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

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Abstract

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 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*$^{-/\Delta vav}$) or both SOCS3 and IL-6 (*IL-6*$^{-/-}$/*SOCS3*$^{-/\Delta\ vav}$) and examined responses in models of acute and chronic inflammation. Acute responses to IL-1β were lethal to *SOCS3*$^{-/\Delta\ vav}$ mice but not *IL-6*$^{-/-}$/*SOCS3*$^{-/\Delta\ vav}$ mice, indicating that IL-6 was required for the lethal inflammation induced by IL-1β. Administration of IL-1β to *SOCS3*$^{-/\Delta\ vav}$ 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*$^{-/\Delta\ vav}$ mice and ameliorated spontaneous inflammatory disease developing during adult life. Infection of *SOCS3*$^{-/\Delta\ vav}$ mice with LCMV induced a lethal inflammatory response that was dependent on IL-6, despite *SOCS3*$^{-/\Delta\ vav}$ mice controlling viral replication. We conclude that

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DISCLOSURES

The authors declare no financial conflicts of interest.

SOCS3 is required for survival during inflammatory responses and is a critical regulator of IL-6 *in vivo*.

Keywords

SOCS3; IL-6; IL-1β; Suppressor of Cytokine Signaling-3

INTRODUCTION

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 production [3, 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 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 gravis [11] 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, GM-CSF and IFN-γ, and enhances production of anti-inflammatory mediators such as IL-10, IRAP, 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 (*gp130*^{F759}) increased IL-6 signalling [24]. Embryonic fibroblasts and T cells derived from gp130$^{F759/F759}$ mutant mice displayed prolonged IL-6/sIL-6R-induced phosphorylation of gp130, Jak1 and STAT3, consistent with a role for SOCS3 in the negative regulation of IL-6-gp130 signalling [24]. Mutant gp130$^{F759/F759}$ mice develop 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 hematopoietic system that succumb to a lethal inflammatory disease characterised by pericarditis and extensive inflammatory lesions in the peritoneal and pleural cavities [25].

While 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 LPS [22]. Consistent with this, mice with a SOCS3 deficiency in macrophages were protected from the lethal effects of galactosamine and LPS administration, a model that is dependent on TNF-induced hepatocyte death and liver failure. It is not known if mice with a SOCS3-deficiency in hematopoietic cells are also resistant to the effects of high doses of LPS [22]. In contrast, Lang et al. and Croker et al. demonstrated that IL-6 delivers an IFNγ-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 [20, 21]. 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 to controls, suggesting that IL-6 is not acting in an anti-inflammatory manner in this model [26]. 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 act in an anti-inflammatory manner but rather can promote lethal acute and chronic inflammatory diseases.

MATERIALS AND METHODS

Mice

C57BL/6J, *IL-6*$^{-/-}$, *vav-Cre/SOCS3*$^{-/loxP}$ (*SOCS3*$^{-/\Delta\ vav}$) [25] and *IL-6/SOCS3*$^{-/\Delta\ vav}$ 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) 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 hematoxylin and eosin. Serum cytokines were analysed using Luminex beads according to the manufacturer's instructions (Biorad).

Bone marrow chimeras

For reconstitution experiments, congenic C57BL/6.SJL *(Ptprc*^{a} *Pep3*^{b} *(Ly5.1))* mice were reconstituted with 5×10^6 C57BL/6 (Ptprc^{b} Pep3^{a} (Ly5.2)) bone marrow cells from either *vavCre*$^{+}$*SOCS3*$^{-/fl}$ or *vavCre*$^{-}$*SOCS3*$^{+/+}$ genotype after two 5.5 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

Hematopoietic 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) and analysed using an Advia 120 analyser (Bayer).

LCMV infection

Mice were infected with 2×10^6 plaque forming units (pfu) LCMV clone 13.[27] Staining of LCMV-specific T cells with tetramers was performed as described previously[28]. For virus quantification, organs were weighed and homogenized using the Qiagen TissueLyser and LCMV titers were determined by focus forming assays using MC57 fibroblast cells as previously described [29].

