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Amplifying IFNγ signaling in DC by CD11c-specific loss of SOCS1 increases innate immunity to infection while decreasing adaptive immunity

Alejandro F. Alice[1], **Gwen Kramer**[1], **Shelly Bambina**[1], **Jason R. Baird**[1], **Keith S. Bahjat**[1,2], **Michael J. Gough**[1], and **Marka R. Crittenden**[1,3,4]

[1]Earle A. Chiles Research Institute, Robert W. Franz Cancer Center, Providence Portland Medical Center, 4805 NE Glisan St, Portland, OR, 97213, USA

[3]The Oregon Clinic, Portland, OR, 97213, USA

Abstract

While prophylactic vaccines provide protective humoral immunity against infectious agents, vaccines that elicit potent CD8 T cell responses are valuable tools to shape and drive cellular immunity against cancer and intracellular infection. In particular, IFNγ-polarized cytotoxic CD8 T cell immunity is considered optimal for protective immunity against intracellular antigens. SOCS1 is a cross-functional negative regulator of TLR and cytokine receptor signaling via degradation of the receptor-signaling complex. We hypothesized that loss of SOCS1 in dendritic cells would improve T cell responses by accentuating IFNγ-directed immune responses. We tested this hypothesis using a recombinant *Listeria monocytogenes* vaccine platform that targets $CD11c^+$ dendritic cells in mice where SOCS1 is selectively deleted in all $CD11c^+$ cells. Unexpectedly, in mice lacking SOCS1 expression in $CD11c^+$ cells, we observed a decrease in $CD8^+$ T cell response to the *Listeria monocytogenes* vaccine. NK cell responses were also decreased in mice lacking SOCS1 expression in $CD11c^+$ cells, but did not explain the defect in $CD8^+$ T cell immunity. We found that DC lacking SOCS1 expression were functional in driving antigen-specific $CD8^+$ T cell expansion *in vitro*, but that this process was defective following infection *in vivo*. Instead monocyte-derived innate TNFα and iNOS-producing DC (TipDC) dominated the anti-bacterial response. Thus, loss of SOCS1 in $CD11c^+$ cells skewed the balance of immune response to infection by increasing innate responses while decreasing antigen-specific adaptive responses to infectious antigens.

Keywords

SOCS1; Dendritic cell; IFNγ; *Listeria monocytogenes*; vaccine

[4]Correspondence to: marka.crittenden@providence.org.

[2]Current address: Bristol-Myers Squibb, Biologics Discovery California, Redwood City, CA, 94063, USA

Introduction

L. monocytogenes is a ubiquitous Gram-positive facultative intracellular pathogen typically found in soil and food. We and others have been developing live-attenuated *L. monocytogenes*-based vaccine platforms for application to both cancer and infectious disease. The systemic infection model of listeriosis in mice has provided important insights into host-pathogen interactions and the adaptive immune response. Both a functional innate response and an adaptive immune response are critical for eradicating the pathogen (1-3). *L. monocytogenes* elicits a potent CD8+ T cell response in mice, attributed to direct infection of dendritic cells (DC) in the spleen and delivery of *L. monocytogenes*–associated antigen directly to the host-cell cytosol (4, 5). $CD8\alpha^+$ DC are the primary reservoir for live bacteria within the first few hours of systemic infection (6), and these cells play a critical role in priming *L. monocytogenes*-specific T cells (6, 7). These DC are an early source of interleukin-12 (IL-12), which in turn induces interferon-γ (IFNγ) release by NK, NKT, and T cells (8). Importantly, these inflammatory cytokines also elicit negative feedback loops through regulatory proteins that limit cellular activation by these potent cytokines.

The suppressor of cytokine signaling (SOCS) family proteins (SOCS1-7 and CIS) are a group of structurally related proteins characterized by a central SH2 docking motif for interaction with tyrosine phosphorylated proteins. SOCS1 is induced by cellular activation and serves as a negative feedback mechanism for cytokines sharing the common gamma chain (IL-2, IL-4, IL-7, IL-15), IFNα, IFNγ, and IL-12. While the SH2 domain targets the SOCS proteins to specific molecules within the JAK-STAT pathway, the SOCS-box functions as an E3 ubiquitin ligase, promoting degradation of the cytokine receptor complex. SOCS1 knockout ($SOCS1^{-/-}$) mice are normal at birth; however, they exhibit slow growth and die within 3 weeks of birth, with activation of peripheral T cells, necrosis of the liver, and macrophage infiltration of major organs (9, 10). The neonatal defects exhibited by $SOCS1^{-/-}$ mice appear to occur primarily as a result of unchecked IFNγ signaling, since $SOCS1^{-/-}$ mice that also lack the IFNγ gene or the IFNγ receptor gene avoids neonatal lethality (11). The major source of this IFNγ has been shown to be T cells, since $Rag^{-/-}$ mice do not display $SOCS1^{-/-}$ lethality (12). SOCS1 is involved in the suppression of inflammation by regulating cytokine signaling in innate immune cells, including macrophages and DC, as well as non-immune cells. Deficiency of SOCS1 in macrophages was shown to result in hyper-responsiveness to lipopolysaccharide (LPS) (13-16), and silencing SOCS1 in DC was shown to enhance antigen presentation, T cell priming, lupus-like autoimmune diseases and anti-tumor immunity (17, 18).

For these reasons, we hypothesized that SOCS1 knockout in DC would be a means to increase antigen-specific T cell responses to *L. monocytogenes*-based vaccines and therefore potentiate their ability to generate therapeutic T cells targeting infectious diseases or cancer. We tested our primary hypothesis using $Socs1^{fl/fl}$ mice crossed with mice expressing Cre recombinase under the control of the CD11c promoter. This resulted in a strain where $CD11c^+$ cells selectively lack SOCS1 expression and activity. DC demonstrated prolonged signal transduction following IFNγ stimulation, but surprisingly vaccination of mice where DCs lack SOCS1 resulted in a deficient $CD8^+$ T cell response to bacterial antigens. While NK cells were also negatively affected in these mice, NK cells were not responsible for the

poor $CD8^+$ T cell responses generated in these animals. Instead, infection in these mice instead resulted in an increase in the number of iNOS producing DC (TipDC) in the spleen resulting in increased innate control of the bacterium. Our data demonstrates that blocking negative feedback of cytokine signaling via deletion of SOCS1 in DC, rather than increasing CD8 T cell responses to a pathogen, instead suppressed T cell responses and increased innate control of bacterial infection.

Materials and Methods

Murine models

To generate mice in which SOCS1 was specifically deleted in $CD11c^+$ cells, CD11c-Cre-GFP transgenic mice (19) were obtained from The Jackson Laboratory (Stock#007567, Bar Harbor, ME) and bred with $SOCS1^{fl/fl}$ mice (15) (generously provided by Dr. Yoshimura, Keio University). Five to ten week old sex-matched $Cre^-SOCS1^{f/f}$ and $CD11c\text{-}Cre^+$ $SOCS1^{f/f}$ littermates were used for all experiments. C57BL/6 mice, OT-1 mice bearing a TCR specific for the SIINFEKL epitope of ovalbumin on a $Rag1^{-/-}$ background, and $Rag1^{-/-}$ knockout mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animal protocols were approved by the Earle A. Chiles Research Institute IACUC (Animal Welfare Assurance No. A3913-01).

Bacterial and viral strains

L. monocytogenes strains used for these studies, wt and *ΔactA* ActA-QV (*ΔactA*-QV, expressing the class I-restricted vaccinia virus derived epitopes $B8R_{20–27}$, $C4L_{125–132}$, $A42R_{88–96}$ and $K3L_{6–15}$, in addition to $OVA_{257–264}$), (20) were grown to stationary phase in brain-heart infusion broth, washed in PBS, and injected intravenously (retro-orbital route) in 200μL total volume. Unless expressed in the text, the following infectious doses were used for *L. monocytogenes* strains: wt - 1×10^4 for survival and infectious studies and 1×10^5 for challenge and cell sorting; *ΔactA*-QV - 1×10^5 for infection and immune response. When bacterial counts were determined in various cell types, mice were infected with 1×10^5 cfu wt *L. monocytogenes*. 15 hr post-infection spleens were harvested and half of each spleen was directly homogenized, while the other half was dissociated and flow sorted for specific cell subpopulations. All spleen samples were then lysed and plated on BHI plates to calculate bacterial cfu in the source material. Vaccinia virus WR expressing full-length chicken ovalbumin (VV-OVA) were grown in HeLa cells and frozen. Thawed cell lysates were treated for 30-minutes with 1.25 μg/mL trypsin at 37°C. Virus was diluted in HBSS and injected intraperitoneally as 1×10^6 PFU in 200μL. For non-infectious vaccination, mice were immunized intraperitoneally with 5μg of anti-DEC-205-OVA (generously provided by CellDex Therapeutics, Hampton, NJ) together with 25μg of anti-CD40 (clone FGK4.5, BioXCell, West Lebanon, NH) in 200μL total volume.

