating expected band sizes of 11 kb (WT) and 9 kb (knockout). C, Real-time PCR with primers specific for exon 5 of murine IL-6ra indicating an absence of IL-6ra RNA in homozygous knockout mice. Data are expressed as fold change relative to WT mice. D, Western blot analysis of liver and spleen tissue collected from mice (two of each genotype) with WT, heterozygous, and homozygous alleles for IL-6Rα. E, Absence of sIL-6Ra in plasma of homozygous Il6ra–/– mice (n = 12) but not in Il6ra+/- (n = 11) or Il6ra+/- mice (n = 12), as measured by ELISA. *p < 0.05. sIL-6R, soluble IL-6R.

**FIGURE 2.** Il6ra–/– and Il6–/– mice share a functional deficit in IL-6 signaling. A dramatic reduction in acute-phase protein production in response to turpentine challenge was seen in Il6ra–/– and Il6–/– mice by real-time RT-PCR of liver RNA (A) and SAA ELISA of plasma (B). C and D, Differential counts were performed on Kwik-diff–stained cells withdrawn from a sterile inflammatory air pouch lesion induced by carrageenan. Resolution of neutrophils (C) and increased influx of monocyte/macrophages (D) occurs in WT mice by day 7 but are delayed in Il6–/– and Il6ra–/– mice. Data are expressed as a percentage of total cells.
in Il6−/− mice. Instead, Il6ra−/− mice with large excision wounds healed almost as well as WT mice upon gross examination (Fig. 4A). Il6−/− mice exhibited reduced wound closure rates and frequently had open wounds for several days longer than WT or Il6ra−/− mice (Fig. 5A). Differences in small biopsy punch wounds (4 mm in diameter) were subtle, as wounds healed rapidly (data not shown). However, large wounds of Il6−/− mice frequently became expanded beyond the boundaries of the original wound (Fig. 4A), presumably by scratching or dragging of the wound with the mouse’s limbs. This assumption was made because the extended edge of the wound was always on the caudal edge, which is more accessible to the legs of mice, and because this action was occasionally observed. Ulcers generated from this activity were seen in only one WT mouse (1 of 12 mice; 8%) with resolution within 24 h.

Il6−/− mice all developed ulcers (12 of 12 mice, 100%), which had not healed at 8 d but were healing in some mice by the 11th day. Only 2 of 11 Il6ra−/− mice (18%) developed ulcers adjacent to the wound area, and both were resolved within 24 h. Whether the formation of these ulcers was prompted by prolonged irritation from an open wound, psychological/stress-induced defects in Il6−/− mice (47), or phenotypic defects in wound contraction was not determined.

Histological evaluation of the wound was performed to quantify differences in the multiple processes involved in successful wound healing. Re-epithelialization was markedly delayed in both Il6−/− and Il6ra−/− mice compared with WT mice in both large (Figs. 4B, 5C) and small wounds (Fig. 5D). Separate experiments performed in mice with conditional expression of IL-6Rα indicated that hepatic expression of IL-6Rα was more important for re-epithelialization than that of myeloid cells (Fig. 5E).

Delayed re-epithelialization in Il6ra−/− mice was an unexpected finding because macroscopic wound closure rates were similar to WT mice. Instead, wound contraction rates were similar in WT and Il6ra−/− mice but delayed in Il6−/− mice, accounting for the macroscopic observation of a smaller defect in Il6ra−/− mice than in Il6−/− mice (Fig. 5A, 5B). Deficits in the formation of granulation tissue were seen in both Il6−/− and Il6ra−/− mice but were more pronounced in Il6−/− mice. Deficits included delays in macrophage infiltration (Fig. 4C, 4D), fibrin clearance (Fig. 4E), and vascularization (Fig. 4F). These deficits have previously been
reported in Il6ra−/− mice (37, 48). Both fibrin clearance and granulation tissue formation can be attributed to macrophage activity in healing wounds. Granulation tissue formation in Il6ra−/− and Il6−/−/Il6ra−/− mice was variable and ranged from being similar to WT to the severe deficits seen in Il6−/− mice. However, large wounds of Il6−/− mice consistently displayed the most severely impaired wound healing with regard to all parameters studied except re-epithelialization (Figs. 4, 5A, 5B).