RESULTS

IL-6 drives chronic inflammatory disease in mice lacking SOCS3

SOCS3$^{-/\Delta\ vav}$ mice lack SOCS3 in hematopoietic and endothelial cells, and develop a lethal chronic inflammatory disease characterised by pericarditis, splenomegaly, hepatitis and severe fibrinous inflammation in the peritoneal and pleural cavities [25]. Our studies and those of other investigators have demonstrated that SOCS3 negatively regulates IL-6, LIF and 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*$^{-/\Delta\ vav}$ mice and *IL-6*$^{-/-}$/*SOCS3*$^{-/\Delta\ vav}$ mice, and followed their survival and tissue pathology. *IL-6*$^{-/-}$/*SOCS3*$^{-/\Delta\ vav}$ mice survived for a significantly longer period than did *SOCS3*$^{-/\Delta\ vav}$ 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*$^{-/\Delta\ vav}$ mice revealed a similar degree of pathology at the time of death in the liver, heart, lung, spleen and fibrinous inflammation surrounding organs in both the peritoneal and pleural cavities, as observed in *SOCS3*$^{-/\Delta\ vav}$ mice (Table 1). To delineate the contribution of hematopoietic and non-hematopoietic cells to this chronic inflammatory disease, we established bone marrow chimeras by reconstituting lethally-irradiated wild-type recipients with wild-type or *SOCS3*$^{-/\Delta\ vav}$ bone marrow cells. As Figure 1B demonstrates, no significant differences in survival were evident in mice receiving *SOCS3*$^{-/\Delta\ vav}$ bone marrow cells compared to mice receiving WT bone marrow cells, indicating that non-hematopoietic cells also play a key role in the development of chronic inflammatory disease in *SOCS3*$^{-/\Delta\ vav}$ mice.

IL-6 does not induce acute inflammatory disease in mice lacking SOCS3

Several lines of evidence prompted us to examine if IL-6 administration could initiate acute inflammatory disease as a single agent or whether additional inflammatory cofactors are required for inflammatory disease. Firstly, as Figure 1 demonstrates, IL-6 plays a key role in development of lethal inflammatory disease. Secondly, serum IL-6 levels are approximately 20-fold higher in arthritic SOCS3-deficient animals than in arthritic control animals [26]. To address the role of IL-6 in acute inflammatory disease, we injected *SOCS3*$^{-/\Delta\ vav}$ 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*

[30]. However, no wild-type or *SOCS3*$^{-/\Delta\ vav}$ mice developed illness during the course of IL-6 and no histopathological features were noted in response to IL-6 injections. These data 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-1β in SOCS3-deficient mice

