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PRINCIPAL INVESTIGATOR: Keith S. Bahjat, Ph.D.

CONTRACTING ORGANIZATION: Providence Portland Medical Center
Portland, OR 97213

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Keith S. Bahjat, Ph.D.

E-Mail: keith.bahjat@providence.org

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14. ABSTRACT
Cells with DNA mutations can be recognized by the immune system and many times, eliminated before causing disease. When these cells have lost the ability to control their own proliferation, and when the immune system can no longer recognize them, a tumor may occur. The objective of cancer immunotherapy is to retrain the immune system to recognize tumor cells, leading to control of tumor growth or even complete eradication of the tumor. Vaccines capable of teaching the immune system to recognize cancer cells must be extremely potent. Many researchers are exploring the use of live-attenuated microbes as vaccines for the treatment of cancer. Because the immune response elicited by these microbes is extremely potent, the immune system responds vigorously before being shut down by regulatory pathways pre-programmed in the immune system. By modifying how these regulatory pathways function in specific cells of the immune system, we can improve the tumor-specific immune response without causing additional risk to the patient. The goal of our proposal is to modify a live-attenuated vaccine vector based on the food-borne pathogen Listeria monocytogenes to promote a tumor-specific immune response while concurrently removing the brakes from a portion of the immune system. We believe this will increase the magnitude and quality of the tumor-specific immune response and improve the effectiveness of these cancer vaccines.

15. SUBJECT TERMS
T Cells, Listeria monocytogenes, cancer vaccines

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INTRODUCTION

We have been working with live-attenuated *Listeria (L.) monocytogenes*-based vaccine platforms for cancer as a way to elicit a potent CD8+ T cell response, an effect attributed to the enhanced delivery of antigen directly to the host-cell cytosol. However, while *Listeria* vaccines elicit inflammatory cytokines acutely (e.g. type I IFN, IL-12p70 and IFN-γ), they also elicit a compensatory negative feedback loop that acts to limit cellular activation. Unfortunately, this feedback also acts to limit the effectiveness of anti-tumor vaccines, including those containing *L. monocytogenes.* The suppressor of cytokine signaling (SOCS) protein 1 (SOCS1) is induced by cellular activation and serves as a one of these negative feedback mechanism for cytokines sharing the common gamma chain (i.e. IL-2, IL-4, IL-7, IL-15), IFN-α, IFN-γ, and IL-12). Importantly, silencing SOCS1 in dendritic cells (DC) enhances antigen presentation, T cell priming, and anti-tumor immunity. Therefore, our overarching hypothesis was that SOCS1-mediated negative feedback limits the potency of live-attenuated *L. monocytogenes*-based vaccines, particularly via the role of SOCS1 expressed in CD11c+ immune cells.

Unexpected challenges. As described in our grant proposal, we originally obtained Socs1^{floxp} mice from Dr. Yoshimura.; however, an unfortunate and unavoidable situation within our institution's vivarium resulted in the loss of this mouse strain. This incident has caused delays in the conduct of animal studies and necessitated a reduced scope of work due to funds that were lost breeding the original colony of mice. As such, Aim 2 will not be completed as described. We did obtain new Socs1^{floxp} animals from Dr. Subburaj Ilangumaran at University of Sherbrooke as his mice that originated from the Yoshimura lab. These animals were crossed with B6.CD11c-Cre-EGFP, to obtain mice lacking SOCS1 specifically in CD11c+ cells denoted with the strain name B6.CD11c-Cre-EGFP Socs1^{floxp}. Despite challenges in generating mice, we have re-established a Socs1^{floxp} colony and characterized B6.CD11c-Cre-EGFP Socs1^{floxp} mice in order to begin to address our hypothesis by the completion of Aim 1 and partial completion of Aim 3.

STUDIES AND RESULTS

Aim 1: Test the hypothesis that DC-intrinsic SOCS-1 expression limits DC maturation, and ultimately the potency of the antigen-specific CD8+ T cell response after vaccination with a live-attenuated *L. monocytogenes*.

Aim 3: Test the hypothesis that secretion of a SOCS-1 small peptide antagonist by a live-attenuated L. *monocytogenes* vaccine can enhance DC maturation, T cell priming and anti-tumor efficacy.

Aim 1 Results

Mouse strain generation. We generated the CD11c-Cre-EGFP Socs1^{floxp} strain by crossing CD11c-Cre-EGFP Socs1^{floxp} homozygous (hom) males with SOCS^{floxp} females (since homozygous females had problems giving birth and 30% of these animals developed dermatitis) generating mice deficient in Socs1 in CD11c+ cells, primarily comprising DCs. For our experiments we included wild type Socs1^{floxp} (SOCS1 f/f) and CD11c-Cre-EGFP Socs1$^{floxp/+}$ heterozygous (het) mice as control strains. Using these strains we tested whether DC-intrinsic SOCS-1 expression altered the innate immune response (Milestone 1.1) or T cell response (Milestone 1.2) to *L. monocytogenes.*

Immune response to *L. monocytogenes* challenge. To determine whether differences existed between innate responses elicited by *Listeria monocytogenes* challenge in Socs1^{floxp} wild type (SOCS1 f/f), CD11c-Cre-EGFP Socs1$^{floxp/+}$ heterozygous (cd11c CRE SOCS1 (het)), or CD11c-Cre-EGFP Socs1 ^{floxp} homozygous (cd11c CRE SOCS1 (homo)) mice, serum cytokines from infected mice were measured in serum obtained 24, 48 and 72 hours after infection to examine cytokine kinetics. Mice were challenged with ActA-QVac at 1 x10^5 cfu per mouse, representing a priming dose. Cytokines sharing the common gamma chain (γ chain), and thus anticipated to be disrupted by SOCS1 ablation (Interleukin (IL)12p70 and Interferon gamma (IFN-γ)), as well as others not requiring γ chain (tumor necrosis factor alpha (TNF-α) and IL-6) were measured.

