IL-6 promotes acute and chronic inflammatory disease in the absence of SOCS3

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The lack of expression of the suppressor of cytokine signalling-3 (SOCS3) or inactivation of the negative regulatory capacity of SOCS3 has been well documented in rheumatoid arthritis, viral hepatitis and cancer. The specific qualitative and quantitative consequences of SOCS3 deficiency on interleukin-6 (IL-6)-mediated pro- and anti-inflammatory responses remain controversial in vitro and unknown in vivo. Mice with a conditional deletion of SOCS3 in hematopoietic cells develop lethal inflammatory disease during adult life and develop gross histopathological changes during experimental arthritis, typified by elevated IL-6 levels. To clarify the nature of the IL-6 responses in vivo, we generated mice deficient in SOCS3 (SOCS3−/−/C0−vav) or both SOCS3 and IL-6 (IL-6−/−/SOCS3−/−/C0−vav), and examined responses in models of acute and chronic inflammation. Acute responses to IL-1β were lethal to SOCS3−/−/C0−vav mice but not IL-6−/−/SOCS3−/−/C0−vav mice, indicating that IL-6 was required for the lethal inflammation induced by IL-1β. Administration of IL-1β to SOCS3−/−/C0−vav mice induced systemic apoptosis of lymphocytes in the thymus, spleen and lymph nodes that was dependent on the presence of IL-6. IL-6 deficiency prolonged survival of SOCS3−/−/C0−vav mice and ameliorated spontaneous inflammatory disease developing during adult life. Infection of SOCS3−/−/C0−vav mice with LCMV induced a lethal inflammatory response that was dependent on IL-6, despite SOCS3−/−/C0−vav mice controlling viral replication. We conclude that SOCS3 is required for survival during inflammatory responses and is a critical regulator of IL-6 in vivo.

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Interleukin-6 (IL-6) is a multifunctional cytokine, regulating diverse physiological and pathological phenomena including granulocyte development,1 T-cell differentiation,2 hepatocyte generation, acute-phase protein production3,4 and autoimmune disease.5 The expression of IL-6 can be induced in multiple cell types following infection or in response to cytokines including IL-1β and tumour necrosis factor alpha (TNFα).6 IL-6 has a range of pro- and anti-inflammatory activities. IL-6 promotes multiple experimental inflammatory and autoimmune diseases, including autoimmune myocarditis,7 experimental autoimmune encephalomyelitis,8 experimental autoimmune arthritis,9,10 experimental autoimmune myasthenia gravis11 and pristane-induced lupus.12 In contrast, IL-6 prevents chronic autoimmune myocarditis following viral infection,13 suppresses neutrophilia and production of pro-inflammatory cytokines such as TNFα, granulocyte-monocyte colony stimulating factor and interferon-γ, and enhances production of anti-inflammatory mediators such as IL-10, IL-1 receptor antagonist, TNF-soluble receptor and protease inhibitors.4,14–16 Control of IL-6 production and its signalling is therefore critical during inflammation, given such a broad spectrum of activities. The central role of IL-6 in acute and chronic inflammatory diseases is demonstrated by the successful introduction of tocilizumab, a neutralising humanised antibody to the IL-6 receptor, in patients with Castleman’s disease, rheumatoid arthritis and systemic-onset juvenile idiopathic arthritis.17–19

The suppressor of cytokine signalling 3 (SOCS3) is an essential negative regulator of IL-6–gp130 signal transduction.20–22 SOCS3 activity in this context is dependent upon binding to phosphorylated Tyr759 (Tyr 757 in the mouse gp130 receptor);23 An inactivating mutation of Y759 (Y759F) on gp130 (gp130F759) increased IL-6 signalling.24 Embryonic fibroblasts and T cells derived from gp130F759/F759 mutant mice developed autoimmune arthritis, splenomegaly, lymphadenopathy and display defects in B- and T-lymphocyte function.24 This phenotype is similar, but not identical, to mice with a
SOCS3-deficient haematopoietic system that succumb to a lethal inflammatory disease characterised by pericarditis and extensive inflammatory lesions in the peritoneal and pleural cavities.