Bone marrow dendritic cell culture and stimulation

Bone marrow derived dendritic cells (BMDC) were generated according to a standard protocol (21). Briefly, 2×10^6 bone marrow cells were seeded per 100mm Petri dish in RPMI 1640 supplemented with 10% fetal bovine serum and 20ng/ml recombinant murine GM-CSF (R&D Systems, Minneapolis, MN) with or without 10ng/ml IL-4 (PeproTech, Rocky Hill,

NJ). On day 3, 10 ml of an RPMI1640 medium containing 20ng/ml mGM-CSF was added to the plates. On day 6 half of the culture supernatant was collected and centrifuged, and the cell pellet was resuspended in 10 ml of a fresh RPMI1640 medium containing 20ng/ml mGM-CSF and returned to the original plate. When needed 10ng/ml IL-4 was added at the same time. In general 7-8 day cultured BMDCs were used for the experiments unless otherwise specified. Flow cytometric analysis showed that these DC subsets contained >90% $CD11c^+$ cells, data not shown.

BMDC (5×10^4) were stimulated with 100ng/ml lipopolysaccharide (LPS, InvivoGen, San Diego, CA), 10ng/ml IFN-γ (R&D Systems, Minneapolis, MN), or a combination of both reagents for 18hs at 37°C. Supernatants were removed and used for cytokine analysis, and cells were washed and stained for flow cytometric analysis as described. For western blot analysis and RNA extraction, cells were lysed and processed as described below.

For *in vitro* antigen presentation studies, BMDC were plated in 96-well plates (Costar-Corning) at 5×10^3 cells per well with α-DEC-205-OVA, soluble Endo-Free OVA (InvivoGen, San Diego, CA) or $OVA_{257–264}$ (SIINFEKL) synthetic peptide for 45 min at 37°C in complete medium. BMDC were washed three times and resuspended in 200μl of complete medium containing 5×10^4 CFSE-labeled OT-1 $CD8^+$ T cells. Proliferation was analyzed after 65–72 h of culture by flow cytometry (22). For isolation of splenic $CD11c^+$ cells, spleens were dissociated and CD11c+ cells purified by positive selection (EasySep™ Mouse CD11c Positive selection isolation kit, StemCell Technologies, Vancouver, Canada) and purity check for flow cytometry. Each determination was performed in triplicate.

For RNA extraction and quantitative Real time-PCR (qRT-PCR), BMDCs were plated in a 6-well plate (2×10^6 cells per well) and stimulated as described above. At 18 hours, cells were harvested and RNA was purified using Qiazol and RNeasy Mini kit (Qiagen, Valencia, CA). DNase-treated RNA was used as template for cDNA synthesis using SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and qRT-PCR was performed using PowerUP SYBR Green Master Mix (Applied Biosystems, Foster City, CA) and the following primers: β-Actin-For; 5′-CCCTGTGCTGCTCACCGA-3′, β-Actin-Rev; 5′-ACAGTGTGGGTGACCCCGTC-3′, SOCS1-For; 5′-CACCTTCTTGGTGCGCG-3′, SOCS1-Rev; 5′-AAGCCATCTTCACGCTGAGC-3′. Reactions were carried out and analyzed in a StepOnePlus™ Real-Time PCR system (Applied Biosystems, Foster City, CA). Fold change was expressed as $2^{-\Delta\Delta Ct}$, where the internal control is the β-Actin gene and the gene of interest is SOCS1.

For western blot analysis, cells were lysed in RIPA buffer in the presence of protease and phosphatase inhibitor (Thermo Fisher Scientific, Waltham, MA) and denatured in SDS loading buffer containing β 2-mercaptoethanol, electrophoresed on 10% SDS-PAGE gels and transferred to PVDF membrane (EMD Millipore, Billerica, MA). Blocked blots were probed overnight at 4°C with anti-STAT-1 (Cell Signaling Technology, Danvers, MA), anti-Phospho-STAT-1 (Tyr701) (#9171, Cell Signaling Technology) or anti-β-actin (#A2228, Sigma-Aldrich, St. Louis, MO) primary antibodies (Cell Signaling Technology, Danvers, MA) diluted 1:1000 followed by goat α-rabbit HRP-conjugated secondary antibody (1:20000) (Sigma, St. Louis, MO). Binding was detected using SuperSignal West Pico

Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA) and images acquired with FluorChem E System (ProteinSimple, San Jose, CA).

Flow cytometry and cytokine analysis

Fluorochrome-conjugated antibodies specific for CD11c (clone N418), CD11b (clone M1/70), Ly-6C (clone HK1.4), Ly-6G (clone 1A8), MHCII I-A/I-E (clone M5/114.15.2), CD90.1 (clone HIS51), CD3 (clone 17A2), iNOS (clone CXNFT), IL12-p40 (clone C17.8), CD19 (clone eBio1D3), IL-2 (clone JES6-5H6), CD86 (clone GL1), CD27 (clone LG.7F9), NK1.1 (clone PK136), CD49b (clone DX5), NKp46 (clone 29A1.4), CD45.1 (clone A20), CD45.2 (clone 104), IFN-γ (clone XMG1.2), (eBioscience, San Diego, CA) CD4 (clone RM4-4), CD8α (clone 53-6.7), TNF (clone MP6-XT22) (BD Bioscience) and XCR1 (clone ZET) (Biolegend, San Diego, CA) were used at optimal titers as determined in our laboratory.

Serum cytokines were determined using the Mouse Inflammation BD Cytometric Bead Array (CBA, BD Biosciences, San Jose, CA). Samples were acquired on an LSRII flow cytometer and the exported data were analyzed using the CBA Analysis Plugin for Excel.

T cell function and analysis

For analysis of T cell responses, spleens were dissociated and filtered through a 70μm cell strainer. Red blood cells were lysed with Red Blood Cell Lysing Buffer (Sigma, St. Louis, MO). For peptide stimulation assays, splenocytes were stimulated for 4 hours with 1μM $OVA_{257–264}$ (SIINFEKL), $B8R_{20-27}$, $A42R_{88–96}$ or $LLO_{190–201}$ peptide in the presence of brefeldin A (GolgiPlug, BD Biosciences, San Jose, CA). Peptides for stimulation were obtained from A&A Labs (San Diego, CA, USA) and reconstituted in DMSO. Unstimulated controls (DMSO only) were used to assess nonspecific protein production for each animal. Cells were stained for surface antigens, and then fixed (Cytofix/Cytoperm buffer, BD Bioscience) and stored at -80C (in Cytofix/Cytoperm buffer) until further analysis. For intracellular cytokine staining, frozen cells were thawed, permeabilized (Perm Wash buffer, BD Biosciences), and stained for intracellular IFN-γ. To assess TipDC/DC activation, splenocytes were processed as described above and incubated 4hs at 37C, 5% CO_2 with or without 10^7 cfu/ml heat killed *L. monocytogenes* (HKLm). Cells were stained and iNOS intracellular staining was performed as described above. Samples were acquired on an LSRII flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo™ 10.2 (FlowJo LLC, Ashland, OR).

Spleens were harvested from donor mice and either $CD8^+$ or total T cells purified by negative selection (EasySep™ Mouse $CD8^+$ T cell and EasySep™ Mouse T cell isolation kits, StemCell Technologies, Vancouver, Canada). Prior to adoptive transfer, cells were stained to confirm purity of $CD8^+$ T cells and total T cells (>90%).

For *in vivo* antigen presentation experiments, single-cell suspensions of purified OT-1 $CD8^+$ T cells were stained with 5, 6-carboxy-succinimidyl-fluoresceine-ester (CFSE, Molecular Probes, Eugene, OR) 10 minutes at 37C. Reactions were stopped with cold PBS and resuspended in the desired volume. Mice were injected with the CFSE-$CD8^+$ T cells and

spleens removed and processed after 3 days. Staining was performed as described and analysis conducted with FlowJo 10.2. Mitotic events were determined as described (22).