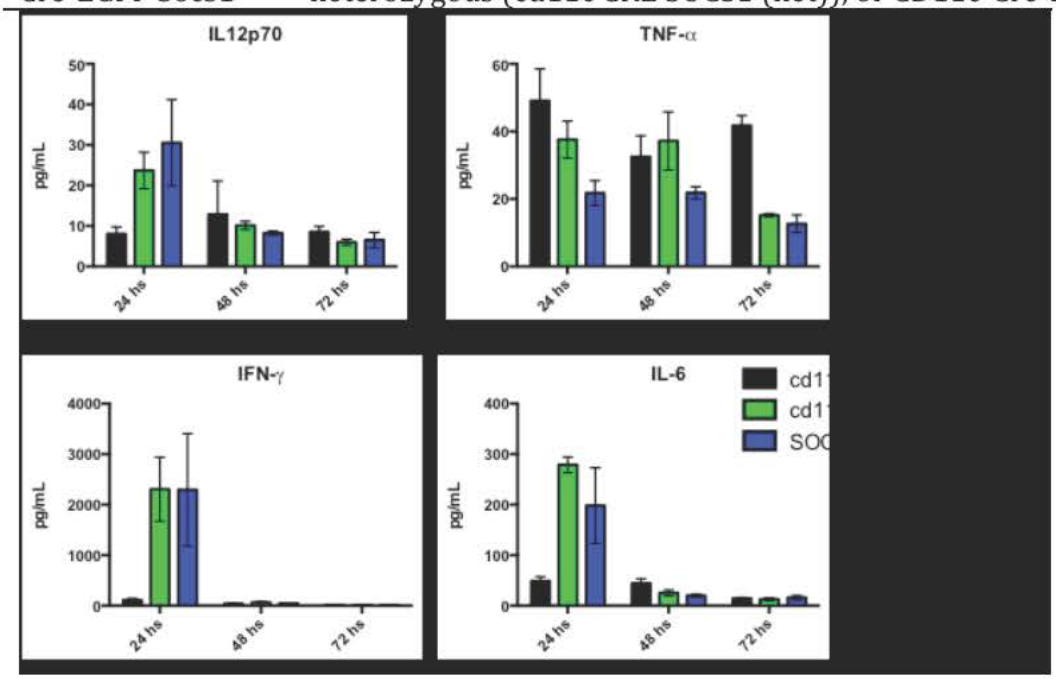

Fig 1. In vivo systemic cytokine response to bacterial challenge in mice lacking SOCS1 in CD11c+ cells. Serum cytokines (indicated above each graph) from mice infected with Lm. Mice were primed with ActA-QVac (1 x10^5 cfu) and serum samples obtained after the indicated time points (x-axis) and cytokine levels (y-axis; pg/ml serum) were determined using Cytokine Bead Array assay kit (BD Biosciences). Data represent mean ± SEM, n = 4 Data represent results from an individual experiment.

As shown in **Fig 1**, IL12p70, IFN-γ, and IL-6 were induced acutely at least 24hrs after bacterial challenge in Socs1^{floxp} wild type (WT or SOCS1 f/f) mice, while these cytokines were attenuated in homozygous mice lacking SOCS1 specifically in CD11c+ cells (cd11c CRE SOCS1 (Hom)). CD11c-Cre-EGFP Socs1$^{floxp/+}$ heterozygous (cd11c CRE SOCS1 (Het)) mice had systemic cytokine levels comparable to WT with the exception of TNF-α (**Fig 1**). TNF-α levels remained elevated in homozygous mice deficient in SOCS1 on CD11c+ cells at 72 hrs compared to WT, while heterozygous mice displayed an intermediate phenotype with augmented TNF-α at 24 and 48 hours that returned to levels similar to wild type by 72 hrs post-bacterial challenge (**Fig. 1,** upper right panel). <u>These results suggest that the absence of SOCS1 specifically in CD11c+ cells may lead to an attenuation of γ chain cytokines despite the absence of this putative negative regulator.</u>

We further examined antigen-specific immunity following priming with ActA-QVac bacterial strain. Socs1^{floxp} wild type (WT or SOCS1 f/f) and CD11c-Cre-EGFP Socs1$^{floxp/+}$ heterozygous (cd11c CRE SOCS1 (Het)) mice were able to mount OVA-specific T cell responses, as determined by tetramer staining (CD8+ T cells in **Fig 2A**)and/or IFN-γ intracellular cytokine staining (CD8+ and CD4+ T cells in **Fig 2B&C,** respectively). However, the strain with specific deletion of the *socs1* gene in CD11c+ cells showed significantly less OVA-specific CD8 and CD4 T cell expansion seven days post-priming ($p<0.05$ vs. Het and WT).

