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Article

SENP1 regulates IFN-γ–STAT1 signaling through STAT3–SOCS3 negative feedback loop

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Interferon-γ (IFN-γ) triggers macrophage for inflammation response by activating the intracellular JAK–STAT1 signaling. Suppressor of cytokine signaling 1 (SOCS1) and protein tyrosine phosphatases can negatively modulate IFN-γ signaling. Here, we identify a novel negative feedback loop mediated by STAT3–SOCS3, which is tightly controlled by SENP1 via de-SUMOylation of protein tyrosine phosphatase 1B (PTP1B), in IFN-γ signaling. SENP1-deficient macrophages show defects in IFN-γ signaling and M1 macrophage activation. PTP1B in SENP1-deficient macrophages is highly SUMOylated, which reduces PTP1B-induced de-phosphorylation of STAT3. Activated STAT3 then suppresses STAT1 activation via SOCS3 induction in SENP1-deficient macrophages. Accordingly, SENP1-deficient macrophages show reduced ability to resist *Listeria monocytogenes* infection. These results reveal a crucial role of SENP1-controlled STAT1 and STAT3 balance in macrophage polarization.

Keywords: IFN-γ, macrophage, SENP1, SUMOylation

Introduction

Microenvironment signals including cytokines, growth factors, and microorganism-associated molecular patterns drive macrophage polarization. These signals activate stimulus-specific transcription factors, which is likely to dictate the functional polarization of macrophages through effects on inducible gene promoters with specific features (Stark and Darnell, 2012). For example, type II interferon (IFN-γ) signaling activates the canonical JAK–STAT1 pathway leading to macrophage function toward the M1 phenotype, whereas interleukin (IL-4) or IL-13 activates the JAK–STAT6 pathway leading to macrophage function toward the M2 phenotype (Levy and Darnell, 2002; Ramana et al., 2002). In addition, IL-10 activates the JAK–STAT3 pathway that is associated with M2-like macrophages (Yu et al., 2009). Thus, STATs are pivotal factors in regulating macrophage polarization.

The STAT family consists of seven members (Darnell et al., 1994; Stark and Darnell, 2012). Among them, STAT1 is activated by IFN-γ (Levy and Darnell, 2002; Ramana et al., 2002; Varinou et al., 2003) during M1 macrophage activation. As a downstream target of cytokine or growth factor receptors, STAT3 always induces expressions of genes (Il10, Tgfb1, Mrc1) associated with M2-like macrophage phenotype to counteract inflammation induced by STAT1 (Hong et al., 2002; Qing and Stark, 2004; Regis et al., 2008). Interestingly, STAT1 and STAT3 activation can be regulated reciprocally (Regis et al., 2008). For example, IFN-γ activates macrophages through STAT3 signaling in *Stat1−/−* cells. STAT1 can thus function as a dominant regulator to suppress STAT3 activity in response to IFN-γ signaling.

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The mechanisms by which STAT1 inhibits STAT3 remain unclear but may include the competition for binding to docking sites of IFN-γ receptors, tyrosine kinases, or other protein factors that required for STAT activation. The disruption of STAT1:STAT3 balance may lead to certain pathological conditions.

IFN produced by activated T cells or NK cells is responsible for M1 macrophage activation (Hu et al., 2002). Biochemical and genetic studies showed that IFN-γ signal is mainly mediated by JAK1/2–STAT1 signaling pathway (Fu et al., 1990; Flynn et al., 1993; Darnell et al., 1994; Durbin et al., 1996; Meraz et al., 1996; Platanias, 2005; Yu et al., 2009; Stark and Darnell, 2012). IFN-γ binds to its cell-surface receptor, triggers receptor-associated JAK1 and JAK2 auto-phosphorylation, and followed by phosphorylation of an IFNγR1 tyrosine residue, which serves as a docking site predominantly for STAT1 (Darnell et al., 1994; Sakatsume et al., 1995; Bach et al., 1996; Kaplan et al., 1996; Bach et al., 1997; Pestka et al., 2004; Platanias, 2005; Stark and Darnell, 2012). IFNγR1-recruited STAT1 is phosphorylated on tyrosine 701 by JAK, dimerizes, and then translocates to the nucleus where it can bind to a regulatory DNA element termed gamma-activated sequence (GAS), which is important for regulating gene expression (Levy and Darnell, 2002; Varinou et al., 2003; Qing and Stark, 2004; Stark and Darnell, 2012). Major mechanisms that are responsible for negative regulation of IFN-γ signaling in cells include STAT dephosphorylation by tyrosine phosphatases and JAK catalytic inhibition by suppressor of cytokine signaling 1 (SOCS1) protein (David et al., 1993; Alexander et al., 1999; Marine et al., 1999; Chen et al., 2000; Kinjyo et al., 2002; ten Hoeve et al., 2002). SOCS1 is a target gene in IFN-γ signaling. Thus, IFN-γ induces SOCS1 feedback inhibition of JAK to restrain its own activity (Alexander et al., 1999; Marine et al., 1999; Chen et al., 2000; Kinjyo et al., 2002).

SUMO (also called Sentrin) is a novel ubiquitin-like protein that can covalently modify a large number of proteins. SUMO modification has now emerged as an important regulatory mechanism in many signaling pathways through alternations of its targeting protein functions. SUMOylation is catalyzed by activating enzyme (E1), conjugating enzyme (E2), as well as ligating enzyme (E3). It can be reversed by a family of Sentrin/SUMO-specific proteases (SENPs) (Hay, 2007; Yeh, 2009). Mounting evidence demonstrates that SENP family members play crucial roles in determining the protein SUMOylation status and activity (Cheng et al., 2007; Hay, 2007; Yeh, 2009). We previously reported that SENP1 is involved in hypoxia signaling, angiogenesis, T and B cell development, and mitochondrial biogenesis via de-SUMOylation of distinct targets (Cheng et al., 2007; Cai et al., 2012; Van Nguyen et al., 2012).

Here, we report SENP1 as a crucial regulator in IFNγ–STAT1 signaling as well as M1 macrophage polarization. SENP1-deficient macrophages show defects in IFN-γ signaling and M1 macrophage activation. We further observe that protein tyrosine phosphatase 1B (PTP1B) is highly SUMOylated in SENP1-deficient macrophages, which reduces its phosphatase activity in STAT3 dephosphorylation. Activated STAT3 in SENP1-deficient macrophages then suppresses STAT1 activation through SOCS3 induction, and consequently induces the downregulation of IFN-γ signaling as well as M1 polarization.