As differences were seen in wound healing between Il6−/− and Il6ra−/− mice, we wanted to determine whether IL-6 uses another receptor in the absence of IL-6Rα or whether the absence of the receptor itself may account for the improved wound-healing phenotype in Il6ra−/− mice. Some promiscuity exists in the IL-6 family of cytokines as high concentrations of CNTF or the IL-27 subunit p28 have been shown to induce a low level of signaling through IL-6Rα (1, 2, 49), and differing phenotypes have been reported between receptor and ligand knockouts for CNTF. However, mammalian IL-6 is not known to have any activity independent of IL-6Rα. We hypothesized that if IL-6 could signal through another receptor, then wound healing would be expected to be delayed in mice with a combined deficit of IL-6 and IL-6Rα. Instead, closure of large excisional wounds in Il6−/−/Il6ra−/− mice occurred on a time frame most similar to Il6ra−/− mice, as did the formation of granulation tissue (Fig. 4). Apparently, absence of the receptor is more important for rescuing wound healing in Il6ra−/− mice than is promiscuous IL-6 activity.

To confirm that the depletion of IL-6Rα improves wound contraction in Il6−/− mice, we treated mice with Abs specific for IL-6Rα and gp130. As expected, depletion of gp130 delayed wound contraction in WT mice (Fig. 5F, 5G), consistent with its previously defined role in wound healing (50) but had no effect on Il6−/− mice (data not shown). However, although depletion of circulating IL-6Rα did not affect wound contraction in WT mice, it dramatically improved wound contraction in Il6−/− mice (Fig. 5F, 5G), similar to data obtained in mice with a combined genetic deficit of IL-6 and IL-6Rα. These data are consistent with the notion that IL-6Rα both facilitates wound healing in an IL-6–dependent fashion but also impairs wound healing in a manner that does not involve IL-6. To determine whether IL-27 p28 binding to IL-6Rα accounts for this negative effect of IL-6Rα in wound contraction, we treated mice with an Ab specific for murine p28. The dose used was sufficient to prevent exogenous p28 from inducing primary murine macrophages from expressing IL-10 and suppressing IL-17 in vitro (data not shown). No difference in
wound contraction was seen with this treatment in either WT or Il6ra−/− mice (data not shown).

To identify a mechanism that could explain the timely wound healing observed in the absence of IL-6Rα regardless of the presence or absence of IL-6, we investigated signaling pathways known to be induced by IL-6Rα signaling. Stat3 phosphorylation was reduced to a similar extent in Il6−/−, Il6ra−/−, and Il6−/−/Il6ra−/− mice (Fig. 6A) compared with WT mice at 30 min after wounding and is therefore unlikely to explain differences between genotypes. At later time points, Stat3 phosphorylation in knockout mice was not different from that in WT mice (data not shown). However, phosphorylation of the MAPK ERK1/2 was undetectable in Il6−/− mice yet increased in Il6ra−/− relative to WT mice 1 d after wounding (Fig. 6A). ERK phosphorylation in Il6−/−/Il6ra−/− was similar to that of WT mice but notably higher than that of Il6−/− mice. Given the importance of ERK activation in promoting wound healing, we wondered whether ERK activation could account for the difference seen between mice lacking IL-6 or IL-6Rα. To test this hypothesis, we treated mice with a MEK1/2 inhibitor (U0126) to prevent ERK activation. Topical application of U0126 reduced ERK phosphorylation in wounds of both WT and Il6ra−/− mice compared with vehicle-treated controls (Fig. 6B). The difference in wound contraction rates was significantly delayed in Il6ra−/− mice treated with U0126 compared with vehicle control-treated mice but did not reach significance in WT mice (Fig. 6C). These data suggest that, despite diminished Stat3 activation in Il6−/− and Il6ra−/− mice, enhanced ERK activation in Il6ra−/− mice may rescue wound contraction.

Discussion

In this paper, we provide the first report of mice with a complete deficiency of IL-6Rα and of mice with conditional deletion of IL-6Rα. Il6ra−/− mice share many of the phenotypic abnormalities reported in Il6−/− mice. However, Il6ra−/− mice do not display the dramatic delay in wound healing seen in Il6−/− mice. Wound healing in mice with a combined deficit of IL-6 and IL-6Rα resembles wound healing in WT and Il6ra−/− mice more than that in Il6−/− mice, suggestive of a previously unknown role for IL-6Rα that does not involve IL-6.