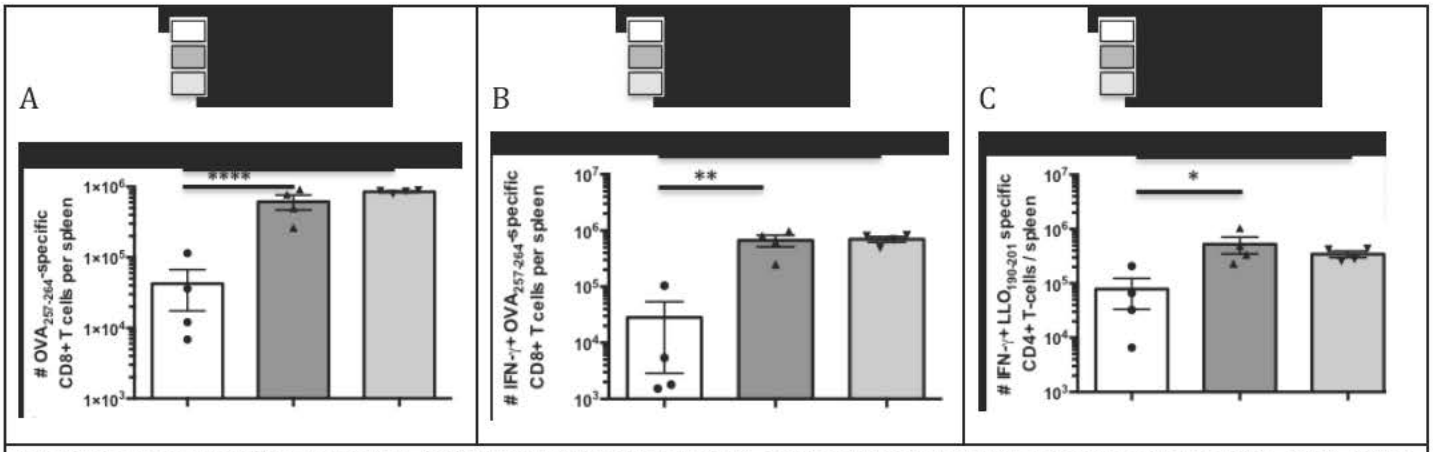

Fig 2. In vivo T cell expansion following priming with *L. monocytogenes*. Mice were primed with ActA-QVac and seven days later $OVA_{257\ 264}$-specific CD8+ T and $LLO_{90\ 20}$ - CD4+ T specific cells frequencies and numbers were determined from spleens by tetramer staining (**A.** CD8+ T cells) or by IFN-γ intracellular cytokine staining (**B.** CD4+ and CD8+ T cells). Experiments were repeated at least twice, indicated are the mean **±** SEM, n = 4. * p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001.

When we characterized the antigen-specific recall response in splenocytes from mice removed 5 days following a second challenge (boost) with *L. monocytogenes*, we observed a similar reduction in the expansion of OVA-specific memory CD8 T cells in mice lacking socs1 in CD11c+ cells (c11c CRE SOCS1 (Hom)) as compared to WT or Het controls (**Fig 3**). The LLO-specific CD4 T cell response during recall was also affected in the knockout strain but the change was more dramatic in CD8+ T cells (**Fig 3**).

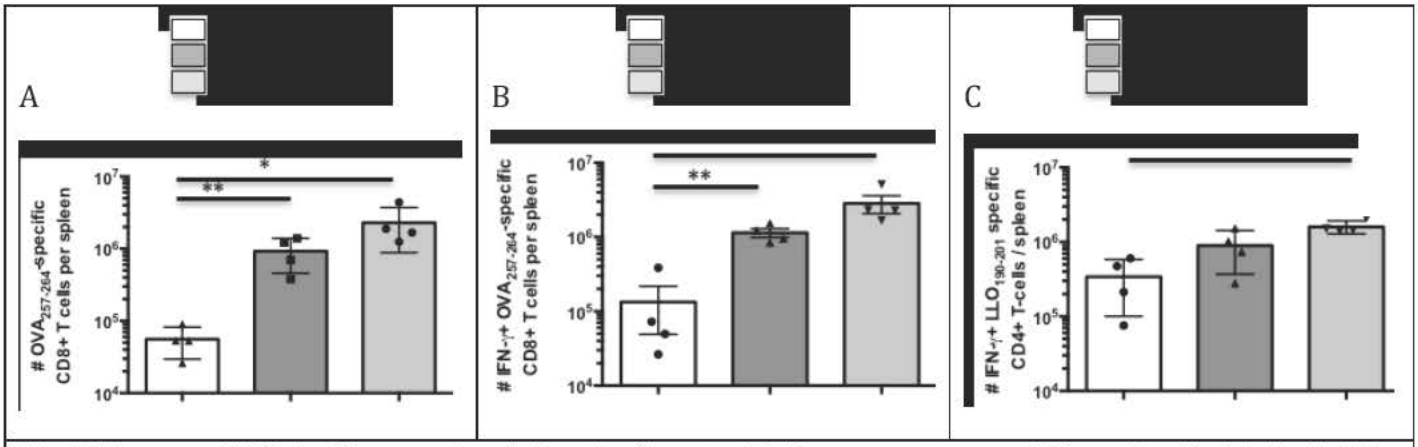

Fig 3. Memory CD8 T cell expansion following boost with *L. monocytogenes*. Mice primed with ActA-QVac were boosted 21 days later with the same *Lm* strain, 5 days post-boost $OVA_{257\ 264}$-specific CD8+ T and $LLO_{90\ 20}$ - CD4+ T specific cells frequencies and numbers were determined from spleens by tetramer staining (**A.** CD8+ T cells) or by IFN-γ intracellular cytokine staining (**B.** CD8+ and CD4+ T cells). Experiments were repeated at least twice, indicated are the mean **±** SEM, n = 4. * p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001.