Results

SENP1 is essential for IFN-γ signaling in macrophages

To examine whether SENP1 plays a role in macrophage activation, we first treated the macrophages, which were isolated from *Senp1*+/+ or *Senp1*−/− fetal livers, with IFN-γ, an inducer for M1 macrophage activation. The induction of IFN-γ-dependent genes was impaired in *Senp1*−/− macrophages compared with *Senp1*+/+ macrophages (Figure 1A). The peritoneal macrophages obtained from *Senp1*+/− mouse (Supplementary Figure S1) or *Senp1*-silenced RAW264.7 cells (Supplementary Figure S2) also showed similar defects in the expression of these genes (Figure 1B and Supplementary Figure S3), suggesting an essential role of SENP1 in IFN-γ-induced gene expression. We then overexpressed SENP1 and SENP1 catalytic mutant in THP-1 cells. The expression of SENP1 but not its catalytic mutant induced the expression of IFN-γ-dependent genes comparable to IFN-γ treatment (Figure 1C), indicating that de-SUMOylation activity is requested for SENP1 to promote IFN-γ activation. We further analyzed the activation status of STAT1, a key transcription factor activated by IFN-γ in SENP1-deficient macrophages. As shown in Figure 1D, IFN-γ-induced phosphorylation of STAT1 (Y701) was significantly reduced in *Senp1*−/− macrophages or *Senp1*+/− macrophages compared with *Senp1*+/+ macrophages. We observed similar changes in si-SENP1-RAW264.7 cells in comparison with the control macrophages (Supplementary Figure S4).

STAT3–SOCS3 mediates an alternative feedback inhibition of IFN-γ signaling in SENP1-deficient macrophages

To understand how IFN-γ signaling is impaired in SENP1-deficient macrophages, we determined the IFN-γ-induced JAK1 and JAK2 activation. We observed that JAK2 but not JAK1 activation decreased in SENP1-deficient macrophages (Figure 2A and Supplementary Figure S4). However, the impaired JAK2 activation was not attributed to defective IFN-γ receptor expression in SENP1-deficient macrophages, as IFNγR1 and IFNγR2 expressions in *Senp1*−/− macrophages were elevated (Supplementary Figure S5), which might be a compensation response to the defect in IFN-γ signaling.

Given the fact that JAK activity is negatively regulated by SOCS-mediated feedback loop (Chen et al., 2000; Krebs and Hilton, 2001; Kinjyo et al., 2002), we explored whether SOCS activity contributed to the downregulation of JAK2 in SENP1-deficient macrophages. SOCS1, a well-known negative regulator of the JAK–STAT1 pathway (Alexander et al., 1999; Krebs and Hilton, 2001), was decreased upon IFN-γ treatment in these *Senp1*-silenced macrophages (Figure 2B). In contrast, SOCS3, a negative feedback regulator of the JAK–STAT3 pathway, markedly increased in these *Senp1*-silenced macrophages upon treatment (Figure 2B and C). Since SOCS3 negatively regulates JAK activity in macrophages in response to IL-6 stimulation (Croker et al., 2003), we speculated that increased expression of SOCS3 might be responsible for the downregulation of JAK2 activity in SENP1-deficient macrophages. To verify this, we silenced *Socs3* in si-SENP1-RAW264.7 cells (Supplementary Figure S6) and examined