Although these data collectively demonstrate a critical role for SOCS3 in the negative regulation of IL-6, the precise physiological consequence of SOCS3 deficiency on IL-6-dependent cellular responses remains unknown. Particularly, it is unknown whether SOCS3 determines whether a cellular response to IL-6 is pro- or anti-inflammatory. Yasukawa et al. have proposed that IL-6 delivers an anti-inflammatory signal in the absence of SOCS3, decreasing TNF production from macrophages stimulated with lipopolysaccharide. Consistent with this, mice with a SOCS3 deficiency in macrophages were protected from the lethal effects of galactosamine and lipopolysaccharide administration, a model that is dependent on TNF-induced hepatocyte death and liver failure. It is not known whether mice with a SOCS3 deficiency in haematopoietic cells are also resistant to the effects of high doses of lipopolysaccharide. In contrast, Lang et al. and Croker et al. demonstrated that IL-6 delivers an interferon-γ-like signal in the absence of SOCS3, suggesting that IL-6 delivers a pro-inflammatory signal in the absence of SOCS3, and that SOCS3 thereby modulates the quality of the cellular response to IL-6. In support of this view, mice lacking SOCS3 in blood cells develop severe antigen-induced arthritis and display massive increases in serum IL-6 compared with controls, suggesting that IL-6 is not functioning in an anti-inflammatory manner in this model. To specifically address these opposing models of SOCS3 regulation of IL-6 signalling, we examined the pathophysiological consequences of a deficiency in both SOCS3 and IL-6 using both acute and chronic models of inflammatory disease. We find that in the absence of SOCS3, IL-6 does not function in an anti-inflammatory manner but rather can promote lethal acute and chronic inflammatory diseases.

RESULTS
IL-6 drives chronic inflammatory disease in mice lacking SOCS3
SOCS3−/−/Δαvβ3 mice lack SOCS3 in haematopoietic and endothelial cells, and develop a lethal chronic inflammatory disease characterised by pericarditis, splenomegaly, hepatitis and severe fibronous inflammation in the peritoneal and pleural cavities. Our studies and those of other investigators have demonstrated that SOCS3 negatively regulates IL-6, leukaemia inhibitory factor and granulocyte colony-stimulating factor (G-CSF) signal transduction, but the relative contributions of these cytokines to chronic inflammatory disease is unknown. To determine the role of IL-6 in the development of lethal chronic inflammatory disease in mice deficient in SOCS3, we established cohorts of SOCS3−/−/Δαvβ3 mice and IL-6−/−/SOCS3−/−/Δαvβ3 mice, and followed their survival and tissue pathology. IL-6−/−/SOCS3−/−/Δαvβ3 mice survived for a significantly longer period than did SOCS3−/−/Δαvβ3 mice (Figure 1a), indicating a role for IL-6 in the development of lethal inflammatory disease in mice lacking SOCS3. However, ultimately the majority of mice succumbed to inflammatory diseases. Histological examination of tissues from IL-6−/−/SOCS3−/−/Δαvβ3 mice revealed a similar degree of pathology at the time of death in the liver, heart, lung, spleen and fibronous inflammation surrounding organs in both the peritoneal and pleural cavities, as observed in SOCS3−/−/Δαvβ3 mice (Table 1). To delineate the contribution of haematopoietic and non-haematopoietic cells to this chronic inflammatory disease, we established bone-marrow chimeras by reconstituting lethally irradiated wild-type recipients with wild-type or SOCS3−/−/Δαvβ3 bone marrow cells. As Figure 1b demonstrates, no significant differences in survival were evident in mice receiving SOCS3−/−/Δαvβ3 bone marrow cells compared with mice receiving wild-type bone marrow cells, indicating that non-haematopoietic cells also have a key role in the development of chronic inflammatory disease in SOCS3−/−/Δαvβ3 mice.