Reduced innate immune reactivity (**Fig 1**) and reduced naïve (**Fig 2**) / memory T cell expansion (**Fig 3**) in mice lacking SOCS1 in DCs were unexpected findings given that our postulate was that deletion of *socs1* in DCs would promote T cell activation and expansion, While these data do not fit with SOCS1 being a negative regulator of DC-induced T cell priming in this system, we postulated that differences in bacterial clearance between these strains could explain the differences in immune response that we observed, irrespective of SOCS1 status. We measured bacterial clearance in spleen and liver after infection with $1x10^5$ cfu of ActA-QVac (the same amount of bacteria used in our prime/boost experiments) in each of these strains. We determined that bacterial clearance was enhanced in the liver acutely (**Fig 4A**) and at 3

days following infection (**Fig 4B**) in mice lacking SOCS1 in CD11c+ cells as compared to WT controls, with an intermediate phenotype being observed by day 3 in livers from infected heterozygotes (**Fig 4B**).

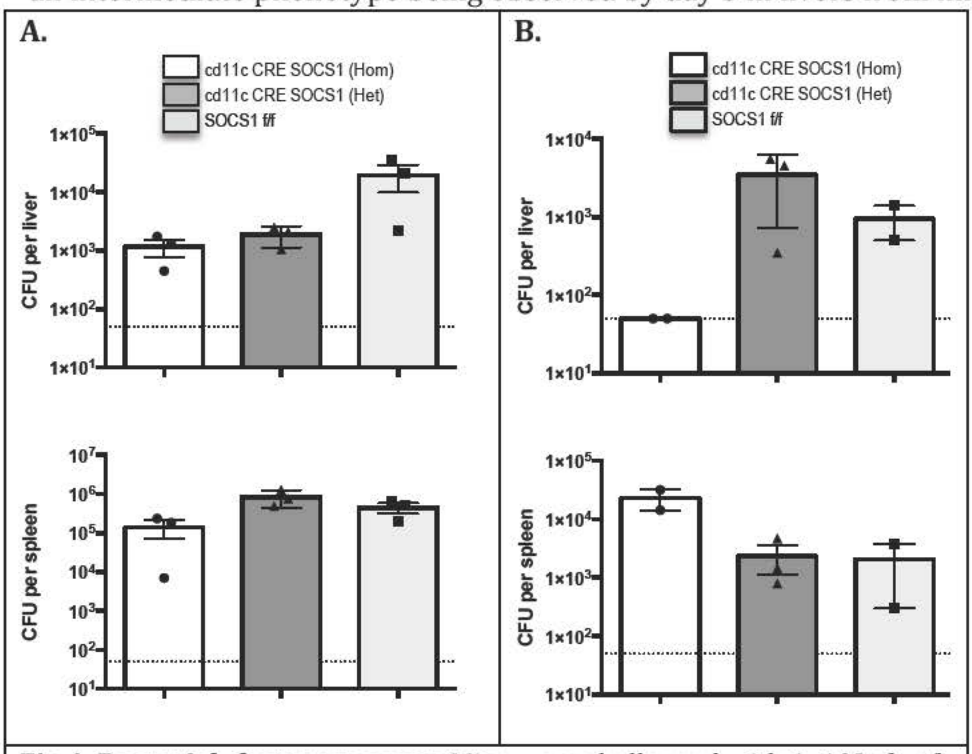

Fig 4. Bacterial clearance assay. Mice were challenged with 1x10^5 cfu of ActA-QVac and spleens and livers were obtained, homogenized and bacteria plated on BHI plates daily for 3 days and values for Day 1 (**A**) and Day 3 (**B**) are shown. Bacterial colonies were counted and mean +/- SEM are shown for n=2-3 animals per group. Results are from individual expt.

Additionally, at 3 days following infection bacterial clearance in the liver remained enhanced compared to both WT and heterozygous control strains (**Fig 4A**). In contrast, clearance was reduced in the spleen of homozygous SOCS1 deficient mice compared to controls. Bacterial counts from spleens from all three mouse genotypes analyzed were comparable at 24 hrs post-infection (**Fig 4A**); however, by 3 days there were more bacteria in spleens from the homozygous strain (**Fig 4B**). These findings along with lower levels of IFN-γ, IL-6 and Il-12p70 measured in serum from these mice (**Fig 1**), correlate with the observed absence of specific CD8 T cell expansion (**Figs 2&3**).