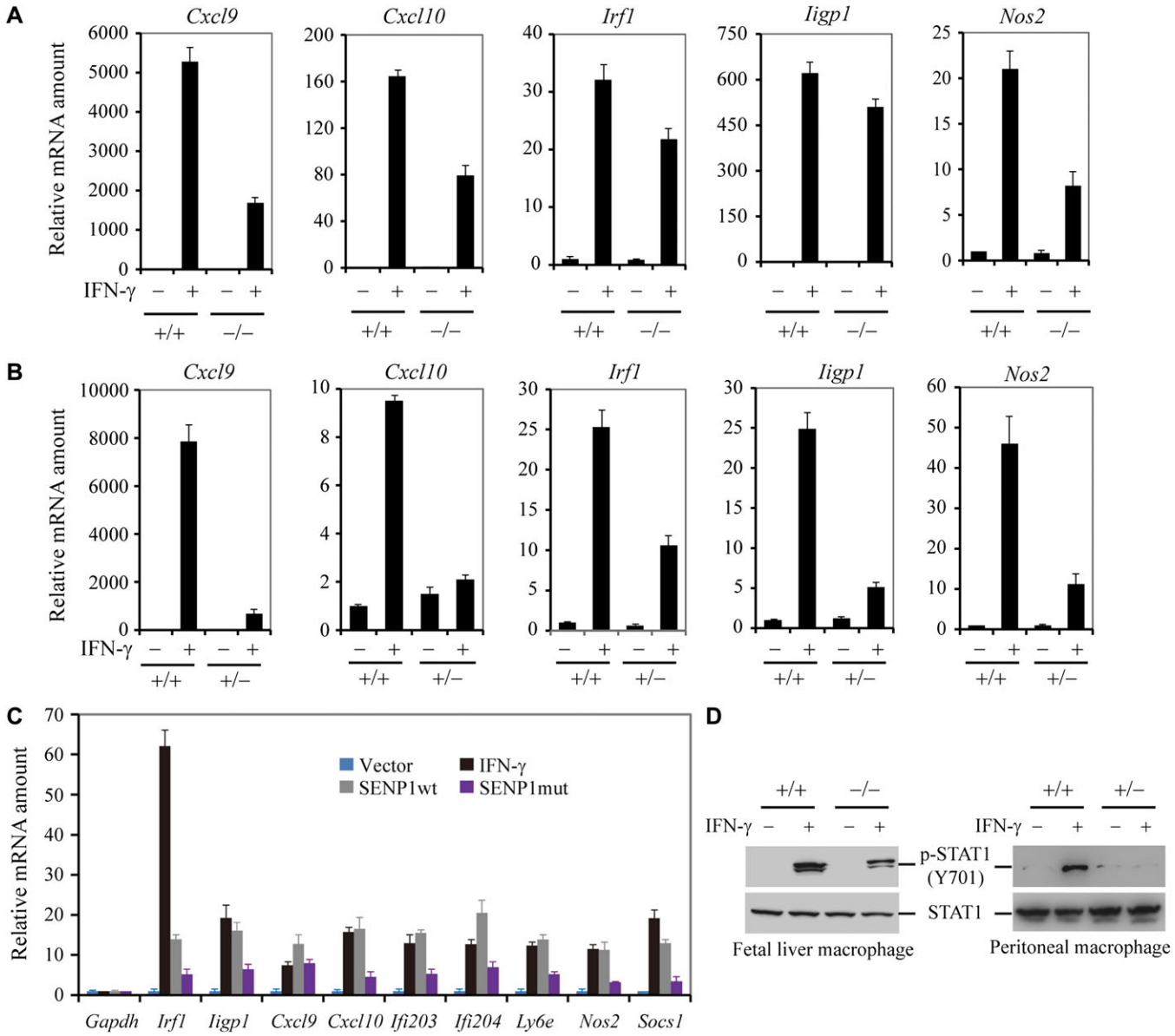

Figure 1 SENP1 is essential for the activation of IFN-γ signaling. (**A**) IFN-γ-induced gene expression in *Senp1–/–* macrophages was lower than that in *Senp1+/+* cells. The data were measured by real-time PCR and presented as mean ± SD of three independent experiments. Differences between *Senp1+/+* and *Senp–/–* macrophages were significant ($P < 0.005$, *t*-test). (**B**) IFN-γ-induced gene expression in *Senp1+/–* macrophages was lower than that in *Senp1+/+* cells. The data were measured by real-time PCR and presented as mean ± SD of three independent experiments. Differences between *Senp1+/+* and *Senp1+/–* macrophages were significant ($P < 0.005$, *t*-test). (**C**) SENP1 overexpression upregulated IFN-γ target genes. Expression of IFN-γ target genes in THP-1 cells transfected with SENP1 wild-type (SENP1wt) or SENP1 mutant (SENP1mut) or treated with IFN-γ (10 ng/ml) was measured by real-time PCR. The data are presented as mean ± SD of three independent experiments. Differences between SENP1wt- and SENP1mut-transfected THP-1 cells, SENP1wt- and empty vector-transfected THP-1 cells, or IFN-γ-treated and empty vector-transfected THP-1 cells were significant ($P < 0.005$, *t*-test). (**D**) SENP1 deficiency reduced STAT1 activation by IFN-γ. STAT1 in fetal liver macrophages from *Senp1+/+* and *Senp1–/–* mice (left panel) and peritoneal macrophages from *Senp1+/+* and *Senp1+/–* mice (right panel) were analyzed by blotting with anti-total or phosphotyrosine antibody as indicated.

the gene induction by IFN-γ in these cells. As shown in Figure 2D, silencing *Socs3* significantly increased the expression of IFN-γ-induced target genes in si-SENP1-RAW264.7 cells. JAK2 phosphorylation was also restored in these cells with silenced *Socs3* (Figure 2E). These results indicate that SOCS3 is responsible for IFN-γ-induced reduction of JAK2–STAT1 activity in SENP1-deficient macrophages.

We further testified whether SOCS3 expression resulted from IFN-γ-induced STAT3 activation in SENP1-deficient macrophages. Indeed, we found that IFN-γ robustly induced STAT3 tyrosine