IL-6 does not induce acute inflammatory disease in mice lacking SOCS3
Several lines of evidence prompted us to examine whether IL-6 administration could initiate acute inflammatory disease as a single agent or whether additional inflammatory cofactors are required for inflammatory disease. First, as Figure 1 demonstrates, IL-6 has a key role in the development of lethal inflammatory disease. Second, serum IL-6 levels are ~20-fold higher in arthritic SOCS3-deficient animals than in arthritic control animals. To address the role of IL-6 in acute inflammatory disease, we injected SOCS3−/−/Δαvβ3 mice (Table 2) with 1 µg IL-6, twice daily for up to 8 days. IL-6 induced mild splenomegaly in wild-type mice (Table 2), consistent with previous reports on its biological activity in vivo. However, no wild-type or SOCS3−/−/Δαvβ3 mice developed illness during the course of IL-6, and no histopathological features were noted in response to IL-6 injections.

Table 1 IL-6-deficiency in SOCS3−/−/Δαvβ3 mice delays the onset but not the severity of inflammatory disease

|                      | SOCS3−/−/Δαvβ3 | IL-6−/−/SOCS3−/−/Δαvβ3 |
|----------------------|---------------|------------------------|
| Pneumonitis          | 3/3           | 10/10                  |
| Hypergranulocytic marrow | 3/3       | 10/10                  |
| Excess granulocytes in spleen | 3/3      | 10/10                  |
| Leukocytic foci in liver | 3/3       | 7/10                   |
| Pericarditis         | 2/3           | 3/10                   |

Abbreviation: SOCS3, suppressor of cytokine signalling-3.

The proportion of mice affected by inflammatory changes at killing are shown for various organs. Detailed descriptions of the lesions in SOCS3−/−/Δαvβ3 mice are provided in ref. 25. Inflammatory lesions found in the liver, lung, heart, spleen and peritoneal cavity of adult IL-6−/−/SOCS3−/−/Δαvβ3 mice are indistinguishable from SOCS3−/−/Δαvβ3 mice at the time of death.

Figure 1 (a) Survival of SOCS3−/−/Δαvβ3 mice is prolonged in the absence of IL-6. P<0.05, SOCS3−/−/Δαvβ3 versus IL-6−/−/SOCS3−/−/Δαvβ3 by log-rank test. (b) Survival of mice reconstituted with SOCS3−/−/Δαvβ3 bone marrow is not different to mice reconstituted with wild-type bone marrow. Wild-type mice were reconstituted with SOCS3−/−/Δαvβ3 or wild-type bone marrow. P>0.05, SOCS3−/−/Δαvβ3 versus wild type, by log-rank test.
Table 2 IL-6 challenge does not induce pathology in SOCS3\(^{-/-}\) vav mice

| Peripheral blood (\(\times 10^6\) cells per ml) | SOCS3\(^{+/+}\) | SOCS3\(^{-/-}\) vav |
|-----------------|----------------|------------------|
| Platelets       |                |                  |
| Saline          | 1272 ± 399     | 1313 ± 177       |
| IL-6            | 1863 ± 289     | 1564 ± 428       |
| Leukocytes      |                |                  |
| Saline          | 3 ± 2          | 6 ± 5            |
| IL-6            | 5 ± 2          | 5 ± 1            |
| Granulocytes    |                |                  |
| Saline          | 0.3 ± 0.1      | 0.4 ± 0.5        |
| IL-6            | 0.4 ± 0.2      | 0.4 ± 0.2        |
| B cells         |                |                  |
| Saline          | 2 ± 2          | 4 ± 3            |
| IL-6            | 3 ± 1          | 3 ± 1            |
| T cells         |                |                  |
| Saline          | 0.7 ± 0.3      | 2 ± 1            |
| IL-6            | 2 ± 0.4        | 1 ± 1            |
| Spleen (\(\times 10^6\) cells) |                |                  |
| Weight (mg)     |                |                  |
| Saline          | 73 ± 6.4       | 133 ± 77         |
| IL-6            | 121 ± 18*      | 169 ± 26         |
| Leukocytes      |                |                  |
| Saline          | 75 ± 3         | 142 ± 94         |
| IL-6            | 150 ± 28       | 164 ± 18         |
| Granulocytes    |                |                  |
| Saline          | 0.7 ± 0.3      | 6 ± 7            |
| IL-6            | 0.8 ± 0.2      | 4.3 ± 0.9        |
| Erythroid cells |                |                  |
| Saline          | 0.4 ± 0.1      | 4.1 ± 4.2        |
| IL-6            | 14 ± 4         | 16 ± 6           |
| B cells         |                |                  |
| Saline          | 46 ± 5         | 88 ± 61          |
| IL-6            | 87 ± 18        | 87 ± 10          |
| T cells         |                |                  |
| Saline          | 23 ± 2         | 34 ± 15          |
| IL-6            | 33 ± 5         | 38 ± 5           |
| BM (\(\times 10^6\) cells) |                |                  |
| Total cells     |                |                  |
| Saline          | 19 ± 0.1       | 14 ± 6           |
| IL-6            | 22 ± 3         | 16 ± 3           |
| Granulocytes    |                |                  |
| Saline          | 6 ± 0.7        | 7 ± 2            |
| IL-6            | 9 ± 1          | 11 ± 2           |
| Erythroid cells |                |                  |
| Saline          | 6 ± 1          | 3 ± 2            |
| IL-6            | 6 ± 1          | 2 ± 1            |
| B cells         |                |                  |
| Saline          | 4 ± 0.3        | 2 ± 1            |
| IL-6            | 5 ± 2          | 1 ± 0.3          |