Summary of Milestone Accomplishments. *Milestone 1.1: Determination of the innate immune response to ActA-QVac in B6.CD11c-Cre-EGFP.Socs1^{floxp} mice. Milestone 1.2: Determination of the antigen-specific cellular immune response to ActA-QV in B6.CD11c-Cre-EGFP.Socs1^{floxp} mice.* We achieved these milestones by showing here that the innate response to *L. monocytogenes* in mice lacking expression of *socs1* in CD11c+ cells was attenuated, as demonstrated by reduced systemic levels of a host of inflammatory cytokines with the exception of TNF-alpha.

We also show that the antigen-specific immune response to *L. monocytogenes* was also attenuated as demonstrated by reduced expansion of CD4+ and CD8+ antigen-specific T cells. We also observed altered bacterial clearance, which could contribute to the unanticipated immune responses in homozygous animals lacking SOCS1 in CD11c+ cells.

Table1. Summary of in vivo findings.

	Phenotype vs. WT SOCS1 f/f control strain							
Experimental Outcome	**1 day**		**2 days**		**3 days**		**7 days**	
	Hom	Het	Hom	Het	Hom	Het	Hom	Het
IL-12p70, IFN, & IL-6	↓	=	=	=	=	=	NA	NA
TNF-alpha	↑	↗	↑	=	↑	=		
bacterial clearance liver	↓	↓			↓	=		
bacterial clearance spleen	↓	=			↑	=		
CD8+ primary expansion							↓	=
CD4+ primary expansion							↓	=
CD8+ recall							↓	↘
CD4+ recall							↓	↘

= denotes no d fference

↗ ↘ denote ntermed ate phenotype vs. Hom and WT

Clonally expanded T cell populations acquire a restricted set of effector functions that aid in clearance of the bacteria at a given time post-infection in a given tissue. The observed increased acute clearance could have altered cell trafficking and thus reduced the antigen specific responses generated at later timepoints. The fact that bacteria remained in the spleen for longer periods, could suggest priming of T cells was inefficient and thus animals were not able to clear the remaining bacteria.

The induction of adaptive immune responses of appropriate strength, kinetics and effector function occurs via efficient internalization of pathogens and dying cells, processing of this material into peptide antigens that are presented in the context of major histocompatibility complex (MHC) molecules to conventional T cells, and integration of inflammatory cues leading to proper polarization of T cells.

We postulate that altered bacterial clearance may reflect alteration of one or more of these parameters leading to reduced priming. The persistent inflammatory stimulation demonstrated by the prolonged TNF-alpha secretion observed in infected homozygous mice suggests that progression to a productive T cell response did not occur in the absence of SOCS1 in CD11c+ cells in these mice. Increased acute clearance could have altered cell trafficking and thus delaying or reducing the potential for antigen specific responses generated at later timepoints.

Aim 3 Results

Bacterial strain generation. We first set out to optimize the molecular biology of the candidate vaccine strains to support the proposal. In order to eventually test our hypothesis that secretion of a SOCS-1 small peptide antagonist by a live-attenuated *L. monocytogenes* vaccine can enhance DC maturation, T cell priming and anti-tumor efficacy **(Aim 3),** we needed to construct novel listeria strains and screen them for potential to secrete a fusion protein containing the JAK2 catalytic domain, JH1 (JAK homology 1), as residues 843-1132 of this domain are described as essential for the interaction with SOCS1 and SOCS3. We first amplified the 5' region of the *actA* gene including the promoter region and fused it by SOE-PCR to JH1 domain. This construct codes for the following: *1)* ActAN100 domain coding for the first 100 amino acids of the ActA protein, a sequence that allows for an efficient secretion of the new protein into the host-cell cytosol and *2)* the JH1 domain of JAK2 (pJAK2) including both the Tyr_{1007} residue that is phosphorylated in the wild type protein after interacting with the cytokine receptor as wells as the GQM motif recently described as important for interaction with SOCS3 (and possibly with SOCS1). We then integrated the gene encoding the engineered ActA-pJAK2 (ActA-X-pJAK2) protein in the chromosome of the *L. monocytogenes* strain DPL4029 (*ΔactA*) by using the vector pPL2e. This novel strain (ActA-X-pJAK2) showed low levels of secretion in macrophages when compared to the ActA-QVac strain. Importantly, the *ΔactA* deletion was maintained in all strains to allow the bacteria to infect the antigen presenting cells (APC) and escape from the vacuole, but not spread to other cells due to their inability to polymerase actin. We subsequently generated the **5 unique bacterial strains** described in **Table 1** (unpublished). While the immunogenicity of the strains remains intact, their pathogenicity has been reduced using *ΔactA* deletion and for this reason they are all excellent vaccine candidates.