Patients with rheumatoid arthritis display high levels of expression of pSTAT3 in synovial tissue [31]. Adenoviral expression of *SOCS3* in the intra-articular joint alleviated arthritis in experimental models [31], and in mouse models of rheumatoid arthritis initiated by IL-1β, lack of SOCS3 results in exacerbated synovitis, pannus formation, cartilage and bone destruction and inflammatory exudate in joints [26]. We therefore postulated that SOCS3 was a key negative regulator of IL-6 responses downstream of acute inflammation induced by IL-1β. To test this possibility, we administered a 1 μg dose of IL-1β on two consecutive days and monitored the incidence of inflammatory disease in *SOCS3*$^{-/\Delta\ vav}$ mice or in *IL-6*$^{-/-}$/*SOCS3*$^{-/\Delta\ vav}$ mice. *SOCS3*$^{-/\Delta\ vav}$ mice became moribund following injections with IL-1β in contrast to *IL-6*$^{-/-}$/*SOCS3*$^{-/\Delta\ vav}$ mice, *IL-6*$^{-/-}$ mice or wild-type controls, which survived the systemic administration of IL-1β (Figure 2A). This lethal systemic inflammatory response in *SOCS3*$^{-/\Delta\ vav}$ mice injected with IL-1β was reflected by a dramatic increase in levels of MIP1α, MIP1β, MCP1 and G-CSF in *IL-6*$^{-/-}$/*SOCS3*$^{-/\Delta\ vav}$ mice compared to *IL-6*$^{-/-}$ controls (Figure 2B). A histological survey of tissues from IL-1β-injected *SOCS3*$^{-/\Delta\ vav}$ 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), that was not apparent in *IL-6*$^{-/-}$/*SOCS3*$^{-/\Delta\ vav}$ mice, *IL-6*$^{-/-}$ mice or wild-type controls (Figure 3 and Supplementary Figure 1). Analysis of haemopoietic cells in the bone marrow of IL-1β-injected *SOCS3*$^{-/\Delta\ vav}$ mice indicated that the reduction in bone marrow cellularity could be attributed to a 50% reduction in $CD11b^{+}Gr1^{+}$ cells, $B220^{lo}IgM^{-}$ precursor B cells and $B220^{hi}IgM^{+}$ 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 signaling during chronic active viral infection, we challenged mice with lymphocytic choriomeningitis virus (LCMV) clone 13. This infection causes persistent high level viremia and it mimics several human chronic active viral infections [32]. IL-1β and IL-6 are highly expressed during the course of LCMV infection [28]. Consistent with the sensitivity of *SOCS3*$^{-/\Delta\ vav}$ mice to IL-1β, *SOCS3*$^{-/\Delta\ vav}$ mice were moribund at day 7 of LCMV clone 13 infection in contrast to *IL-6*$^{-/-}$/*SOCS3*$^{-/\Delta\ vav}$ mice, of which only one of five became moribund, and wild-type mice which 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*$^{-/\Delta\ vav}$ mice compared to *IL-6*/*SOCS3*$^{-/\Delta\ vav}$ mice (Figure 4B). No differences in viral titres were evident in the liver, lung, kidney and spleen of *SOCS3*$^{-/\Delta\ vav}$ or *IL-6*/*SOCS3*$^{-/\Delta\ vav}$ mice compared to controls (Figure 4C).

DISCUSSION

Here 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*$^{-/\Delta vav}$ mice to viral infection is not attributable to an inability to contain viral replication. Rather, the data supports 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$^{-/\Delta\ vav}$ mice develop a lethal inflammatory disease during adult life [25]. We demonstrate that the survival of adult *SOCS3*$^{-/\Delta\ vav}$ 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 hematopoietic cells indicated key roles for non-hematopoietic cells in the development of inflammatory disease in *SOCS3*$^{-/\Delta\ vav}$ mice. These data are consistent with previous observations that non-hematopoietic tissues contribute to the pathological effects of G-CSF administration in *SOCS3*$^{-/\Delta\ vav}$ mice [25]. Because IL-6 alone appears unable to induce a lethal inflammatory response when injected in *SOCS3*$^{-/\Delta\ vav}$ 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*$^{-/\Delta\ vav}$ 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, 33]. 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, 33], 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 leukemia inhibitory factor (LIF) [34]. These *in vivo* studies suggest that SOCS3 may play key roles in regulating a hallmark condition of sepsis, the systemic apoptosis of lymphocytes [35]. The data support key roles for SOCS3 in the regulation of inflammatory responses instigated by viral infection and IL-1β-driven inflammatory responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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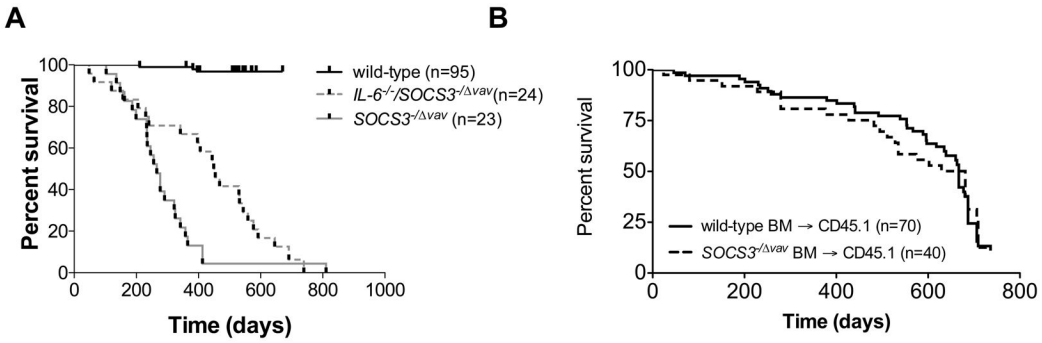


Figure 1.