Abbreviations: BM, bone marrow; IL, interleukin; SOCS3, suppressor of cytokine signaling 3. Mice were injected with 1 \(\mu\)g IL-6 twice daily for 7 days and analysed at day 8. B220, Ter119, B220 and Thy1 were used as markers for granulocytes, erythroid cells, B cells and T cells, respectively. Leukocyte number from bone marrow is from 2 femurs. Figures represent mean and s.d. from 2 (saline-treated) or 4 (IL-6-treated) mice per group.

\(P<0.05\), saline-treated versus IL-6-treated mice.

demonstrate that, at least at the concentration used here, IL-6 does not induce acute inflammatory disease by itself. Rather, IL-6 seems likely to require the input of other upstream and parallel cytokine signalling pathways regulated directly or indirectly by SOCS3 for initiation and progression of pathology.

IL-6 is required for the lethal effects of IL-1\(\beta\) in SOCS3-deficient mice

Patients with rheumatoid arthritis display high levels of expression of pSTAT3 in synovial tissue. Adenoviral expression of SOCS3 in the intra-articular joint alleviated arthritis in experimental models, and in mouse models of rheumatoid arthritis initiated by IL-1\(\beta\), lack of SOCS3 results in exacerbated synovitis, pannus formation, cartilage and bone destruction and inflammatory exudate in joints. We therefore postulated that SOCS3 was a key negative regulator of IL-6 responses downstream of acute inflammation induced by IL-1\(\beta\). To test this possibility, we administered a 1 \(\mu\)g dose of IL-1\(\beta\) on 2 consecutive days and monitored the incidence of inflammatory disease in SOCS3\(^{-/-}\) vav mice or in IL-6\(^{-/-}\)/SOCS3\(^{-/-}\) vav mice. SOCS3\(^{-/-}\) vav mice became moribund following injections with IL-1\(\beta\) in contrast to IL-6\(^{-/-}\)/SOCS3\(^{-/-}\) vav mice, IL-6\(^{-/-}\) vav mice or wild-type controls, which survived the systemic administration of IL-1\(\beta\) (Figure 2a). This lethal systemic inflammatory response in SOCS3\(^{-/-}\) vav mice injected with IL-1\(\beta\) was reflected by a dramatic increase in levels of MIP1\(\alpha\), MIP1\(\beta\), MIP1\(\alpha\) and MIP1\(\beta\) in serum (Figure 2b)
IL-1β induces loss of cells in the bone marrow (a) and apoptosis of leukocytes in the thymus (b) of SOCS3−/−Δvav mice but not IL-6−/−SOCS3−/−Δvav mice. Tissues were stained with haematoxylin and eosin. (c) Flow-cytometrical analysis of haemopoietic cells in the bone marrow from IL-1β-injected wild-type and SOCS3−/−Δvav mice, 36 h after injection. *P<0.05, mean ± s.d., wild-type versus SOCS3−/−Δvav, n=4 mice per genotype.