TABLE 2. List of *Listeria monocytogenes* strains generated

Strain name	Genotype / Characteristics	Reference
DPL4029	*ΔactA*	Lauer *et al*. (2002)
ActA-QVac	*ΔactA pPL2e:actA-A42R*$_{(88-96)}$*-C4L*$_{(125-132)}$*-K3L*$_{(6-15)}$*-B8R*$_{(20-27)}$*OVA*$_{(257-264)}$	Lauer *et al*. (2008)
ActA-Lm-OVA	*ΔactA pPL2:LLO-OVA*	Bahjat *et al*. (2006)
ActA-X-pJAK2	*ΔactA pPL2e:actA-jh1*	This work
ActA-L-pJAK2	*ΔactA pPL2e:actA-L-jh1 with a linker*	This work
ActA-pJAK2-HA	*ΔactA pPL2e:actA-jh1 with a linker, SIINFEKL and HA C-term*	This work
ActA-pJAK2	*ΔactA pPL2e:actA-jh1 with a linker, HA and SIINFEKL C-term*	This work
ActA-pJAK2-AA	*ΔactA pPL2e:actA-jh1,same as ActA*-pJAK2 *with a* Y_{1007} Y_{1008} *to AA mutation*	This work

To allow the two domains (ActAN100 and JH1 domains) to move independently of one another and increase the probability of interaction between JH1 domain and SOCS1, we included a 12 amino---- acid "linker" [(Gly---- Gly---- Ser)$_4$] between them. This new strain (ActA---- L---- pJAK2, **Table 1**) showed better secretion than ActA---- X---- pJAK2. A SIINFEKL sequence was next added to effectively study the MHC---- I presentation for dendritic cells *in vitro* as well as *in vivo*. However, when we also placed the SIINFEKL ($OVA_{257-264}$) epitope upstream of the human influenza hemagglutinin motif (HA), a motif that allows us to perform immunoprecipitations to confirm the interaction with SOCS1/3 (ActA---- pJAK2---- HA; **Table 1**), we could not detect antigen presentation by using the B3Z reporter cell system designed to detect SIINFEKL antigen specific responses (K^b---- SIINFEKL---- specific T cell hybridoma with LacZ under control of the IL---- 2 promoter, courtesy of N. Shastri).

In the newest version of this strain, we placed a sequence corresponding to the HA motif before the SIINFEKL ($OVA_{257-264}$) epitope that now is located at the very end of the C---- terminus (ActA---- pJAK2, **Table 1** & **Fig 5**). Lastly, Ahmed *et al.* (J. Immunol, 2010) described that a small 13---- amino acid peptide containing the sequence surrounding the Tyr_{1007} residue of the JAK2 protein can antagonize the SOCS1 function both *in vitro* and *in vivo*. When the Tyr_{1007} and Tyr_{1008} residues were replaced by two Alanine residues (AA), the function of the peptide was abrogated meaning that SOCS1 activity was no longer inhibited. Taking advantage of this construct, we generated a strain containing the AA residues to assess specificity of the ActA---- pJAK2 response for SOCS1 inhibition (ActA---- pJAK2---- AA, **Table 1** & **Fig 5**).

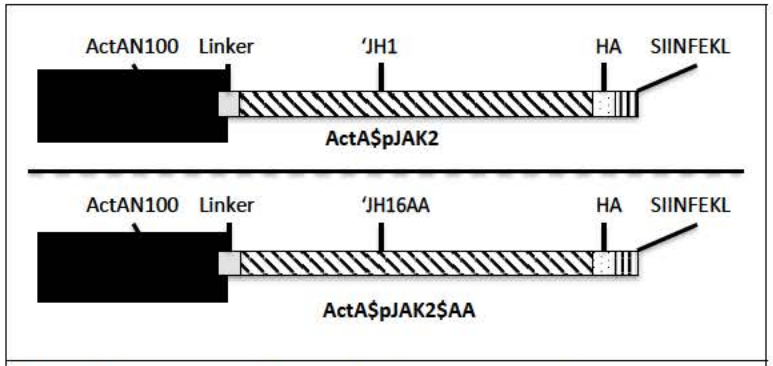

Fig 5. Schematic of the two leading candidate constructs for further study (see Table 1 for more information).

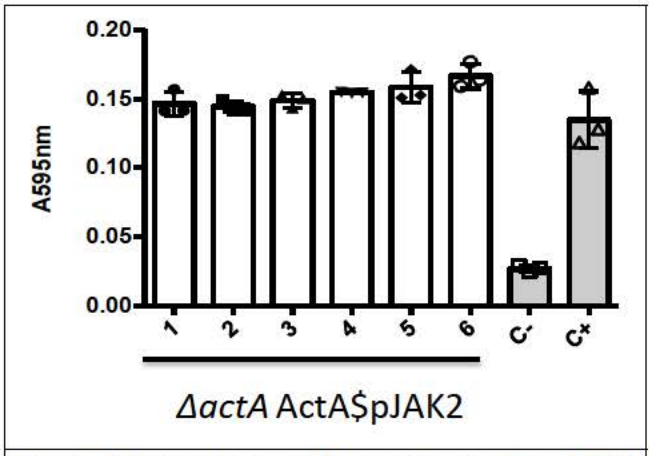

Fig 6. B3Z reporter cell assay results. DC2.4 dendritic cells were infected with various *L. monocytogenes* strains were incubated for 1hr at 37degreesC. Extracellular bacteria were washed away with PBS and B3Z T---- cells (K^b---- SIINFEKL---- specific T cell hybridoma with IL---- 2 driven LacZ) were added in gentamycin medium for 16hrs then lysed and β---- galactosidase activity measured (A595nm). X-axis numerals 1 to 6 denote different clones. C---- :negative control (*ΔactA*), C+: positive control (*ΔactA* ActA---- QVac).