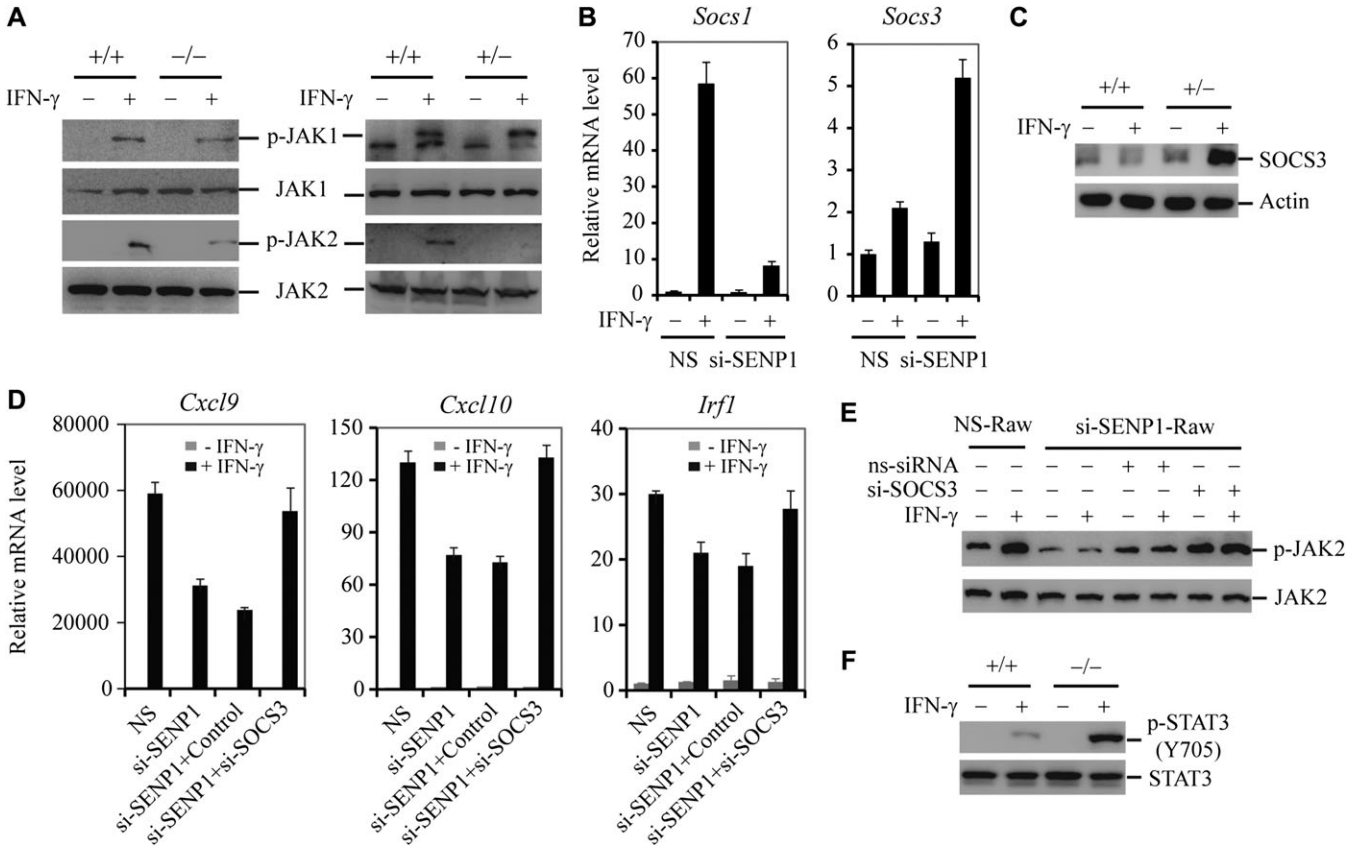

Figure 2 STAT3–SOCS3 mediates an alternative feedback inhibition in IFN-γ signaling. (**A**) IFN-γ-induced JAK2 but not JAK1 phosphorylation was reduced in SENP1-deficient macrophages. JAK1 and JAK2 in *Senp1*+/+ and *Senp1*−/− macrophages (left panel) or *Senp1*+/+ and *Senp1*+/− macrophages (right panel) were analyzed by blotting with JAK1, JAK2, pY-JAK1, or pY-JAK2 antibody as indicated. (**B**) SENP1 deficiency decreased *Socs1* but enhanced *Socs3* expression. IFN-γ-induced expressions of *Socs1* and *Socs3* in RAW264.7 cells transfected with si-SENP1 or control were measured by real-time PCR. The data are presented as mean ± SD of three independent experiments. Differences between IFN-γ-treated si-SENP1-RAW264.7 and control cells were significant ($P < 0.005$, *t*-test). (**C**) SENP1 deficiency enhanced SOCS3 expression. SOCS3 expression in peritoneal macrophages from *Senp1*+/+ and *Senp1*+/− mice was blotted with anti-SOCS3 antibody. (**D**) Silencing *Socs3* rescued SENP1 deficiency-caused downregulation of IFN-γ target genes. The expression of IFN-γ-induced genes was measured by real-time PCR in RAW264.7 macrophages transfected with si-SENP1, si-SOCS3, or control as indicated. The data are presented as mean ± SD of three independent experiments. (**E**) Silencing *Socs3* rescued SENP1 deficiency-caused downregulation of JAK2. JAK2 protein was analyzed in western blot with JAK2 antibody or phosphotyrosine antibody in RAW264.7 macrophages transfected with either si-SENP1 or si-SOCS3, or treated with IFN-γ. (**F**) IFN-γ induced higher level of STAT3 phosphorylation in *Senp1*−/− macrophages than in *Senp1*+/+ cells. STAT3 expression in macrophages from *Senp1*+/+ and *Senp1*−/− fetal livers was blotted with anti-total or phosphotyrosine antibody as indicated.

phosphorylation in *Senp1*−/− macrophages (Figure 2F). Similarly, IFN-γ-induced STAT3 phosphorylation was significantly increased in *Senp1*+/− macrophages as well as in *Senp1*-silenced Raw264.7 cells (Supplementary Figure S7). All together, these data support that the STAT3–SOCS3 axis tightly controls IFN-γ signaling in SENP1-deficient macrophages.

SENP1 negatively regulates STAT3 activity by deSUMOylating PTP1B

STAT3 phosphorylation status in cells is an indicator of the balance between JAK tyrosine kinase and the phosphatases responsible for STAT3 de-phosphorylation (Regis et al., 2008). Since SENP1-deficient macrophages showed downregulation of JAK2 specifically in response to IFN-γ, we postulated that IFN-γ-induced STAT3 activation might result from the reduction of phosphatase-mediated de-phosphorylation in SENP1-deficient macrophages. PTP1B and TCPTP are two well-recognized tyrosine phosphatases, which are important for STAT3 de-phosphorylation (ten Hoeve et al., 2002; Zabolotny et al., 2002; Xu and Qu, 2008). Cells were treated with either PTP1B-specific inhibitor or TCPTP-specific inhibitor. As shown in Supplementary Figure S8, IFN-γ-induced STAT3 phosphorylation was significantly increased in PTP1B-specific inhibitor-treated macrophages but not in TCPTP inhibitor-treated samples, indicating that PTP1B is a tyrosine phosphatase in response to STAT3 de-phosphorylation in macrophages.

Previous studies have reported that PTP1B is a SUMOylated protein (Dadke et al., 2007). To reveal whether PTP1B is a SENP1 target in STAT3 regulation, we analyzed PTP1B SUMOylation status in *Senp1*−/− cells in comparison with *Senp1*+/+ cells. We observed an accumulation of SUMOylated PTP1B protein in *Senp1*−/− but not in *Senp1*+/+ cells (Figure 3A), indicating that SENP1 functions as a specific de-SUMOylation protease for PTP1B. SUMOylated PTP1B impairs its intrinsic phosphatase activity (Dadke et al., 2007). We treated cells with PTP1B-specific inhibitor, and found that PTP1B inhibition augmented STAT3 phosphorylation but reduced STAT1 activation, which mimicked the response of SENP1-deficient macrophages to IFN-γ signal (Figure 3B). We next performed *in vitro* de-phosphorylation assay, which showed that SUMOylated PTP1B had less phosphatase activity to STAT3 (Supplementary Figure S9). We further transfected PTP1B wild-type (WT) or PTP1B SUMO mutant (KR) plasmids into SENP1-deficient Raw264.7 cells. Although the expression of STAT1 target genes Cxcl9, Cxcl10, and Irf1 was upregulated in both PTP1B WT and PTP1B KR-transfected cells, PTP1B KR certainly demonstrated a stronger activity in upregulating the expression of these genes than PTP1B WT (Figure 3C). These data together indicate that the accumulation of SUMOylated PTP1B contributes to STAT3–SOCS3 activation and subsequently inactivates IFN-γ–STAT1 signaling in SENP1-deficient macrophages.

IFN-γ induces SENP1 expression

Since SENP1 acts as a positive regulator in IFN-γ signaling via inhibition of a STAT3–SOCS3-mediated negative feedback loop, we thought that IFN-γ would induce SENP1 expression to promote macrophage activation. As expected, *Senp1* mRNA level was significantly increased in IFN-γ-treated macrophages (Figure 4A). We further validated this result by detecting the elevated SENP1 protein level in THP-1 cells post IFN-γ treatment (Figure 4B). Analysis of sequence upstream to *Senp1* promoter showed a GAS element located at −68 to −59 bp of *Senp1* promoter. We verified this GAS element in a SENP1 promoter-luciferase reporter construct, and found that IFN-γ significantly induced the transcription of *Senp1* promoter reporter gene (Figure 4C). In contrast, the mutation in the GAS sequence of *Senp1* promoter completely abolished IFN-γ-induced *Senp1* transcription. We then performed ChIP assay to confirm that STAT1 was bound to the *Senp1* promoter in response to IFN-γ signal (Figure 4D).