MCP1 and G-CSF in IL-6−/−/SOCS3−/−Δvav mice compared with IL-6−/− controls (Figure 2b). A histological survey of tissues from IL-1β-injected SOCS3−/−Δvav mice revealed a dramatically reduced bone-marrow cellularity (Figure 3a), and a high frequency of apoptotic cells in thymus (Figure 3b), spleen and lymph nodes (Supplementary Figure 1), which was not apparent in IL-6−/−/SOCS3−/−Δvav mice, IL-6−/− mice or wild-type controls (Figure 3 and Supplementary Figure 1). Analysis of haematopoietic cells in the bone marrow of IL-1β-injected SOCS3−/−Δvav mice indicated that the reduction in bone-marrow cellularity could be attributed to a 50% reduction in CD11b+Gr1+ cells, B220loIgM- precursor B cells and B220hiIgM+ mature recirculating B cells (Figure 3c).

SOCS3 is required for survival after LCMV challenge

To investigate the role of SOCS3 in the regulation of IL-6 signalling during chronic active viral infection, we challenged mice with LCMV clone 13. This infection causes persistent high-level viremia, and it mimics several human chronic active viral infections. IL-1β and IL-6 are highly expressed during the course of LCMV infection. Consistent with the sensitivity of SOCS3−/−Δvav mice to IL-1β, SOCS3−/−Δvav mice were moribund at day 7 of LCMV clone 13 infection in contrast to IL-6−/−/SOCS3−/−Δvav mice, of which only one of five became moribund, and wild-type mice that remained healthy (Figure 4a).

To assess T-cell responses to LCMV, we used tetramers specific for the LCMV epitopes (GP33-41, GP276-286 and NP396-404). A significant increase in total numbers of NP396-specific CD8+ T cells was found in SOCS3−/−Δvav mice compared with IL-6−/−SOCS3−/−Δvav mice (Figure 4b). No differences in viral titres were evident in the liver, lung, kidney and spleen of SOCS3−/−Δvav or IL-6−/−SOCS3−/−Δvav mice compared with controls (Figure 4c).

DISCUSSION

In this study, we demonstrate a key role for SOCS3 in the regulation of IL-6-dependent inflammatory responses in vivo. The absence of SOCS3 expression promotes a pathophysiological response to IL-6 during viral infection, acute inflammation induced by IL-1β and adult life. The data indicate that the hypersensitivity of SOCS3−/−Δvav mice to viral infection is not attributable to an inability to contain viral replication. Rather, the data support a role for SOCS3 in the pathogenesis of viral infection by regulating responses to IL-6. We propose that an evaluation of the role of SOCS3 in regulating responses to cytokines, in addition to overall cytokine levels, will provide novel insight into the pathogenesis of acute and chronic inflammatory diseases.

SOCS3−/−Δvav mice develop a lethal inflammatory disease during adult life. We demonstrate that the survival of adult SOCS3−/−Δvav
mice is prolonged in the absence of IL-6, supporting a pathological role of IL-6 in the absence of SOCS3. Monitoring of bone-marrow chimeras reconstituted with wild-type or SOCS3-deficient haematopoietic cells indicated key roles for non-haematopoietic cells in the development of inflammatory disease in SOCS3−/−/Dvav mice. These data are consistent with previous observations that non-haematopoietic tissues contribute to the pathological effects of G-CSF administration in SOCS3−/−/Dvav mice.25 Because IL-6 alone seems unable to induce a lethal inflammatory response when injected in SOCS3−/−/Dvav mice, we suggest that IL-6 synergises with IL-1β or that the biological effects of IL-6 require the actions of other cytokines induced by IL-1β, such as G-CSF. The data presented in Figure 2b indicate that SOCS3 is a critical regulator of IL-6-independent production of cytokines following IL-1β challenge, and we suggest that the synergistic actions of IL-6, G-CSF, MCP1, MIP1α and MIP1β drive the lethal systemic inflammatory response in SOCS3−/−/Dvav mice. We have previously demonstrated that G-CSF, rather than being well tolerated, becomes toxic in the absence of SOCS3, causing neutrophilic infiltration and destruction of multiple tissues.25 Our previous studies also indicate that SOCS3 regulates both the quality and the quantity of signalling downstream of the IL-6 and G-CSF receptors.20,31 The enhanced activation of STAT3 and STAT1, and the profound changes in gene transcription profiles induced by IL-6 and G-CSF in SOCS3-deficient cells,20,22,25,31 may underlie this lethal systemic inflammatory response by preventing appropriate resolution of inflammation triggered by IL-1β or LCMV.