Candidate Strain Characterization. We first examined the expression and presentation of ActA---- pJAK2 by infecting DC2.4 dendritic cells with this *Lm* strain. Using a B3Z reporter cell system (K^b---- SIINFEKL---- specific T cell hybridoma with LacZ under control of the IL---- 2 promoter, courtesy of N. Shastri) we demonstrated that dendritic cells presented the SIINFEKL epitope at similar levels to those observed in our positive control, *ΔactA* actA---- QVac a *Lm* strain which secrets the ActAN100 domain fused to various immunogenic epitopes, including SIINFEKL (**Fig 6**). To test the hypothesis that this protein can interact with SOCS1/3 and inhibit its function, with the consequence being the induction of the JAK---- STAT pathway, we also cloned the same construct in the pcDNA3.4 vector and transfected RAW 267.4 macrophages. In this construct the gene encoding the fused protein is under the control of the CMV promoter. Transfected macrophages were treated with IFN---- γ and the STAT1 phosphorylation was analyzed by Western blot. The levels of pTyr701---- STAT1 in the macrophages transfected with recombinant plasmid were increased compared to those of the empty vector transfected cells, suggesting that ActA---- pJAK2 protein affects the levels of JAK/STAT activation by inhibiting SOCS1 (**Fig 7**).

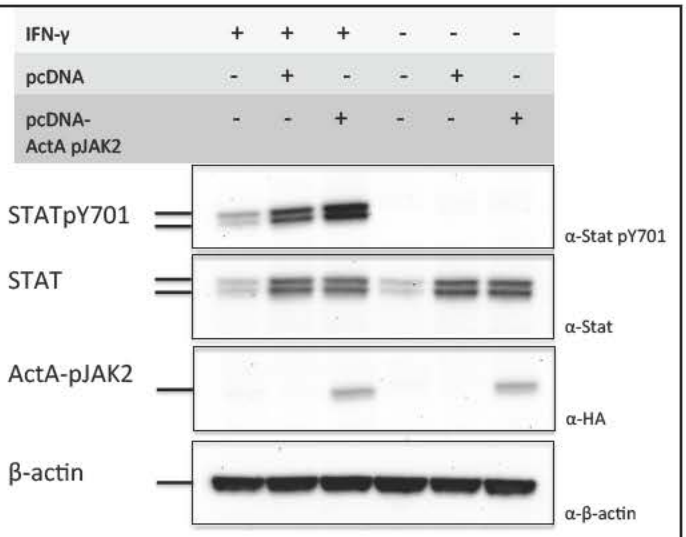

Fig 7. ActA-pJAK2 protein affects the levels of phospho-STAT in Macrophages. Macrophages were transfected with pcDNA3.4 (empty vector) or pcDNA3.4 expressing ActA-pJAK2 or neither vector (-ctrl) for 48 hrs. Then IFN-γ 1ng/ml or vehicle alone was added for 20min. Cells were harvested, lysed and total proteins extracted. Differential protein expression was determined by Western blot with the indicated antibody on the right.

Immunogenicity of the ActA-pJAK2-expressing *Lm* strain in C57Bl/6J mice. Since the construct expressed from the bacterial chromosome contains the SIINFEKL motif, we analyzed the SIINFEKL-specific CD8+ T cell response after prime and/or boost. As a control we used the ActA-QVac-expressing *Lm* strain. We first analyzed the response in splenocytes from animals infected with the aforementioned strains after 7 days of priming. Both tetramer staining (**Fig 8A**) and intracellular cytokine staining (ICS; **Fig 8B**) showed a significant increase in the SIINFEKL-specific CD8+ T cell expansion in animals primed with the ActA- pJAK2 strain as compared to ActA-QVac primed animals indicating that, as expected, the antigen presentation was enhanced when the SOCS1 protein was inhibited by the ActA-pJAK2 construct. The same result was observed when an ActA-OVA expressing strain (where the whole OVA protein is expressed fused to ActAN100) was analyzed together with ActA-pJAK2 (data not shown).

We next determined whether this ActA-pJAK2 strain was also able to generate a strong memory CD8+ T cells expansion by priming mice with either ActA-pJAK2- or ActA-QVac- expressing Lm strains followed by boost with same strain 21 days later. We then examined the spleens 5 days following boost to characterize the antigen-specific recall response. We also examined if the bacterial strain used during priming affected the response observed in the recall by injecting the already primed animals with a different strain. A strong memory CD8+ T cell expansion was observed in animals boosted with the ActA-pJAK2 strain irrespective of the strain used at for priming (**Fig 9**). When the response was analyzed in animals boosted with the ActA-QVac strain, only animals that were previously primed with ActA-pJAK2 demonstrated specific-CD8 expansion levels that were closer to those observed for ActA-pJAK2 boosted animals. Notably, every repeat of this experiment showed a heterogeneous response in the ActA-pJAK2 primed / ActA-QVac boosted group (**Fig 9**). Importantly, animals primed with either strain did not show differences in the number or functionality of antigen specific-CD8 T cells at the day of the boost i.e. 21 days post priming (data not shown). Altogether these results suggest a possible role for the pJAK2 domain present in our construct in determining the magnitude of antigen-specific CD8 expansion.