(A) Survival of *SOCS3*$^{-/\Delta\ vav}$ mice is prolonged in the absence of IL-6. $p<0.05$, *SOCS3*$^{-/\Delta\ vav}$ v *IL-6*$^{-/-}$*/SOCS3*$^{-/\Delta\ vav}$, by log-rank test. (B) Survival of mice reconstituted with *SOCS3*$^{-/\Delta\ vav}$ bone marrow is not different to mice reconstituted with wild-type bone marrow. Wild-type mice were reconstituted with *SOCS3*$^{-/\Delta\ vav}$ or wild-type bone marrow. $p>0.05$, *SOCS3*$^{-/\Delta\ vav}$ v wild-type, by log-rank test.

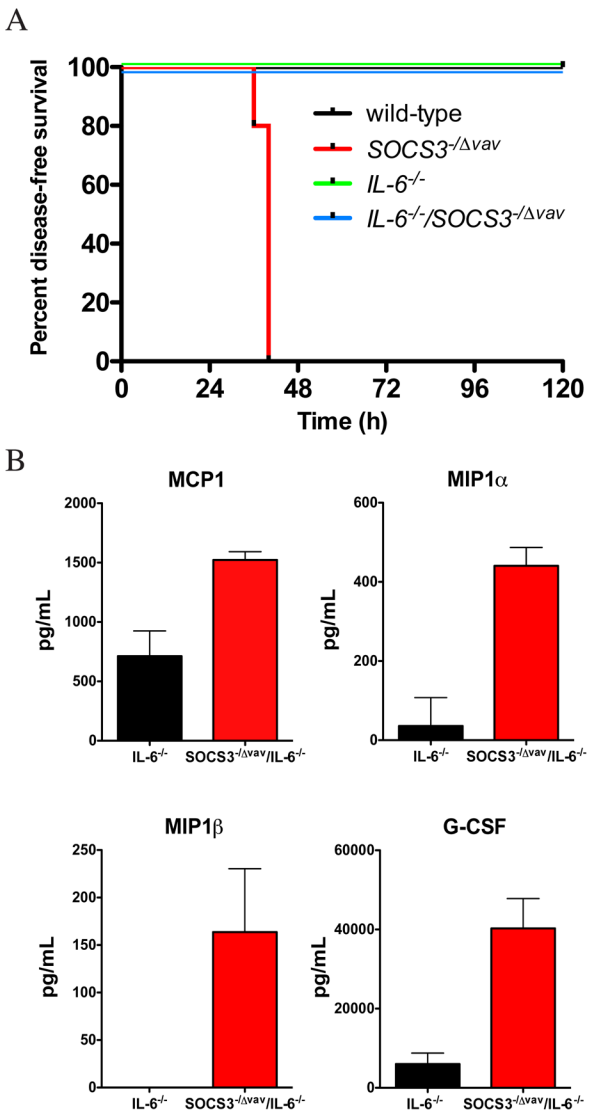


Figure 2.

SOCS3 regulates responses to IL-1β *in vivo*. (A) *SOCS3*$^{-/\Delta\ vav}$ mice are hypersensitive to IL-1β, resulting from loss of regulation of IL-6. n=4–5 per group. (B) SOCS3 regulates cytokine production independently of IL-6 after challenge with IL-1β. Cytokine production was analysed in the serum of mice injected with IL-1β.