To evaluate the immune response, 10,000 purified OT-1 $CD8^+$ T cells were transferred into $Cre^-SOCS1^{f/f}$ and CD11c- Cre^+ $SOCS1^{f/f}$ mice 1 day prior to immunization with *ΔactA*-QV. Spleens were harvested and processed for flow cytometry 7 days later.

For reconstitution of $RAG1^{-/-}$ knockout hosts, 2×10^7 purified total T cells from $Cre^-SOCS1^{f/f}$ or CD11c- Cre^+ $SOCS1^{f/f}$ spleens and the following day they were infected with *ΔactA*-QV. Seven days after the immunization, spleens were removed and processed for staining.

NK cells function and analysis

NK cells were purified from mouse splenocytes by using EasySep Mouse NK cell isolation kit (StemCell Techonologies, Vancouver, Canada) as described by the manufacturer. Cell purity was over 90% as confirmed by flow cytometry. Cells were plated and stimulated with IL-18 (R&D Systems, Minneapolis, MN) and/or IL-12 (PeproTech, Rocky Hill, NJ) for 6 hs before removing the supernatant for CBA analysis and staining the cells for ICS as described above.

For *in vivo* NK depletion, 100μg of α-NK1.1 antibody (Clone PK136, BioXCell, West Lebanon, NH) was intraperitoneally injected and NK cell depletion confirmed by analyzing blood samples 24hr later. Mice were primed with *ΔactA*-QVac, serum IFN-γ levels measured at 24hr post-infection and spleens removed and processed for ICS 7 days later as described.

Statistics

Data were analyzed and graphed using Prism (GraphPad Software, La Jolla, CA). Individual data sets were compared using Student's T-test and analysis across multiple groups was performed using ANOVA with individual groups assessed using Tukey's comparison.

Results

To achieve loss of SOCS1 in dendritic cells, mice expressing Cre recombinase under the control of the CD11c promoter were bred with a floxed *socs1* gene ($SOCS1^{fl/fl}$ mice) to generate $Cre^-SOCS1^{fl/fl}$ and $Cre^+SOCS1^{fl/fl}$ littermates for study. These mice were healthy, avoiding the postnatal lethality of $SOCS1^{-/-}$ mice (9, 10); however, $Cre^+SOCS1^{fl/fl}$ mice exhibited some degree of splenomegaly and females developed psoriatic symptoms at 2-3 months, males after 4-5 months. To confirm cell specific expression of Cre, we took advantage of bicistronic expression of GFP along with Cre under control of the CD11c promoter (19). Consistent with prior reports, GFP was clearly detected in $CD11c^+MHCII^+$ cells in the spleen, including both the $CD8\alpha^-$ and $CD8\alpha^+$ subsets (Figure 1a). $CD11b^+$ cells lacking CD11c were GFP^-, as were $CD19^+$ B cells and $CD3^+$ T cells of both the CD4 and CD8 compartments. NK cell subsets can express CD11c; however, we could detect only weak GFP expression in these cells, mostly in the $NK1.1^+CD49b^+$ subset (Figure 1a). To confirm loss of SOCS1, BMDC derived from $Cre^-SOCS1^{fl/fl}$ and $Cre^+SOCS1^{fl/fl}$ littermates

were stimulated with IFNγ and the *socs1* transcript was detected by qRT-PCR. While Cre^{-}SOCS1$^{fl/fl}$ BMDC robustly increased expression of *socs1* transcript following IFNγ treatment, no *socs1* transcript was detectable in Cre^{+}SOCS1$^{fl/fl}$ BMDC after cytokine priming (Figure 1b). To confirm functional loss of SOCS1 activity, STAT1 phosphorylation was measured in BMDC following prolonged stimulation with LPS, IFNγ or the combination. BMDC lacking SOCS1 activity exhibited increased IFNγ-mediated STAT1 phosphorylation compared to control littermates (Figure 1c). The increased response to IFNγ had phenotypic consequences, since while untreated Cre^{-}SOCS1$^{fl/fl}$ and Cre^{+}SOCS1$^{fl/fl}$ BMDC exhibited similar expression of costimulatory molecules, BMDC lacking SOCS1 activity exhibited a greater upregulation of CD86 following IFNγ treatment (Figure 1d). Naïve Cre^{-}SOCS1$^{fl/fl}$ and Cre^{+}SOCS1$^{fl/fl}$ littermates exhibited similar proportions and absolute numbers of CD11c^{+}MHCII^{+}, CD11c^{+}MHCII^{+}CD8α^{+} and CD11c^{+}MHCII^{+}CD11b^{+}CD8α^{-} splenic DC (Supplementary Figure 1a-b). However, SOCS1 deficient DC showed higher basal levels of expression of MHCII in CD11c^{+} cells (Figure 1e) and higher basal levels of CD86 in CD11c^{+}MHCII^{+}CD8α^{+} and CD11c^{+}MHCII^{+}CD11b^{+}CD8α^{-} DC (Supplementary Figure 1ci-ii). Furthermore, XCR1 expression levels were significantly higher in CD11c^{+}MHCII^{+}CD8α^{+} DC when SOCS1 was ablated (Supplementary Figure 1ci-ii). These data demonstrate that mice lacking SOCS1 are highly responsive to IFNγ treatment and exhibit increased basal activation *in vivo*.

To determine whether vaccine-driven antigen-specific responses are improved in mice with DC-specific loss of SOCS1 expression we used a *L. monocytogenes*–based vaccine. *L. monocytogenes* vaccines directly infects dendritic cells in the spleen following systemic administration, and these dendritic cells are required to initiate CD8^{+} T cell responses to bacterial antigens (23). Cre^{-}SOCS1$^{fl/fl}$ and Cre^{+}SOCS1$^{fl/fl}$ littermates were vaccinated with *ΔactA* QV *L. monocytogenes* expressing 4 different well-characterized T cell epitopes, including the SIINFEKL epitope of ovalbumin. Mice with wild-type SOCS1 expression made a strong antigen-specific T cell response to vaccination, with IFN-γ^{+} SIINFEKL-specific CD8 T cells readily detectable in the spleen 7 days following vaccination; however, unexpectedly mice with DCs lacking SOCS1 demonstrated a significantly lower CD8 T cell response to antigen (Figure 2a). Similarly, CD8 T cell response to the other epitopes in the vaccine B8R and A42R were significantly reduced in Cre^{+}SOCS1$^{fl/fl}$ mice (Figure 2b). To determine whether the T cell response to other infectious agents was similarly compromised in these mice, mice were vaccinated with a recombinant vaccine strain of Vaccinia virus expressing ovalbumin. Again, mice with wild-type SOCS1 expression made a strong response to ovalbumin, while mice with DCs lacking SOCS1 demonstrated a significantly lower CD8^{+} T cell response to ovalbumin (Figure 2a), indicating that this failure is not unique to *L. monocytogenes*. To determine whether this response was specific to infectious agents, mice were vaccinated with α-DEC205-OVA and α-CD40, which delivers ovalbumin to splenic DCs and drives efficient cross-presentation to T cells to generate robust ovalbumin-specific CD8^{+} T cell expansion (24). Cre^{-}SOCS1$^{fl/fl}$ and Cre^{+}SOCS1$^{fl/fl}$ littermates made equivalent responses to vaccination with anti-DEC205-OVA and anti-CD40 (Figure 2a), suggesting that DC function is intact when antigen is delivered via this route. Furthermore, the success of this vaccination strategy was not due to the activity of anti-CD40, since delivery of anti-CD40 to mice vaccinated with *ΔactA* QV *L. monocytogenes*