The widespread induction of apoptosis of SOCS3-deficient lymphocytes in response to IL-1β may be a consequence of excessive STAT3 activation, converting pro-survival signals to pro-apoptotic signals, as demonstrated for SOCS3-deficient murine embryonic fibroblasts stimulated with leukaemia inhibitory factor.32 These in vivo studies suggest that SOCS3 may have key roles in regulating a hallmark condition of sepsis, the systemic apoptosis of lymphocytes.33 The data support key roles for SOCS3 in the regulation of inflammatory responses instigated by viral infection and IL-1β-driven inflammatory responses.

METHODS
Mice
C57BL/6, IL-6−/−, vav-Cre/SOCS3−/Δav (SOCS3−/Δav)25 and IL-6−/−/SOCS3−/Δav mice were bred at The Walter and Eliza Hall Institute of Medical Research with unlimited access to food and water. Experiments were conducted in accordance with institute animal ethics guidelines and approval.

IL-1 and IL-6 challenge
For IL-1 challenge experiments, 1 μg IL-1β (eBioscience, San Diego, CA, USA) in 0.2 ml saline was injected intraperitoneally every 12 h for 36 h. For IL-6 challenge experiments, 1 μg IL-6 was injected intraperitoneally every 12 h for 7 days. The bioactivity of IL-6 was confirmed using clonogenic bone marrow progenitor cell assays.25 Tissues were fixed in 10% buffered formalin, embedded in paraffin and 1 μm (bone) or 2 μm (spleen, thymus and lymph node) sections were stained with haematoxylin and eosin. Serum cytokines were analysed using Luminex beads according to the manufacturer’s instructions (Bio-Rad, Hercules, CA, USA).
Bone marrow chimeras
For reconstitution experiments, congeneric C57BL/6SJL (Ptpα<sup>−/−</sup> Pebp<sup>−/−</sup>Ly5.1<sup>−/−</sup>) mice were reconstituted with 5 × 10<sup>5</sup> C57BL/6 Ptpα<sup>−/−</sup> Pebp<sup>−/−</sup>Ly5.2+ bone marrow cells from either vavCre<sup>−/−</sup>Socs3<sup>−/−</sup> or vavCre<sup>−/−</sup>Socs3<sup>+/+</sup> genotype after 2.5 Gy doses of irradiation given 3 h apart. The reconstitution of recipients with donor cells was consistently greater than 80% in the bone marrow, peripheral blood and lymph node.

Flow cytometry
Haematopoietic cells from the bone marrow, spleen and peripheral blood were analysed using antibodies specific for CD45R (B220), IgM, CD11b, CD45.1, CD45.2, Gr1, Ter119 and Thy1 (provided by Dr A Strasser, Walter and Eliza Hall Institute of Medical Research, Parkville, Australia). Blood was collected into tubes containing EDTA (Becton Dickinson, Franklin Lakes, NJ, USA) and analysed using an Advia 120 analyser (Bayer, Leverkusen, Germany).

Lymphocytic choriomeningitis virus infection
Mice were infected with 2 × 10<sup>6</sup> plaque forming units lymphocytic choriomeningitis virus (LCMV) clone 13 (ref. 34). Staining of LCMV-specific T cells with tetramers was carried out as described previously. For virus quantification, organs were weighed and homogenised using the Qiagen TissueLyser (Qiagen). Mice were killed at the time points indicated, organs were weighed and homogenised using the Qiagen TissueLyser (Qiagen). The number of viral copies was determined by real-time PCR with SYBR Green detection (Qiagen). The number of viral copies was determined by real-time PCR with SYBR Green detection (Qiagen). The number of viral copies was determined by real-time PCR with SYBR Green detection (Qiagen). The number of viral copies was determined by real-time PCR with SYBR Green detection (Qiagen).

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CONFLICT OF INTEREST
The authors declare no conflict of interest.