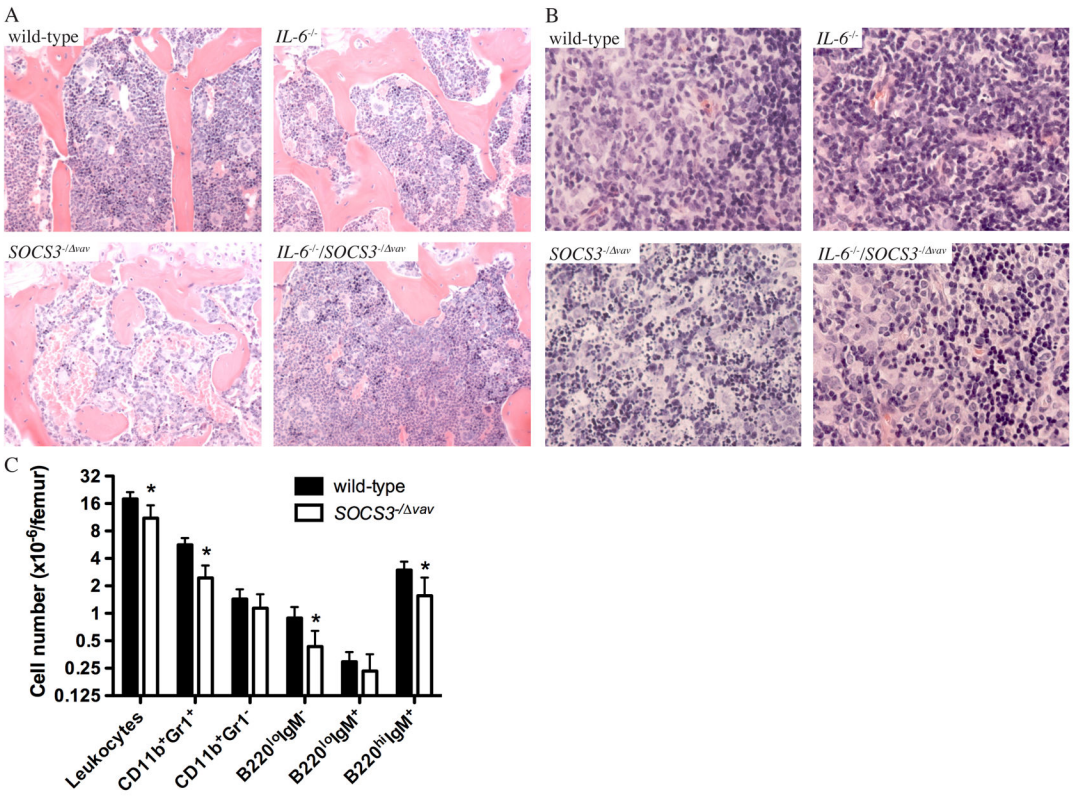


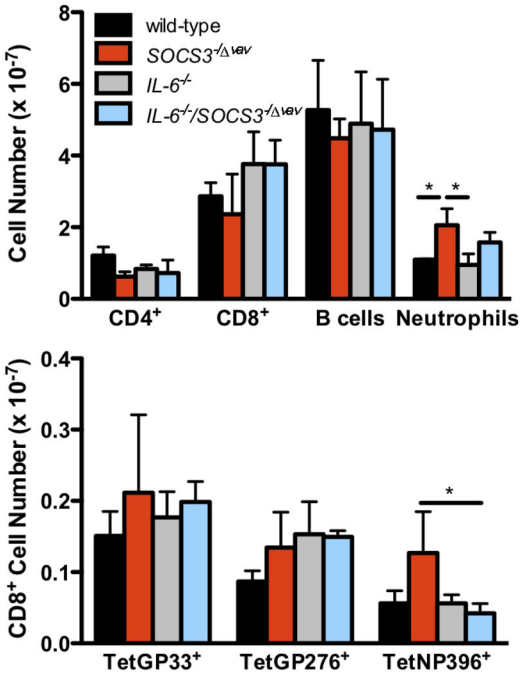
Figure 3.

IL-1β induces loss of cells in the bone marrow (A) and apoptosis of leukocytes in the thymus (B) of *SOCS3*$^{-/\Delta\ vav}$ mice but not *IL-6*$^{-/-}$/*SOCS3*$^{-/\Delta\ vav}$ mice. Tissues were stained with haematoxylin and eosin. (C) Flow cytometric analysis of haemopoietic cells in the bone marrow from IL-1β-injected wild-type and *SOCS3*$^{-/\Delta\ vav}$ mice, 36 h after injection.