was not able to restore T cell responses in $Cre^{+}SOCS1^{fl/fl}$ mice (Supplementary Figure 2). Interestingly, the $CD4^{+}$ T cell response to *Listeria*-associated antigen was not diminished in $Cre^{+}SOCS1^{fl/fl}$ mice (Figure 2c), suggesting that the failure in response was associated with intracellular antigens from the infectious agents that are cross-presented by DC to $CD8^{+}$ T cells. To exclude the possibility that $CD8^{+}$ T cell function was broadly diminished in $Cre^{+}SOCS1^{fl/fl}$ mice, $Cre^{-}SOCS1^{fl/fl}$ and $Cre^{+}SOCS1^{fl/fl}$ littermates were adoptively transferred with a low dose of TCR transgenic $CD8^{+}$ OT1 T cells, which are specific for the SIINFEKL epitope of ovalbumin presented on $H2K^{b}$, and have wild-type SOCS1 expression. Vaccination of these mice with *ΔactA* QV *L. monocytogenes* resulted in significantly lower OT1 $CD8^{+}$ T cell responses in mice where DCs lack SOCS1 expression (Figure 2d). To assess the function of T cells from $Cre^{-}SOCS1^{fl/fl}$ and $Cre^{+}SOCS1^{fl/fl}$ mice independent of DC function, T cells from $Cre^{-}SOCS1^{fl/fl}$ and $Cre^{+}SOCS1^{fl/fl}$ littermates were adoptively transferred to $Rag1^{-/-}$ mice with wild-type SOCS1 expression, and these mice were vaccinated with *ΔactA* QV *L. monocytogenes*. While the proportion of $CD8^{+}$ T cells from $Cre^{+}SOCS1^{fl/fl}$ mice responding to antigens was slightly lower than $CD8^{+}$ T cells from $Cre^{-}SOCS1^{fl/fl}$, there was no difference in the number of responding T cells after vaccination (Figure 2ei-ii). Again, the $CD4^{+}$ T cell response to LLO was equivalent between strains (Figure 2eiii). These data demonstrate that when DCs lack SOCS1 expression, antigen-specific $CD8^{+}$ T cell responses to infectious agents are significantly decreased.

L. monocytogenes generates both an innate and adaptive immune response that each contribute to clearance of the bacterium. To determine whether the innate response was altered in mice where DCs lack SOCS1, we examined cytokine expression in the serum 24 hours following infection with wt *L. monocytogenes*. In mice where DCs lack SOCS1 there was a significantly lower expression of IFNγ, CCL2, and IL-6, but a significantly higher level of TNFα (Figure 3a). Consistent with prior reports (25), infection with *ΔactA* L. monocytogenes did not result in IL-10 secretion in control mice or in mice lacking SOCS1 in DC (not shown). Since NK cells can be an important source of IFNγ in early innate responses to infectious agents including *L. monocytogenes* (8), and NK cells expressed low levels of Cre (Figure 1a), we investigated the role of NK cells in $Cre^{+}SOCS1^{fl/fl}$ mice. NK cells were decreased in number and proportion in $Cre^{+}SOCS1^{fl/fl}$ mice (Figure 3b), mostly as a result of decreased number and proportion of mature $CD11b^{+}CD27^{+}$ and $CD11b^{+}CD27^{-}$ NK cells (Figure 3c). Following infection with *L. monocytogenes*, IFNγ production by NK cells was significantly decreased in $Cre^{+}SOCS1^{fl/fl}$ mice (Figure 3d), indicating a failure of NK activation in response to infection. However, NK cells isolated from $Cre^{-}SOCS1^{fl/fl}$ and $Cre^{+}SOCS1^{fl/fl}$ littermates were equally able to produce IFNγ in response to stimulation *in vitro* (Supplementary Figure 3) suggesting the defect was not intrinsic to the NK cell but rather their response to infection. To determine whether NK cells were required for T cell antigen-specific responses to infection, wild-type mice were depleted of NK cells prior to vaccination with *ΔactA* QV *L. monocytogenes* using a depleting NK1.1 antibody (Figure 3ei and Supplementary Figure 3). These mice demonstrated loss of NK cells and significantly decreased IFNγ in the serum following infection, but antigen-specific T cell responses were unchanged (Figure 3eii). These data demonstrate that *in vivo* NK cell function is changed in $Cre^{+}SOCS1^{fl/fl}$ mice, but that early

NK cell production of IFNγ is not required for $CD8^+$ T cell responses and so does not explain the poor response in mice where DCs lack SOCS1.

Infection of $CD8\alpha^+$ DC is critical to generate antigen-specific responses following infection with *L. monocytogenes*. To determine whether this infection was deficient in $Cre^+SOCS1^{fl/fl}$ mice, we analyzed infection of a range of cell populations at early time points following *in vivo* infection with *L. monocytogenes* wt strain. Similar amounts of bacteria could be detected at this early time point in the spleen of $Cre^-SOCS1^{fl/fl}$ and $Cre^+SOCS1^{fl/fl}$ littermates (Figure 4ai), and as anticipated, the majority of *L. monocytogenes* was present in $CD11c^+CD8\alpha^+$ DC with small amounts of the bacterium in $CD11c^+CD8\alpha^-$ DC and $CD11b^+$ monocytes (Figure 4aii). The number of bacteria in dendritic cells was not different between $Cre^-SOCS1^{fl/fl}$ and $Cre^+SOCS1^{fl/fl}$ littermates (Figure 4aii), indicating that the bacteria is similarly infecting $CD11c^+CD8\alpha^+$ DC despite a failure to generate T cell responses. To determine whether DCs from these animals have a defect in functional antigen presentation, bone marrow-derived DC from $Cre^-SOCS1^{fl/fl}$ and $Cre^+SOCS1^{fl/fl}$ littermates were pulsed with ovalbumin, SIINFEKL peptide or anti-DEC205-OVA then tested for their ability to stimulate proliferation of OT1 $CD8^+$ T cells *in vitro*. In each case, OT1 $CD8^+$ T cell proliferation was equivalent in each group (Figure 4b). To assess antigen presentation *in vivo*, $Cre^-SOCS1^{fl/fl}$ and $Cre^+SOCS1^{fl/fl}$ littermates were challenged with *ΔactA* QV *L. monocytogenes* (which also contains the SIINFEKL epitope) 4, 3, 2 or 1 day prior to adoptive transfer of a high dose of OT1 $CD8^+$ T cells, and assessed for OT1 $CD8^+$ T cell proliferation 3 days later. OT1 $CD8^+$ T cell proliferation was significantly decreased in the $Cre^+SOCS1^{fl/fl}$ mice at each time point following infection (Figure 4c), indicating a significantly decreased capacity to functionally present antigen *in vivo*. To determine whether this functional difference in the cross-presenting capacity of splenic DC was true *ex vivo*, we sorted $CD11c^+$ splenic DC from $Cre^-SOCS1^{fl/fl}$ and $Cre^+SOCS1^{fl/fl}$ littermates and pulsed these with ovalbumin, SIINFEKL peptide or anti-DEC205-OVA then tested for their ability to stimulate proliferation of OT1 $CD8^+$ T cells *in vitro*. $Cre^+SOCS1^{fl/fl}$ DC showed slightly increased capacity to stimulate OT1 proliferation following cross-presentation of ovalbumin and were equally able to stimulate T cells with pulsed peptide and anti-DEC205-OVA (Figure 4d). These data demonstrate that DC lacking SOCS1 are functional in classic *in vitro* assays of cross-presentation, but dysfunctional in stimulating adaptive immunity following infection *in vivo*.

To determine whether the failure in mice lacking SOCS1 in DCs is limited to priming of naïve T cell responses, $Cre^-SOCS1^{fl/fl}$ and $Cre^+SOCS1^{fl/fl}$ littermates were vaccinated with anti-DEC205-OVA plus anti-CD40 to generate an equivalent and functional *in vivo* response, then challenged 21 days later with *ΔactA* QV *L. monocytogenes*. Mice lacking SOCS1 in DCs exhibited a significantly lower $CD8^+$ T cell response following rechallenge (Figure 5a), indicating that $CD8^+$ T cell memory expansion is also impaired following *L. monocytogenes* rechallenge. To determine whether a failure in CD8 T cell responses impacted bacterial clearance, naïve mice or mice that had been vaccinated with *ΔactA* QV *L. monocytogenes* 21 days prior were challenged with $1LD_{50}$ (1×10^5 cfu) wild-type *L. monocytogenes* and bacterial load in the spleen was determined 3 days later. $Cre^-SOCS1^{fl/fl}$ mice that were vaccinated demonstrated significantly improved bacterial clearance compared to naïve mice (Figure 5b). By contrast, $Cre^+SOCS1^{fl/fl}$ littermates were not