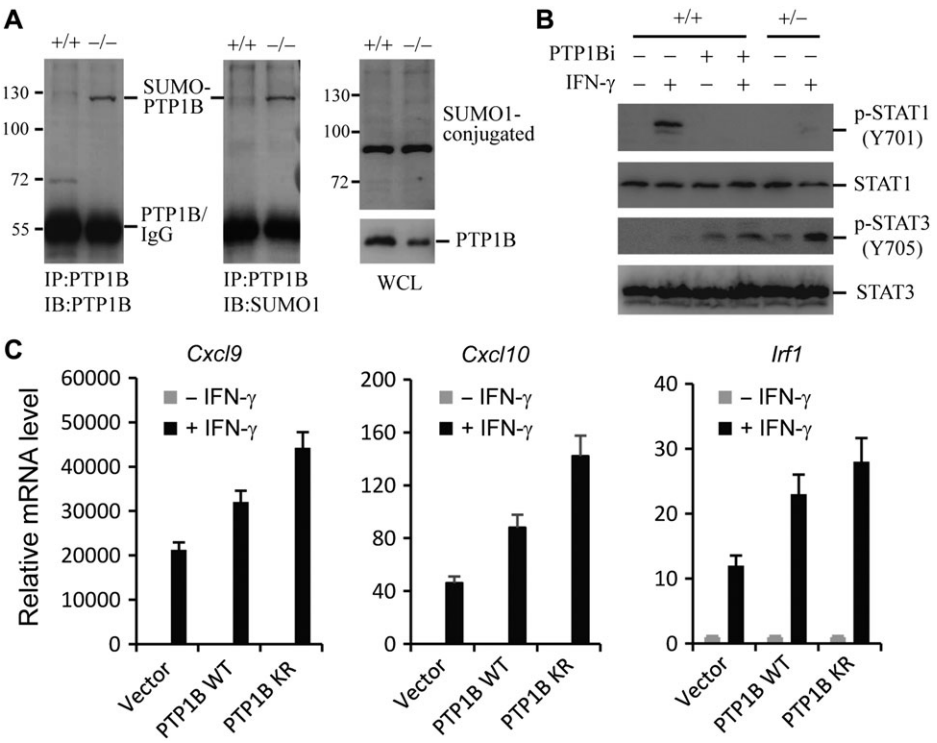

Figure 3 SUMOylated PTP1B is accumulated in SENP1-deficient macrophages and reduces PTP1B inhibition of STAT3 in IFN-γ signaling. (**A**) SUMOylated PTP1B was accumulated in *Senp1*−/− fetal liver macrophages. PTP1B was immunoprecipitated by anti-PTP1B antibody and blotted with anti-PTP1B or anti-SUMO1 antibody. (**B**) PTP1B inhibitor increased STAT3 phosphorylation but decreased STAT1 phosphorylation in macrophages. STAT proteins were analyzed in western blot with STAT1, STAT3, phospho-STAT1, or phospho-STAT3 antibody in *Senp1*+/+ or *Senp1*+/− macrophages treated with PTP1B inhibitor or IFN-γ as indicated. (**C**) PTP1B SUMO mutant (KR) rescued SENP1 deficiency-caused downregulation of IFN-γ target genes. si-SENP1-Raw264.7 cells were infected with retrovirus (pMSCV vector) encoding flag-PTP1B WT or KR (K73, 335, 347, and 389 R). The expression of Cxcl9, Cxcl10, and Irf1 was analyzed by real-time PCR in cells with or without IFN-γ treatment.

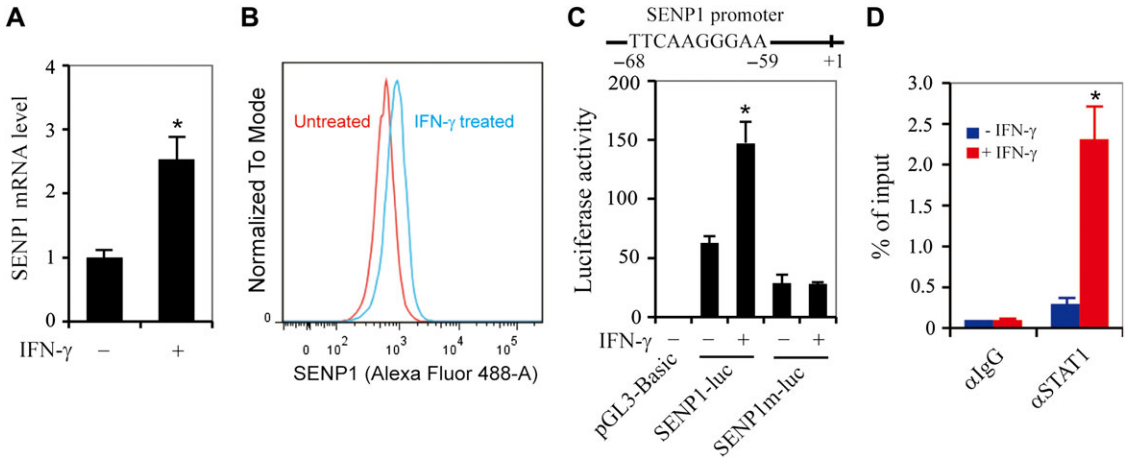

Figure 4 IFN-γ induces SENP1 expression. (**A**) IFN-γ-induced *Senp1* transcripts. *Senp1* mRNA level was measured in macrophages stimulated with IFN-γ (10 ng/ml) for 2 h. The data are presented as mean ± SD of three independent experiments. Difference between macrophages with and without IFN-γ treatment was significant ($P < 0.005$, *t*-test). (**B**) IFN-γ treatment increased SENP1 protein level. Expression of SENP1 was analyzed by flow cytometry in THP-1 cell treated with or without IFN-γ (10 ng/ml) for 24 h. (**C**) GAS site was identified on mouse *Senp1* promoter. RAW264.7 cells were transfected with empty vector, SENP1 promoter luciferase, or GAS site-mutated SENP1 promoter luciferase as indicated. The cells were treated with IFN-γ (10 ng/ml) for 30 min at 24 h after transfection. The luciferase activity was measured, and the data are presented as mean ± SD of three independent experiments. Difference between SENP1-luc-transfected RAW264.7 cells with and without IFN-γ treatment was significant ($P < 0.005$, *t*-test). (**D**) IFN-γ promoted STAT1 occupancy at *Senp1* promoter. STAT1 occupancy at *Senp1* promoter was analyzed by qChIP assays in macrophages treated with or without IFN-γ (10 ng/ml). The data are presented as mean ± SD of three independent experiments. Difference between macrophages with and without IFN-γ treatment was significant ($P < 0.005$, *t*-test).