Similar to Il6−/− mice, Il6ra−/− mice show deficiencies in the modulation of inflammatory responses. Using a model of sterile inflammation, we show that Il6ra−/− mice demonstrate a similar defect in neutrophil resolution and in progression to a macrophage-dominated response as Il6−/− mice. Similarly, mice of both genotypes share a complete inability to generate an acute-phase response after inflammatory challenge with turpentine. Mice with a conditional deletion of IL-6Rα in hepatocytes but not in macrophages or granulocytes shared the inability of Il6ra−/− mice to generate a response to turpentine. These results confirm the importance of the hepatocyte in this response and indicate that.
soluble IL-6Rα expression by inflammatory cells is neither required nor sufficient. Given that mice with IL-6Rα deficiency in hepatocytes still have almost two-thirds of the levels of circulating soluble IL-6Rα as WT mice yet have the same defect in generating an acute-phase response as Il6ra−/− mice, we conclude that membrane-bound IL-6Rα on hepatocytes is essential for the acute-phase response.

Through studies in mice with conditional expression of IL-6Rα, we demonstrated that macrophages and granulocytes produce the majority of soluble circulating IL-6Rα (62.7%) in unchallenged and challenged mice and that hepatocytes produce most of the remainder (30.6%). The greater contribution by immune cells is consistent with immune cell-derived IL-6Rα being secreted and transported to distant inflammatory sites. Soluble IL-6Rα delivered to an inflammatory area allows IL-6 signaling through all cells present and, hence, a rapid response to injury or infection. Hepatic expression of IL-6Rα, however, appears to be more relevant to cell-type–specific rapid acute-phase responses specifically mediated by hepatocytes. However, it should be noted that differences in soluble IL-6Rα expression may exist in mice and humans, because mice are restricted to proteolytic cleavage and do not produce the differentially spliced form. It is of interest that the combined hepatic and granulocytic contribution of IL-6Rα apparently accounts for almost all (93.4%) of the soluble IL-6Rα expression in mice. It is possible that compensation from other compartments may occur in mice lacking IL-6Rα in particular cell types or that incomplete knockdown of IL-6Rα in macrophages and granulocytes with the lysosome M promoter [95–99% efficiency in mature cells; 75–79% efficiency in bone marrow-derived cells (40)] accounts for residual IL-6Rα expression. However, the finding that macrophages, granulocytes, and hepatocytes are the major producers of soluble IL-6Rα is consistent with tissue expression studies (51). Our study did not address lymphocyte production of soluble IL-6Rα, but it is likely that these cells produce the remaining circulating IL-6Rα in unchallenged mice and that their production increases during immune responses (52).

On the basis of the similarities that we found between the phenotypes of Il6−/− and Il6ra−/− mice, it was somewhat surprising to find differences between these genotypes in wound-healing responses. Inflammation is an influential part of wound healing, and early granulation tissue contains many of the inflammatory cell types, cytokines, and angiogenic vessels typical of inflammation. Deficits in macrophage infiltration and subsequent fibrin clearance in Il6−/− mice were not typical in wounds of Il6ra−/− mice. The improved wound-healing phenotype in IL-6Rα−/− mice indicates a divergence in functionality between IL-6 and IL-6Rα and possibly a compensatory mechanism in the absence of IL-6Rα. As mice with a combined deficiency of IL-6 and IL-6Rα demonstrated a wound-healing phenotype closely aligned with that of Il6ra−/− mice, it is likely that the absence of IL-6Rα played a greater role in rescuing the phenotype in Il6ra−/− mice than did IL-6 promiscuity. However, mice with both deficits did not heal as efficiently as Il6ra−/− mice. Therefore, we cannot eliminate the possibility that IL-6 may also signal through a different receptor in these mice.

Many interrelated processes involved in wound healing were improved in Il6ra−/− mice compared with Il6−/− mice, including macrophage infiltration, fibrin clearance, and angiogenesis. That re-epithelialization was delayed in both Il6−/− and Il6ra−/− mice was surprising given the differences in wound closure rates. It seems that restored wound contraction and granulation tissue formation in Il6ra−/− mice accounts for the appearance that wounds are healing
efficiently, despite the delay in keratinocyte migration over the wound bed. Interestingly, experiments in mice with conditional expression of IL-6Rα indicated that hepatocyte IL-6Rα was more important in cutaneous wound re-epithelialization than that of macrophages and granulocytes. This finding is unexpected given that most circulating IL-6Rα comes from macrophages and granulocytes and because these cell types are numerous in healing wounds. Apparently, one or more of the numerous acute-phase reactants or growth factors produced by the liver in response to IL-6 contribute to re-epithelialization.