protected by vaccination (Figure 5b). However, naïve mice lacking SOCS1 in DCs surprisingly appear to exhibit improved innate control of the infection at 3 days post-infection compared to mice with normal SOCS1 expression (Figure 5b). Daily analysis of bacterial load in infected mice demonstrated that wt *L. monocytogenes* was able to infect and replicate in $Cre^{+}SOCS1^{fl/fl}$ mice and while bacterial counts are initially lower, the mice are similarly susceptible to progressive infection as control mice (Supplementary Figure 4a). Similarly, *ΔactA* QV *L. monocytogenes* showed initially lower bacterial counts in the spleen and livers of $Cre^{+}SOCS1^{fl/fl}$ mice, but similar clearance of the attenuated *ΔactA* QV *L. monocytogenes* strain by day 5 post-infection (Supplementary Figure 4b) Thus, the differing T cell response to infection cannot be adequately explained by differential clearance or persistence of bacteria in mice. To examine cell populations that may be participating in the innate clearance of *L. monocytogenes*, $Cre^{-}SOCS1^{fl/fl}$ and $Cre^{+}SOCS1^{fl/fl}$ littermates were analyzed for their monocyte and neutrophil populations. Naïve $Cre^{+}SOCS1^{fl/fl}$ mice have higher numbers of $CD11b^{+}Ly6C^{hi}$ monocytes in the spleen than $Cre^{-}SOCS1^{fl/fl}$ mice, and equivalent numbers of $Ly6G^{hi}Ly6C^{hi}$ neutrophils (Figure 5ci-ii). Monocytes have been shown to be recruited into the spleen of infected mice and differentiate into TNFα and iNOS-producing DC (TipDC), which participate in innate bacterial clearance (26). As we demonstrated, wt *L. monocytogenes* infected $Cre^{+}SOCS1^{fl/fl}$ mice display elevated TNFα compared to control littermates (Figure 3a), suggestive of increased TipDC activity. To determine whether there was increased TipDC activity, iNOS expression was measured in splenic cells following infection. We observed increases in TipDC over time following infection with wt *L. monocytogenes*, and the number of these cells was significantly elevated in $Cre^{+}SOCS1^{fl/fl}$ mice compared to control littermates (Figure 5d). These data suggest that mice lacking SOCS1 in DCs exhibit a dual phenotype. These mice generate an increased innate response associated with increased induction of iNOS in TipDC, but an impaired T cell response due to poor expansion of $CD8^{+}$ T cells.

Discussion

We demonstrate that SOCS1 deficiency in $CD11c^{+}$ cells results in poor activation of CD8 T cell responses to antigens in bacterial and viral vaccines. This deficiency is caused by poor expansion of CD8 T cells by the critical $CD8\alpha^{+}$ DC population. Prior publications have described improved antigen presentation in DC with SOCS1 deficiency; however, these experiments were performed with *ex vivo* derived BMDC and not in an intact animal (18, 27). We demonstrate for the first time that restricted cell-specific loss of SOCS1 in dendritic cells *in vivo* has the function of redirecting the immune response following *L. monocytogenes* infection away from an adaptive and towards an innate response. We found that SOCS1 deficiency causes an increase in TNFα secretion in serum as well as an increase in $iNOS^{+}$ $CD11b^{int}$ $Ly6C^{high}$ $CD11c^{int}$ $MHCII^{+}$ TipDCs in spleens during the infection that could improve innate rather than adaptive control of infection.

L. monocytogenes LLO-mediated entry into the cytoplasm of DC is required for efficient cross-presentation to CD8 T cells (28). *L. monocytogenes* produces cyclic dinucleotides, which are critical for bacterial function (29), but also activate the cytoplasmic sensor STING (STimulator of INterferon Genes) resulting in type I IFN production (30). Archer *et al.* demonstrated that over-activation of STING can result in excess IFN production that limits

CD8 T cell responses to *L. monocytogenes* (31). Similarly, over-activation of STING using exogenous administration of cyclic dinucleotides has resulted in type I IFN-mediated suppression of CD8 T cell responses (32). However, despite decreased adaptive immune responses, *L. monocytogenes* that over-activate STING do not have increased virulence, and in fact can exhibit decreased virulence *in vivo* (33). This would be consistent with our data demonstrating an increased innate control of infection concomitant with decreased adaptive responses. TNFα and iNOS are essential for defense against infection with *L. monocytogenes* (26, 34, 35). In addition, IFNγ secretion from NK cells has been described as crucial for activation of monocytes to differentiate to TipDCs (8). Although in the mice lacking SOCS1 in DC, the levels of NK cell-derived IFNγ are low during the first 24hs post infection, it may be sufficient to induce iNOS production due to the higher sensitivity to IFNγ (17, 18). Thus, the balance of innate and adaptive responses to *L. monocytogenes* infection can be varied and still control the infection, but to achieve strong protective T cell-mediated immunity requires carefully controlled inflammation at challenge (36, 37).

The $CD8a^+$ subpopulation of DC are critical to generate CD8 T cell responses via cross-presentation of viral and bacterial-associated antigens (4, 5, 38). This same DC population is required for cross-presentation of cell-associated antigens (39), and thus these cells direct adaptive immune responses to cancer (40). Importantly, over-exuberant inflammatory responses have similarly been shown to diminish immune control of cancer through “rebound immune suppression” (41). Thus, as with bacterial and viral vaccination approaches, strategies that aim to generate CD8 T cell mediated immunity to tumors may similarly need to avoid over-activation of cross-presenting dendritic cells in order to optimize the adaptive immune response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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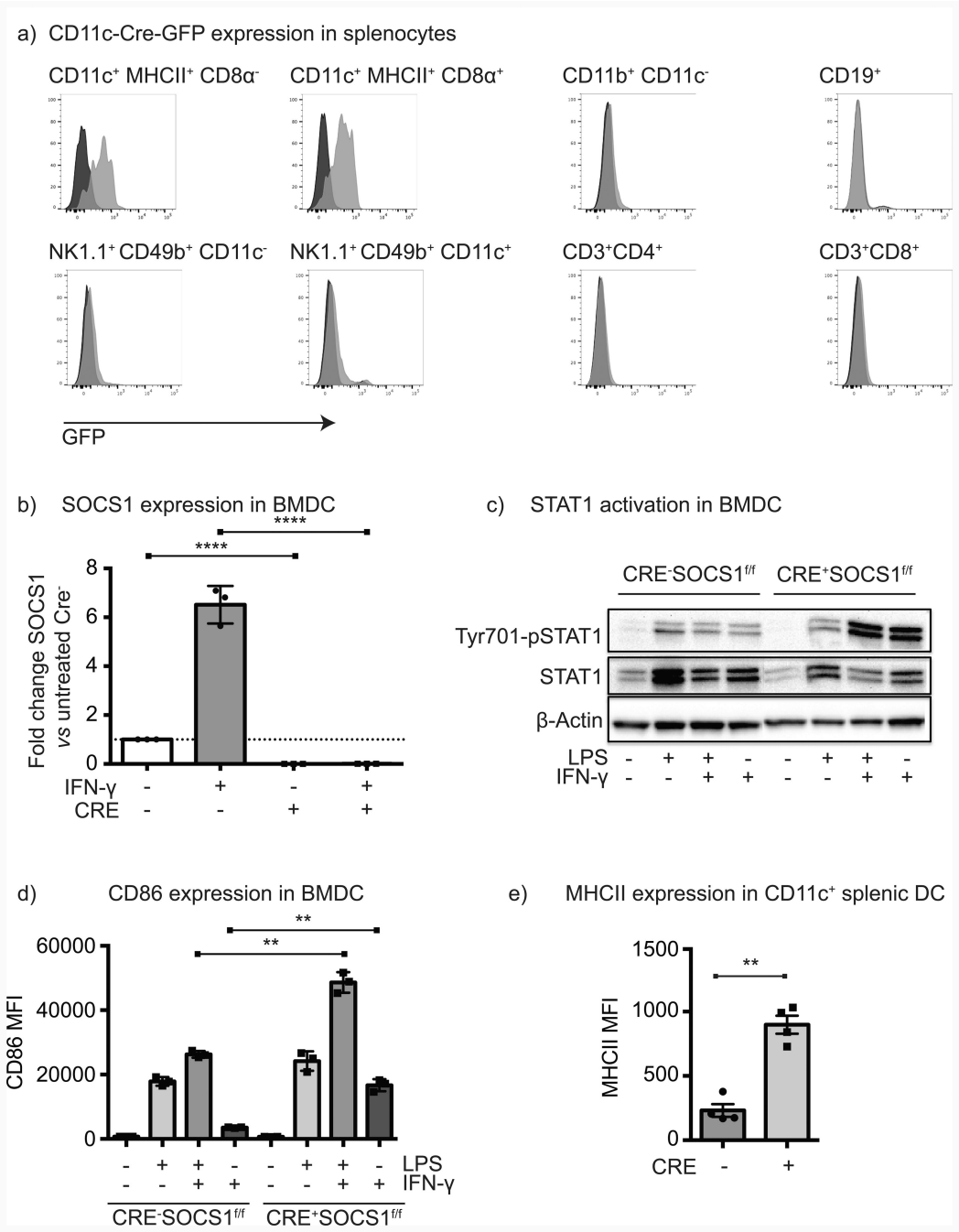