Defect in M1 macrophage polarization in Senp1+/– mice

To further explore the role of SENP1 in regulating macrophage polarization, the bone marrow (BM)-derived macrophages extracted from both *Senp1*+/+ and *Senp1*+/– mice were treated with recombinant IFN-γ (10 ng/ml), lipopolysaccharide (LPS), or IFN-α, respectively (*in vitro*). We stained the macrophages with F4/80, CD11b, CD11c, and CD206 fluorochrome-conjugated antibodies after 24 h culturing. The proportion of M1 ($CD11c^+CD206^-$) or M2 ($CD11c^-CD206^+$) population in total macrophages ($F4/80^+CD11b^+$) was analyzed by flow cytometry. As shown in Figure 5A, *Senp1*+/+ macrophages were activated and polarized to M1 phenotype upon stimulation with IFN-γ or LPS, but not with IFN-α. The percentage of M1 increased to 23.9% after IFN-γ stimulation or 17.7% after LPS stimulation as compared with medium control at 7.52%. In contrast, *Senp1*+/– macrophage stimulated with IFN-γ had only marginal increase in the percentage of M1 with 12.6% compared with control at 7.97%, suggesting that SENP1 is essential for IFN-γ-induced M1 macrophage polarization. Interestingly, we detected similar extent of M1 increase (16.7%) in *Senp1*+/– macrophages as in *Senp1*+/+ cells in response to LPS stimulation, suggesting that LPS-induced M1 phenotype of macrophage activation is not dependent on SENP1 regulation. No changes in M2 proportion in *Senp1*+/+ macrophages were observed after all three stimuli, whereas a little decrease in M2 proportion in *Senp1*+/– was detected upon IFN-γ stimulation but not upon LPS stimulation (Figure 5A and B).

SENP1-deficient macrophages show reduced ability to resist Listeria monocytogenes infection

We performed the bacterial killing assay to testify the defects in M1 activation in *Senp1*+/– macrophages. The numbers of intracellular bacteria were determined in *L. monocytogenes*-infected *Senp1*+/+ and *Senp1*+/– macrophages. As shown in Figure 5C, *Senp1*+/– macrophages had 10 times more bacteria load than *Senp1*+/+ cells (Figure 5C). We then infected *Senp1*+/+ or *Senp1*+/– mice with *L. monocytogenes*, and counted bacterial colony-forming units (CFUs) in livers and spleens from infected mice at 48 h after infection. Figure 5D illustrated that *Senp1*+/– mice had significantly higher CFUs than *Senp1*+/+ mice. These results suggest that SENP1 is essential for M1 activation in macrophages.

We further determined whether the role of SENP1 in anti-bacteria defense relates to STAT1:STAT3 activation in macrophages. As shown in Figure 5E, *Senp1*+/– macrophages pretreated with STAT3 inhibitor JSI-124 showed increased STAT1 activation and became more resistance to *L. monocytogenes* infection than un-pretreated *Senp1*+/– macrophages, suggesting that hyper-activation of STAT3 is a factor leading to the failure of anti-bacteria defense in SENP1-deficient macrophages.

Discussion

In this study, SENP1-deficient macrophages show the reduced IFN-γ signaling for M1 activation. We have identified a STAT3–SOCS3-mediated alternative negative feedback loop in IFN-γ signaling, which is tightly controlled by SENP1 through