In both knockout mice and Ab-depletion studies, we showed that depletion of IL-6Rα rescues wound healing in Il6−/− mice. No difference in Stat3 phosphorylation was seen between any of the wounds in the knockout mice, which in each case was less than the phosphorylated Stat3 observed in WT mice. Similarly, the lack of re-epithelialization in both Il6−/− and Il6ra−/− mice, a Stat3-dependent process, argues against this mechanism alone accounting for the rescued wound healing observed in Il6ra−/− mice. Our data imply that IL-6Rα contributes positively to wound healing in an IL-6– and Stat3-dependent manner but potentially hinders wound contraction in an IL-6–independent fashion. This mechanism is unlikely to involve other IL-6 family ligands because they predominantly induce Jak/Stat signaling. However, because select ligands in the IL-6 family, specifically IL-27 p28 and CNTF, have been shown to mediate low-affinity binding to IL-6Rα in vitro (1, 2), we investigated the effect of blocking IL-27 p28 in vivo. We reasoned that if the interaction of this ligand with IL-6Rα accounted for the inhibitory effects of wound contraction by IL-6Rα, then blocking p28 with a specific Ab should rescue wound contraction in Il6−/− mice. Instead, no differences in wound contraction were seen in either WT or Il6−/− mice treated with Abs to p28. CNTF blocking experiments were not performed because CNTF production is restricted to glial and CNS cells, and CNTF does not possess a signal peptide for secretion. Instead, we investigated ERK activation as both a downstream mediator of IL-6Rα signaling (13, 53, 54) and as an important effector of wound contraction (55). ERK phosphorylation was increased in wounds of Il6ra−/− mice but not Il6−/− mice, and blocking ERK activation with a MEK inhibitor delayed wound healing in Il6ra−/− mice. These data suggest that increased ERK activation increases wound contraction in Il6ra−/− mice but not in Il6−/− mice. We observed that topical application of the MEK inhibitor U0126 inhibited wound contraction to a greater extent in Il6ra−/− mice than in WT mice. Although it is tempting to speculate that wound healing in the absence of IL-6Rα may be more dependent on ERK activation than in WT mice, such a conclusion would be premature, because more studies are needed to determine the contributions of the MAPK or other signaling pathways and mechanisms to the healing process in Il6ra−/− mice. In addition, it should be noted that the importance of such a mechanism remains to be determined, because it may prove to be specific to mice or may occur only in the context of the nonphysiological absence of IL-6Rα.

Questions still remain regarding the mechanism by which depletion of IL-6Rα could lead to increased ERK activation, independently of IL-6 signaling. It could be that IL-6Rα plays a role in gp130 internalization and membrane expression, or a cytoplasmic phosphorylation site of IL-6Rα (56) may be involved in ERK suppression in the wound microenvironment. Sparse data suggest that gp130 and/or IL-6Rα interact with several adaptor proteins upstream of ERK activation, such as Grb1, Src homology region 2 domain-containing phosphatase 2, Vav, and Src family kinases (8, 53, 57–60). The depletion of IL-6Rα protein may deregulate the balance between these positive and negative effectors of the MAPK pathway, leading to increased ERK activation. In support of this idea, treatment of HepG2 cells with soluble IL-6Rα in the absence of IL-6 led to a complete inhibition of early growth response protein 1, a gene downstream of ERK activation (61). Similarly, cross-talk between gp130 and/or IL-6Rα and the EGF family receptors has been reported in several tumor cell lines (62–66) and in primary mammary epithelial cells (67). It is also possible that increased ERK activation in the absence of IL-6Rα is due to indirect effects on the expression or activity of other cytokines or on receptors other than gp130. It is not clear what the effect of IL-6Rα deficiency would have on these interactions. However, given the similarity between healing wounds and the tumor microenvironment and given that Abs against IL-6Rα are already in clinical usage in patients with rheumatic diseases, there is a strong rationale for better understanding this novel role for IL-6Rα in regulating ERK activation.

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Disclosures

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