Figure 1. Selective elimination of SOCS1 activity in dendritic cells

a) CD11c-Cre-GFP expression in splenocytes from Cre^{-}SOCS1$^{fl/fl}$ (black histogram) and Cre^{+}SOCS1$^{fl/fl}$ (grey histogram) littermates gated on key immune cell populations. b) BMDC were stimulated with IFNγ or left untreated for 18hr and RNA extracted and analyzed for SOCS1 expression using qRT-PCR. c) BMDC from the indicated strains were left untreated or stimulated with LPS, IFNγ or the combination for 18 hr and Western blotted forpSTAT1, STAT1 and β-actin as a loading control. d) BMDC stimulated as in c) were analyzed for CD86 expression by flow cytometry. e) Splenic CD11c^{+} DC from naïve

$Cre^{-}SOCS1^{fl/fl}$ and $Cre^{+}SOCS1^{fl/fl}$ mice were analyzed for MHCII expression by flow cytometry. Results shown are representative of two to four independent experiments. Statistics calculated by Student's *t* test; * = $p<0.05$, ** = $p<0.01$, **** = $p<0.0001$.

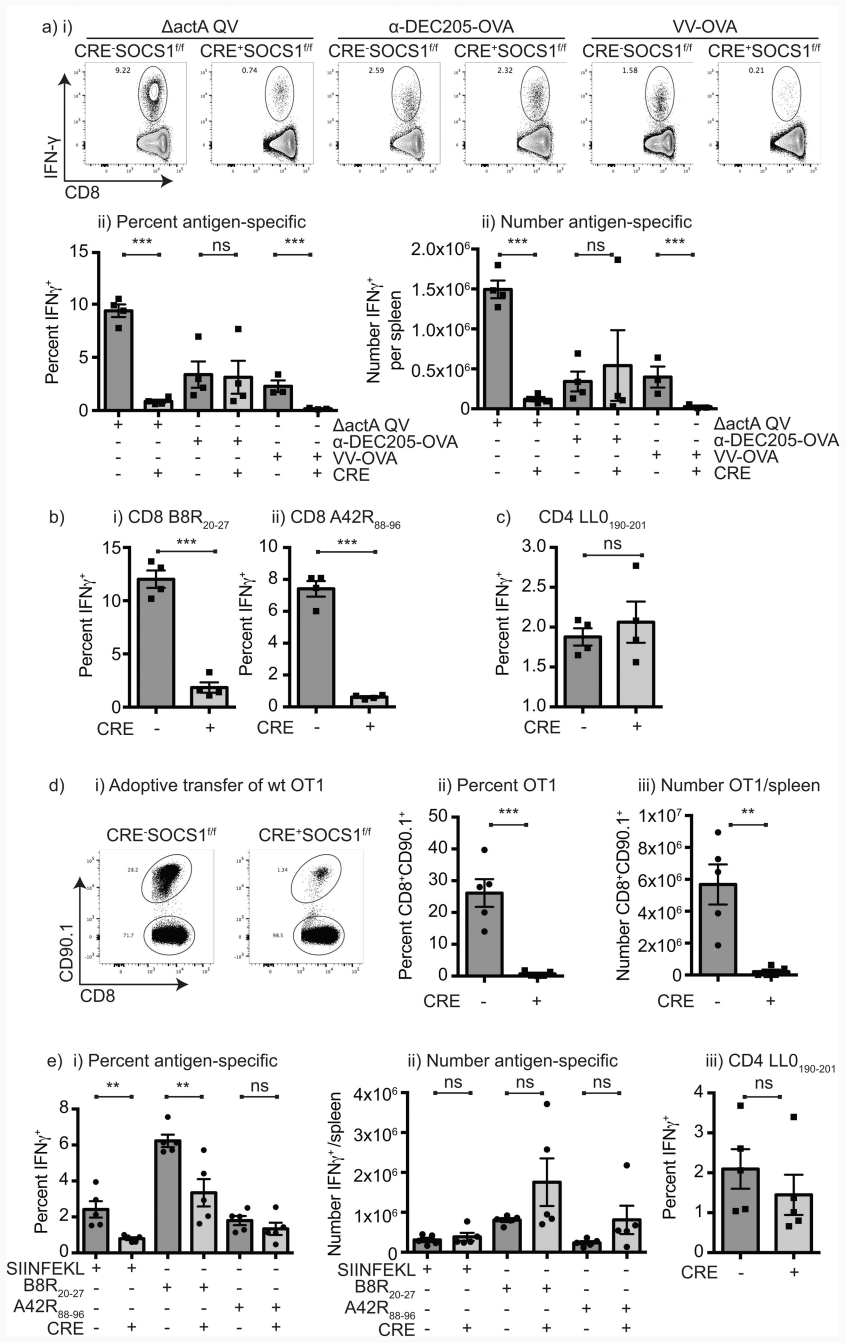

Figure 2. Diminished CD8 T cell responses to infection in mice lacking SOCS1 in DC

a) Cre^{-}SOCS1$^{fl/fl}$ and Cre^{+}SOCS1$^{fl/fl}$ littermates were primed with 1×10^{5} cfu *ΔactA* QV, 5μg anti-DEC205-OVA with 25 μg anti-CD40 or 1×10^{6} pfu VV-OVA. Spleens were harvested 7 days post priming and IFNγ^{+} SIINFEKL-specific CD8 T cells determined by using ICS. i) Representative flow-cytometry plots of IFNγ expression in response to SIINFEKL peptide. Quantitation of ii) the percent and iii) absolute numbers of IFNγ^{+} SIINFEKL-specific CD8 T cells per spleen. b) Quantitation of the percent of IFNγ^{+} i) B8R$_{20–27}$-specific or ii) A42R$_{88–96}$-specific CD8 T cells in spleens from mice immunized

with *ΔactA* QV. c) Percent IFNγ^+ $LLO_{190\text{-}201}$–specific CD4 T cells 7 days post immunization with *ΔactA* QV. d) OVA-specific CD90.1$^+$ OT1 CD8 T cells were isolated from OT1 transgenic mice and 10,000 cells per mouse adoptively transferred to Cre$^-$SOCS1$^{fl/fl}$ and Cre$^+$SOCS1$^{fl/fl}$ mice. The following day mice were immunized with 1×10^5 cfu *ΔactA* QV as above, spleens harvested 7 days later, cells stained and CD90.1$^+$ (OT1) CD8 T cells analyzed. i) Representative flow-cytometry plot of CD90.1$^+$ CD8 T cell expansion in Cre$^-$SOCS1$^{fl/fl}$ and Cre$^+$SOCS1$^{fl/fl}$ mice. ii) The percent and iii) total number of OT1 cells per mouse. e) CD8 T cells from Cre$^-$SOCS1$^{fl/fl}$ and Cre$^+$SOCS1$^{fl/fl}$ mice were purified and transferred to Rag1$^{-/-}$ mice. 24hr later mice were immunized with 1×10^5 cfu *ΔactA* QV. Spleens were harvested 7 post infection and splenocytes stimulated *in vitro* with the indicated peptide. i) Percent and ii) absolute numbers of antigen-specific CD8 T cells as well as iii) percent of $LLO_{190\text{-}201}$ CD4 T cell responses are shown. Each symbol represents one mouse, n=3-5 mice per group. Data represents the mean ± SEM of each group. The displayed experiments are representative of 3 to 5 independent repeats. Statistics calculated by Student's *t* test; ns = no significant differences observed between the groups analyzed; ** = $p<0.01$; *** = $p<0.001$.