A

Genotype	Number moribund at Day 7
wild-type	0/6
IL-6$^{-/-}$	0/6
Socs3$^{-/\Delta vav}$	6/6
IL-6$^{-/-}$/*Socs3*$^{-/\Delta vav}$	1/5

B



C

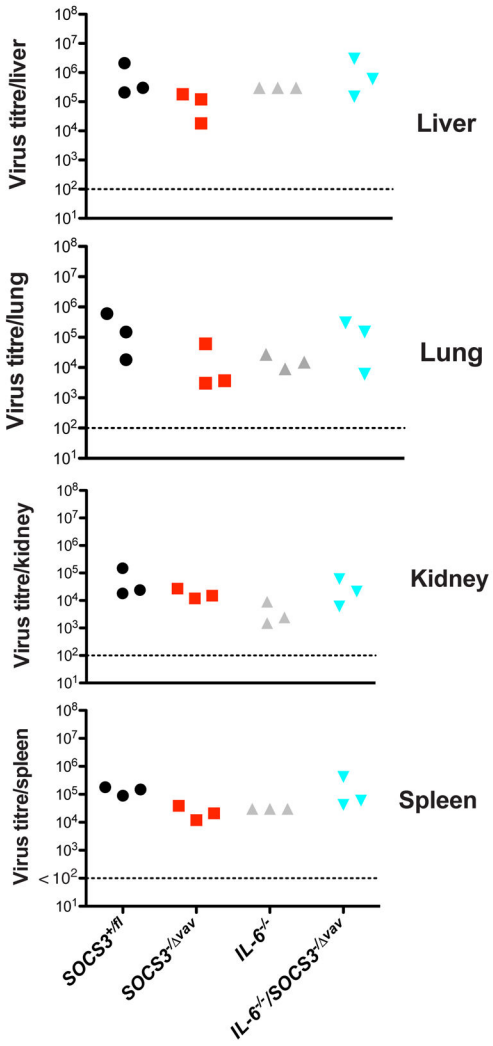


Figure 4.

SOCS3 is essential for survival to LCMV but not essential for the generation of LCMV-specific CD8$^+$ T cell responses or the containment of viral replication. (A) Mice that were moribund at day 7 of LCMV infection. (B) Hematopoietic cell populations were measured by flow cytometry on day 7 of LCMV infection. LCMV-specific CD8+ T cells were identified using tetramers specific for LCMV epitopes GP33-41, GP276-286 and NP396-404. (C) Viral titres in lung, kidney, liver and spleen were assessed on day 7. *p<0.05, ANOVA and SNK.

Table 1

IL-6 deficiency in *SOCS3*$^{-/\Delta\ vav}$ mice delays the onset but not the severity of inflammatory disease. The proportion of mice affected by inflammatory changes at sacrifice are shown for various organs. Detailed descriptions of the lesions in *SOCS3*$^{-//\Delta vav}$ mice are provided in [25]. Inflammatory lesions found in the liver, lung, heart, spleen and peritoneal cavity of adult *IL-6*$^{-/-}$*/SOCS3*$^{-/\Delta\ vav}$ mice are indistinguishable from *SOCS3*$^{-/\Delta\ vav}$ mice at the time of death.

	SOCS3$^{-/\Delta vav}$	**IL-6**$^{-/-}$**/SOCS3**$^{-/\Delta\ vav}$
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

Table 2

IL-6 challenge does not induce pathology in *SOCS3*$^{-/\Delta vav}$ mice

			SOCS3$^{+/fl}$	SOCS3$^{-/\Delta\ vav}$
Peripheral Blood (×10^6/mL)	**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 (×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±4
BM (×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

Mice were injected with 1 μg IL-6 twice daily for 7 days and analysed day 8. Gr1, 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 standard deviation from 2 (saline-treated) or 4 (IL-6-treated) mice per group.

* $p<0.05$, saline-treated versus IL-6-treated.