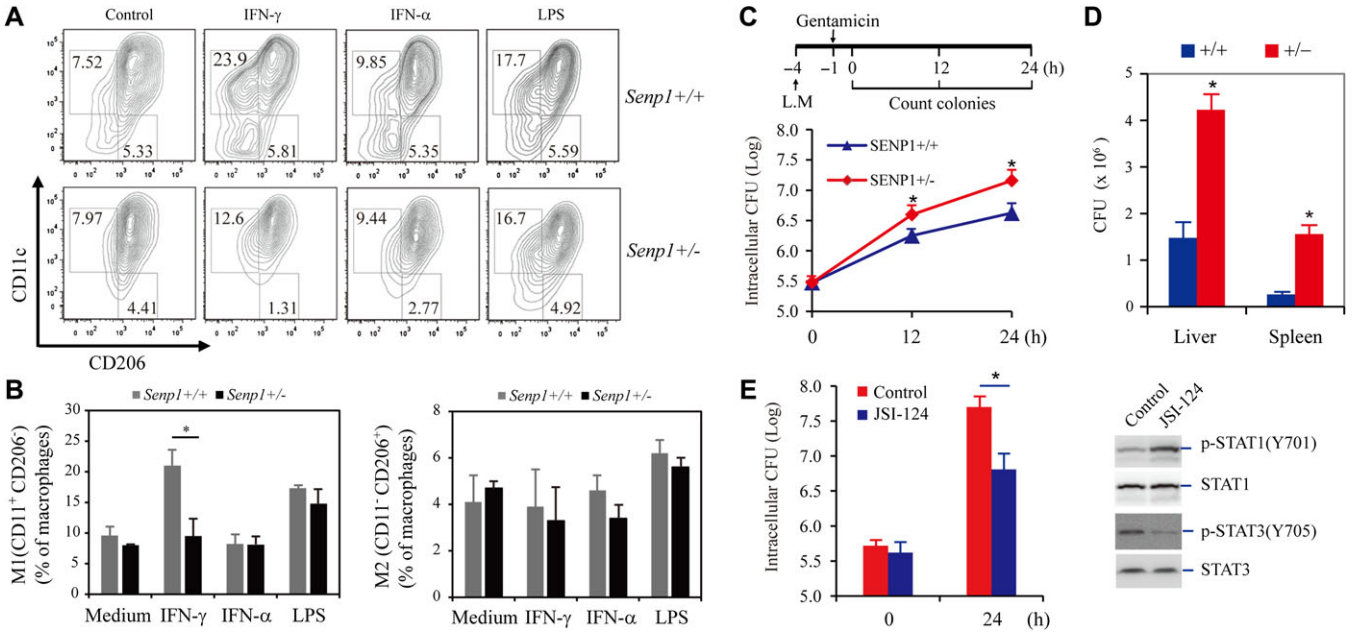

Figure 5 SENP1 is essential for M1 polarized phenotype of macrophages. (**A**) IFN-γ-induced M1 macrophages were reduced in *Senp1*−/− mice. *Senp1*+/+ and *Senp1*+/− macrophages were treated with recombinant IFN-γ, LPS, or IFN-α *in vitro*. After 24 h in culture, the proportion of M1 ($CD11c^+CD206^-$) or M2 ($CD11c^-CD206^+$) population in total macrophages ($F4/80^+CD11b^+$) was analyzed by flow cytometry with CD11c and CD206 fluorochrome-conjugated antibodies. (**B**) Quantification of M1 or M2 population in macrophages from *Senp1*+/+ and *Senp1*+/− mice ($n = 4$) treated with recombinant IFN-γ, LPS, or IFN-α *in vitro*. (**C**) The bacteria killing in *Senp1*+/− macrophages was lower than that in *Senp1*+/+ cells. *Senp1*+/+ and *Senp1*+/− macrophages were infected with *L. monocytogenes* a multiplicity of infection (MOI) of 3. The macrophages were collected at the indicated times (top panel) and lysed and dilution plated to determine bacterial burdens. The data are presented as mean ± SD. Differences between *Senp1*+/+ and *Senp1*+/− macrophages were significant (*$P < 0.005$, *t*-test). (**D**) The bacteria killing in *Senp1*+/− mice was lower than that in *Senp1*+/+ mice. *Senp1*+/+ and *Senp1*+/− mice ($n = 5$) were infected with a dose of *L. monocytogenes* (10000 CFU). Spleens and livers were harvested 2 days later and homogenized and dilution plated to determine bacterial burdens. The data are presented as mean ± SD. Differences between *Senp1*+/+ and *Senp1*+/− mice were significant (*$P < 0.005$, *t*-test). (**E**) STAT3 inhibitor promoted the bacteria killing in *Senp1*+/− macrophages. *Senp1*+/− macrophages were pretreated with STAT3 inhibitor JSI-124 (0.5 μM) or vehicle control. At 6 h after pre-treatment, these cells were infected with *L. monocytogenes* at an MOI of 3. The macrophages were collected at 0 and 24 h and lysed and dilution plated to determine bacterial burdens. The data are presented as mean ± SD. Differences between JSI-124-pretreated and vehicle control macrophages were significant (*$P < 0.005$, *t*-test). Right panel shows the level of STAT1, phospho-STAT3, STAT3, or phospho-STAT3 in the infected macrophages at 24 h after infection.

de-SUMOylation of PTP1B. Furthermore, in SENP1-deficient macrophages, the accumulation of SUMOylated PTP1B induces an increase in phosphorylated STAT3 to promote SOCS3 expression and eventually leads to suppression of STAT1 signaling. Thus, SENP1 determines macrophage fate by balancing STAT1: STAT3 activation (Figure 6).

The STAT1 pathway is the key player in controlling the expression of immune effector genes in classical M1 macrophage activation (Darnell et al., 1994). Since IFN-γ could induce STAT3 activation in *Stat1*−/− cells, STAT1 is supposed to suppress STAT3 activity (Qing and Stark, 2004). In this study, we found that STAT1 activation induces SENP1 expression, which inhibits STAT3–SOCS3 via de-SUMOylation of PTP1B to enhance STAT1 signaling. Therefore, STAT3 can also suppress STAT1 activity through SOCS3.

Our previous studies have identified SENP1 as a critical regulator in erythroid and lymphoid cell development (Cheng et al., 2007;

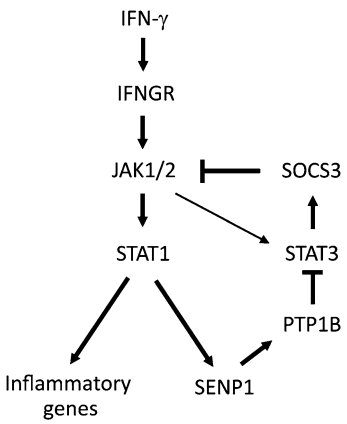

Figure 6 A model depicting the role of SENP1 in modulating IFN-γ–STAT1 signaling.

Van Nguyen et al., 2012). Here, we show a previously unexplored aspect of SENP1 function in the regulation of macrophage activation by targeting a phosphatase PTP1B. SUMOylation represses the de-phosphorylation activity of PTP1B and inhibits the negative effect of PTP1B on insulin receptor signaling as well as transformation by the oncogene *v-crk* (Dadke et al., 2007). This study shows that SUMOylation of PTP1B can reduce its inhibition to STAT3 in IFN-γ signaling. Therefore, our studies support the proposal that the function of SENP1 as a positive regulator in STAT1-induced macrophage activation is mainly through de-SUMOylation of PTP1B, which reduces inhibition of STAT3–SOCS3 on STAT1 signaling. PTP1B engagement is well known for the regulation of insulin-stimulating signaling (Yip et al., 2010). Our study shows that PTP1B is also essential for IFNγ-induced macrophage activation.

We further demonstrate the role of SENP1 in controlling the balance of STAT1:STAT3 activation. SENP1 shows as a suppressor of STAT3 and promotes STAT1 activation. Interestingly, we could not detect any difference in STAT1 activation between LPS or IFN-α-treated *Senp1*+/+ and *Senp1*+/– macrophages, although both stimuli can activate STAT1 signaling. LPS stimulates M1 macrophage phenotype mainly through the TLR–NF-κB pathway and STAT1 signaling through the auto-secretion of IFN-β. IFN-α stimulation promotes the formation of ISGF3γ (STAT1:STAT2:IRF9) complex to regulate the expression of genes with the promoters containing IFN-stimulated response element (IRSE) sequence. Both signaling pathways do not include STAT3 engagement, indicating that STAT3 is essential for SENP1 to target STAT1 pathway activation. Interestingly, STAT3 is an important regulator in tumorigenesis. Whether SENP1 could control tumorigenesis via modulating STAT3 activity is worth investigating in future.