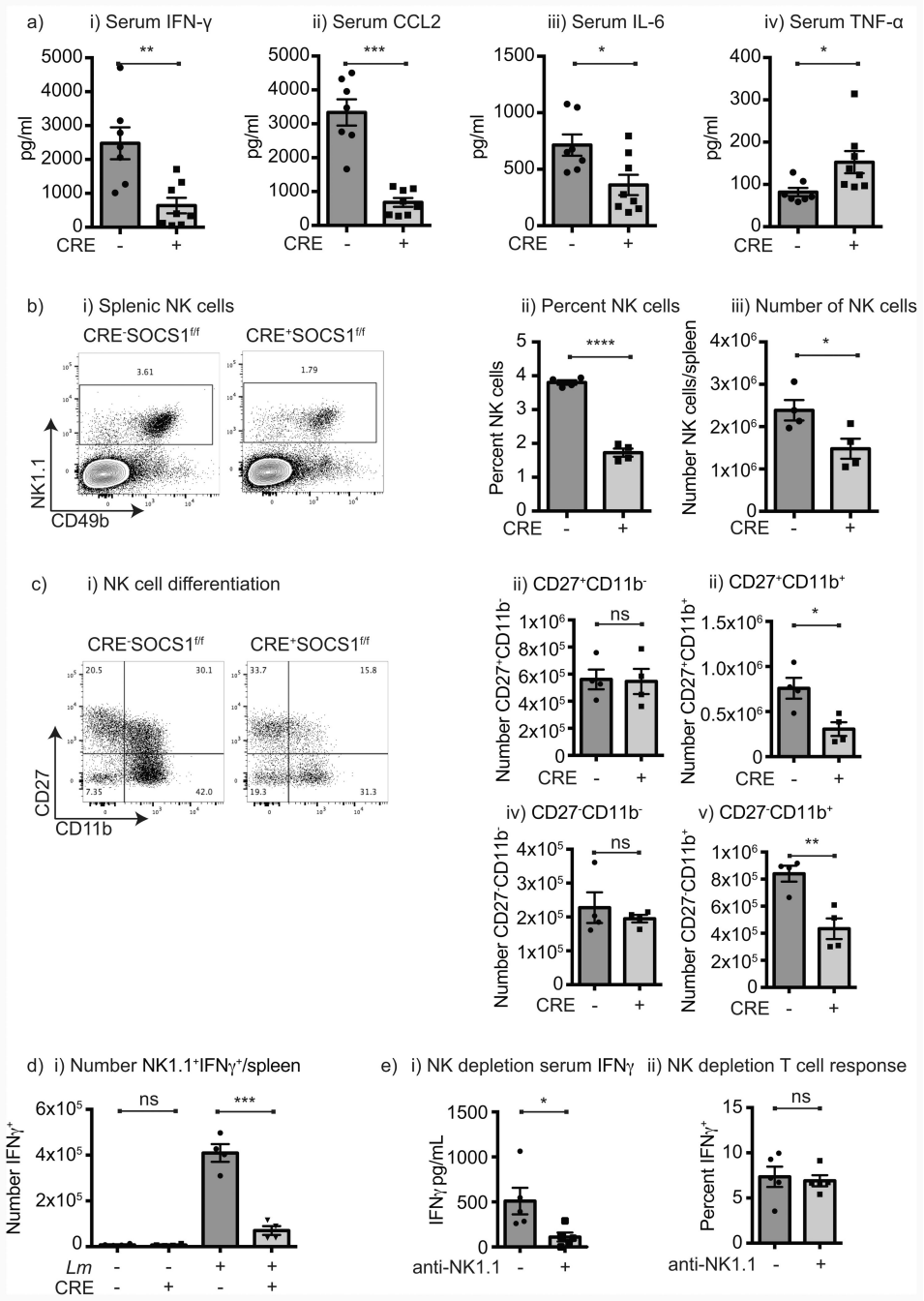

Figure 3. Decreased NK cell activity in mice lacking SOCS1 in DC

a) Cre^{-}SOCS1$^{fl/fl}$ and Cre^{+}SOCS1$^{fl/fl}$ mice were infected with 1×10^{4} cfu wt *L. monocytogenes* and 24hr later serum cytokines analyzed by multiplex cytokine bead assay. Values shown are from two combined experiments. b) i) Representative flow-cytometry plot of the NK1.1^{+}CD49b^{+} NK cells in gated CD3^{-} naïve Cre^{-}SOCS1$^{fl/fl}$ and Cre^{+}SOCS1$^{fl/fl}$ splenocytes; ii) percentages and iii) absolute numbers of splenic NK cells. c) Characterization of NK cell activation/maturation in naïve mice; i) Representative flow-cytometry plot of CD27 and CD11b expressing in gated NK cells in representative

Cre^{-}SOCS1$^{fl/fl}$ and Cre^{+}SOCS1$^{fl/fl}$ mice; ii) absolute numbers of CD27^{+}CD11b^{-}; iii) CD27^{+}CD11b^{+}; iv) CD27^{-}CD11b^{-} and v) CD27^{-}CD11b^{+} NK cells. d) Mice were left untreated or immunized with 1×10^{4} cfu wt *L. monocytogenes*, spleens harvested 24 hr later and splenocytes incubated with brefeldin A for 4 hr before ICS staining to identify absolute numbers of NK1.1^{+} IFNγ^{+} cells. e) Cre^{-}SOCS1$^{fl/fl}$ mice were left untreated or treated with anti-NK1.1 antibody to deplete NK cells and 24hr later immunized with 1×10^{5} cfu *ΔactA* QV. i) One day post-infection serum was removed from both groups and IFNγ determined by cytokine bead assay. ii) Seven days post-infection spleens were removed and IFNγ^{+} SIINFEKL-specific CD8 T cell determined by ICS. Each symbol represents one mouse, n=3-5 mice per group. Data represents the mean ± SEM of each group. The displayed experiments are representative of 2 to 4 independent repeats. Statistics calculated by Student's *t* test; ns = no significant differences observed between the groups analyzed; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$, **** = $p<0.0001$.

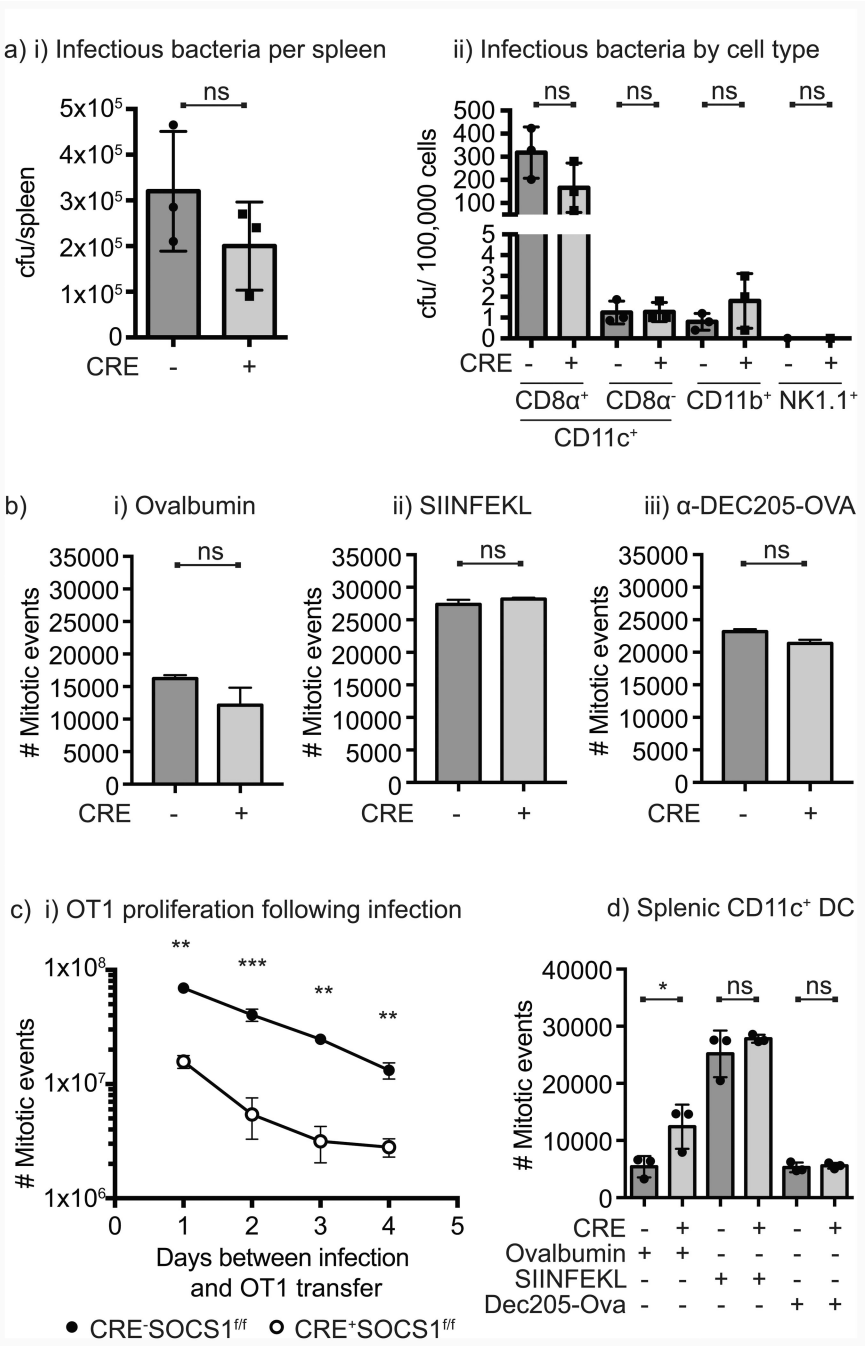

Figure 4. Decreased in vivo but equivalent in vitro T cell activation activity in mice lacking SOCS1 in DC

a) Mice were infected with 1×10^5 cfu wt *L. monocytogenes* and 15 hr post-infection spleens were harvested. i) Half of each spleen was homogenized, cells lysed, samples plated on BHI plates and incubated overnight at 37°C; ii) the other half of the spleens was dissociated, splenocytes stained and the indicated cells flow sorted, lysed and plated on BHI plates. After 24hr incubation at 37°C, cfu were counted. Each symbol represents one mouse, n=3 mice per group. b) BMDC generated with GM-CSF plus IL-4 were pulsed with the indicated antigens for 45 min at 37°C, then washed and co-cultured with purified CFSE-labeled OT1

CD8 T cells. Proliferation was analyzed by CFSE dilution after 3 days of incubation and converted to mitotic events. c) Individual groups of mice were immunized with 1×10^5 cfu *ΔactA* QV for four consecutive days, and on the fifth day CFSE-labeled OT1 CD8 T cells were adoptively transferred into the mice and spleens harvested 72 hs later. Proliferation was analyzed by CFSE dilution and converted to mitotic events. Data represents the mean ± SEM of each group. d) CD11c+ DC were flow sorted from naïve $Cre^{-}SOCS1^{fl/fl}$ and $Cre^{+}SOCS1^{fl/fl}$ mice and pulsed in vitro with the indicated antigens for 45 min at 37°C, then washed and co-cultured with purified CFSE-labeled OT1 CD8 T cells. Proliferation was analyzed by CFSE dilution after 3 days of incubation and converted to mitotic events. Results shown are representative of two to four independent experiments. Statistics calculated by Student's *t* test; ns, no significant differences observed between the groups analyzed; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$, **** = $p<0.0001$.

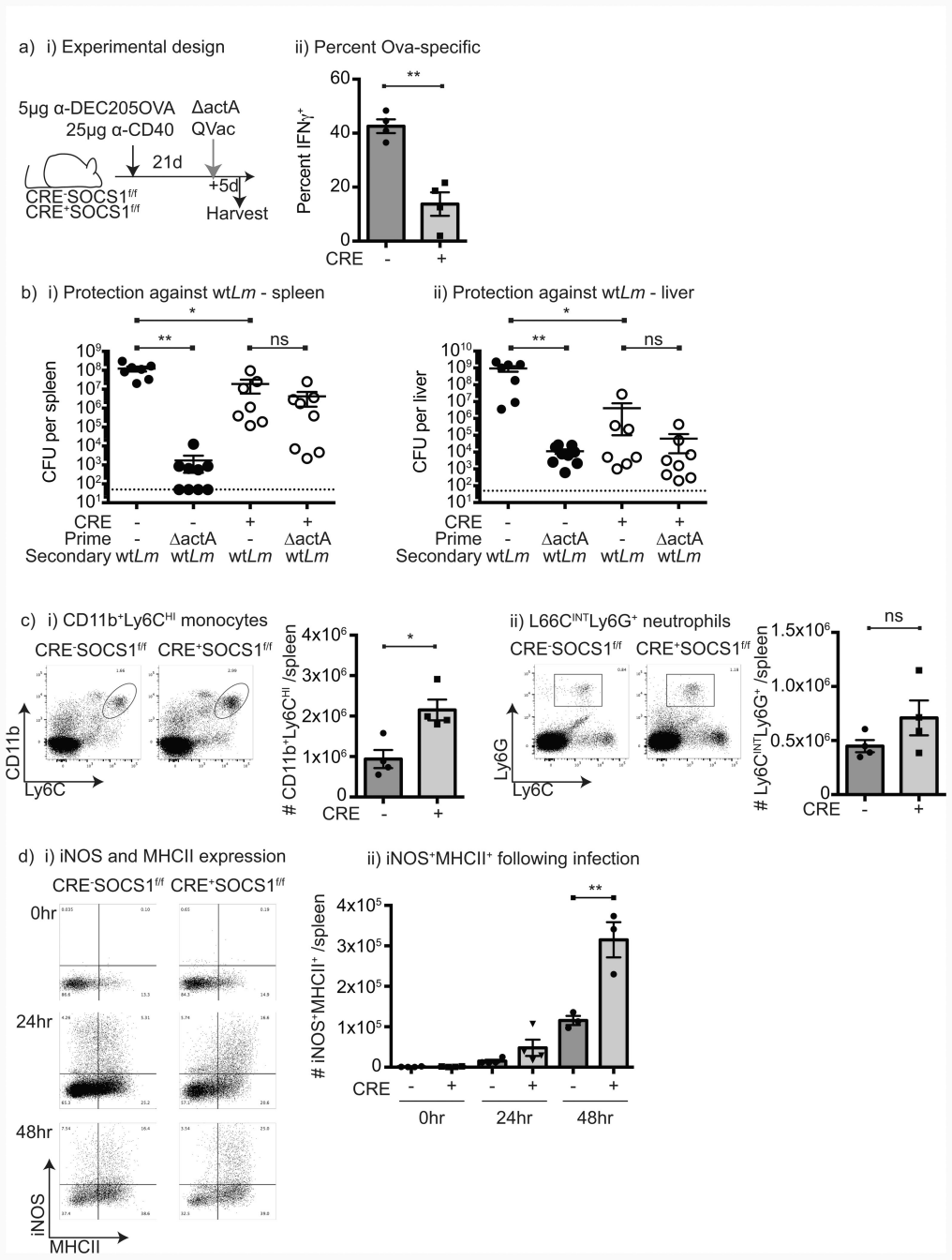

Figure 5. Increased innate response and TipDC activity in mice lacking SOCS1 in DC

a) i) Cre^{-}SOCS1$^{fl/fl}$ and Cre^{+}SOCS1$^{fl/fl}$ mice were immunized with anti-DEC205-OVA and anti-CD40 to generate an equivalent priming event then boosted 21 days later with 1×10^{5} cfu *ΔactA* QV. ii) Spleens were harvested 5 days post-boost and IFNγ^{+} SIINFEKL-specific CD8 T cells determined by using ICS. b) Cre^{-}SOCS1$^{fl/fl}$ and Cre^{+}SOCS1$^{fl/fl}$ mice were left untreated or primed with 1×10^{5} cfu *ΔactA* QV and challenged 21 days later with 1×10^{5} cfu wt *L. monocytogenes*. Three days later i) spleens and ii) livers were harvested, homogenized, plated on BHI plates and cfu numbers determined after overnight incubation

at 37°C. c) Spleens from naïve mice were harvested, stained and analyzed by flow-cytometry. i) Summary of the absolute number of $CD11b^{+}Ly6C^{high}$ cells and ii) the absolute number of $Ly6G^{+}Ly6C^{int}$ cells gated in the population. d) Mice were left untreated (0hr) or immunized with 1×10^{4} cfu wt, spleens removed at 24hr and 48hr post-infection, dissociated, stained and iNOS synthesis determined by ICS. i) Representative expression of iNOS and MHCII in $CD3^{-}CD19^{-}CD11b^{+}Ly6C^{high}$ monocytes over time, and ii) summary of the number of $iNOS^{+}$ $MHCII^{+}$ Tip-DC/spleen. Each symbol represents one mouse. Data represents the mean ± SEM of each group. Results shown are representative of three independent experiments. Statistics calculated by Student's *t* test; ns = no significant differences observed between the groups analyzed; * = $p<0.05$; ** = $p<0.01$.