Materials and methods

Mice

Senp1+/– mice were generated as previously described (Cheng et al., 2007). Mice were maintained at the Shanghai Jiao Tong University School of Medicine animal facility. All animal experiments were performed with the approval of Shanghai Jiao Tong University School of Medicine Committee on Animal Care.

Antibodies and reagents

Antibodies for pY1022/Y1023-JAK1, pY1007/Y1008-JAK2, pY701-STAT1, pY705-STAT3, JAK1, JAK2, STAT1, STAT3, and SOCS3 were purchased from Cell Signaling Technology. SENP1 antibody was generated by immunizing rabbit with human SENP1 peptide (170-183: SPKKTQRRHVSTAE) (Wang et al., 2013). PTP1B inhibitor (CAS 765317-72-4) was purchased from Santa Cruz Biotech. TCPTP inhibitor Compound 8 was from Zhong-Yin Zhang at Indiana University School of Medicine (Zhang et al., 2009). STAT3 inhibitor JSI-124 was purchased from Calbiochem (EMD Biosciences, Inc.), Recombinant mouse IFN-γ was purchased from R&D Systems.

SENP1-silenced RAW264.7 macrophages

RAW264.7 cells were infected by retrovirus encoding non-specific (NS) or *Senp1*-specific shRNA (si-SENP1, 5′-GCAGGAT CCTCTTGCAATA-3′) and selected in medium containing 3 μg/ml puromycin.

Preparation of fetal liver macrophages

Fetal liver macrophages were prepared from the livers of E13.5 embryos as previously described (Morris et al., 1988).

Real-time quantitative PCR

Total RNA was isolated by the TRIzol kit (Invitrogen). RNA was treated with DNase (Promega). Complementary DNA was synthesized using the cDNA synthesis kit (Takara Bio Inc.) according to the manufacturer's instructions. Fluorescence real-time RT-PCR was performed with the SYBR Green PCR Core Reagents of (PE Biosystems) on the ABI PRISM 7300 system (PerkinElmer Life Sciences). All data were analyzed by ABI PRISM SDS software version 2.0 (PerkinElmer Life Sciences). Pairs of PCR primers used to amplify the target genes were listed in Table 1.

Clearance of pathogens in vivo and in vitro

Senp1+/+ and *Senp1*+/– mice (6–8 weeks old) were infected (tail vein) with 1×10^4 CFU of log-phase *L. monocytogenes* strain. After 48 h, livers and spleens were harvested for bacteria CFU counting. Briefly, liver or spleen homogenate was coated on the no antibiotic plate by gradient dilution and culture at 37°C

Table 1 Primers used for RT-PCR.

Gene	Primer
Ifnγr1	5′-TGGGCCAGAGTTAAAGCTAAG-3′ (forward)
	5′-GTGAAATACGAGGACGGAGAG -3′ (reverse)
Ifnγr2	5′-GATTCTAACTTGGGAGCCGTC-3′ (forward)
	5′-ACTTTGTCTCTGTGATGTCCG-3′ (reverse)
Ifnγ	5′-GGCCATCAGCAAAACATAAGCGT-3′ (forward)
	5′-TGGGTTGTTGACCTCAAACTTGGC-3′ (reverse)
Irf1	5′-TGGAGATGTTAGCCCGGACACTTT-3′ (forward)
	5′- ACGGTGACAGTGCTGGAGTTATGT-3′ (reverse)
Iigp1	5′-AAGCGGCAATTTCTGAAGCAGAGG-3′ (forward)
	5′-ATCCACTCCAAACACAGTGCGGTA-3′ (reverse)
Socs1	5′-AGTCGCCAACGGAACTGCTTCTT-3′ (forward)
	5′-ACGTAGTGCTCCAGCAGCTCGA AA-3′ (reverse)
Socs3	5′-CCAGCATCTTTGTCGGAAGACTGT-3′ (forward)
	5′- TCATACTGATCCAGGAACTCCCGA-3′ (reverse)
Cxcl9	5′-ATCTTCCTGGAGCAGTGTGGAGTT-3′ (forward)
	5′-AGGGATTTGTAGTGGATCGTGCCT-3′ (reverse)
Cxcl10	5′-ATATCGATGACGGGCCAGTGAGAA-3′ (forward)
	5′-AATGATCTCAACACGTGGGCAGGA-3′ (reverse)
Ifi203	5′-TGTCAGGTGTGAACCAGGCAGTAA-3′ (forward)
	5′-ACTGAGTCTGGGTTGAGTGGCTTT-3′ (reverse)
Ifi204	5′-GTGCTCACTGCAACAGACCCATTT-3′ (forward)
	5′-TCTCCAGGATGCCTTTGCTCTCAA-3′ (reverse)
Ly6e	5′-ATGTCAACCTTGGCTACACCCTGA-3′ (forward)
	5′-AGAGCCAACAAGCTAAGCAGGAGT-3′ (reverse)
Nos2	5′-CTGCTGGTGGTGACAAGCACATTT-3′ (forward)
	5′-ATGTCATGAGCAAAGGCGCAGAAC-3′ (reverse)
Senp1	5′-AGCTGCCCAAGAAACCAGGAAA-3′ (forward)
	5′-AATGGTCAAGCGGAATGCCT-3′ (reverse)
Gapdh	5′-CATGTTCGTCATGGGTGTGAACCA-3′ (forward)
	5′-AGTGATGGCATGGACTGTGGTCAT-3′ (reverse)

for 12–18 h. Then, 30–300 colonies were selected for counting and the total number of bacteria was calculated according to the dilution fold.

Macrophages differentiated from BM of *Senp1+/+* or *Senp1+/–* mice (6–8 weeks old) were cultured with *L. monocytogenes* for 3 h (MOI = 3, i.e. bacteria number:cell number = 3:1). The medium was altered and gentamicin was added for additional 1-h incubation in order to kill the pathogens adherent to cell membrane or uninfected cells. The cells were cracked by lysis buffer (1% Triton X-100) at various time points, and spread on the plates with a gradient dilution method. Plates of 30–300 colonies were selected for counting and then the total number of the pathogens was calculated according to the dilution fold.

Statistics

Results were shown as mean ± SD. Statistical analyses represent a non-parametric Student's *t*-test, and null hypotheses were rejected at $P < 0.05$.

Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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