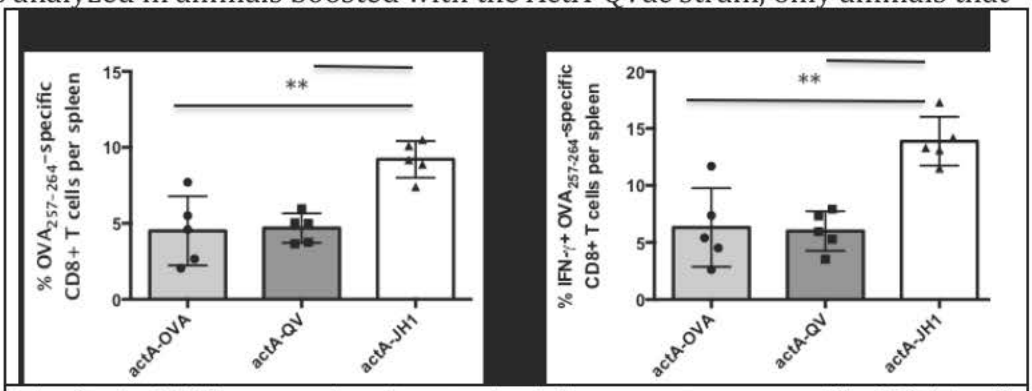

Fig 8. ActA-JH1 expressing Lm strain induces a strong specific-CD8 T cell expansion. Mice were primed with the indicated *Lm* strain and 7 days later $OVA_{257-264}$-specific CD8+ T cells frequencies were determined by tetramer staining (**A**) or by IFN-γ intracellular cytokine staining (**B**). Experiments were repeated at least twice, indicated are the mean ± SEM, n = 5. * $p<0.05$, ** $p<0.01$.

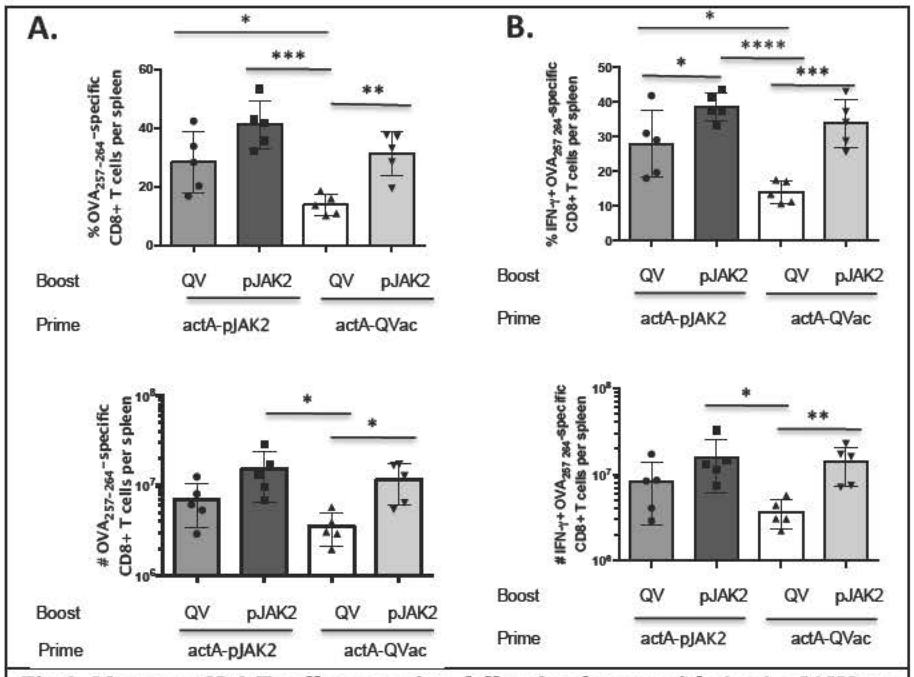

Fig 9. Memory CD8 T cell expansion following boost with ActA-pJAK2 or ActA-QVac. Mice primed with ActA-pJAK2 and ActA-QVac were boosted 21 days later with the indicated *Lm* strain, 5 days post-boost $OVA_{257-264}$-specific CD8+ T cells frequencies and numbers were determined by tetramer staining (**A**) or by IFN-γ intracellular cytokine staining (**B**). Experiments were repeated at least twice, indicated are the mean ± SEM, n = 5. * p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001.

In order to confirm that the strong SIINFEKL specificity of CD8 T cells in mice primed/boosted with the ActA-pJAK2 strain was a result of a specific SOCS1 inhibition during *Lm* infection of APC cells, we tested the ActA-pJAK2-AA strain where we introduced the $Y_{1007}Y_{1008}$ to AA mutations in the pJAK2 domain resulting in the function of the peptide being abrogated meaning that SOCS1 activity was no longer inhibited. B3Z assays and Western blots confirmed that the SIINFEKL epitope was presented by DC2.4 dendritic cells and the recombinant protein was secreted in macrophages at similar levels to those observed when ActA-pJAK2 and ActA-QVac were used as controls (data not shown). The immunogenicity of this control strain was tested in a similar prime/boost setting described above. When animals were primed with ActA-pJAK2-expressing *Lm* strain and boosted with the strain expressing the ActA-pJAK2-AA protein, levels of tetramer specific-CD8 T cells were significantly lower than those observed when the boost was performed with the ActA-pJAK2 strain deficient in SOCS1 activity (**Fig 10**).

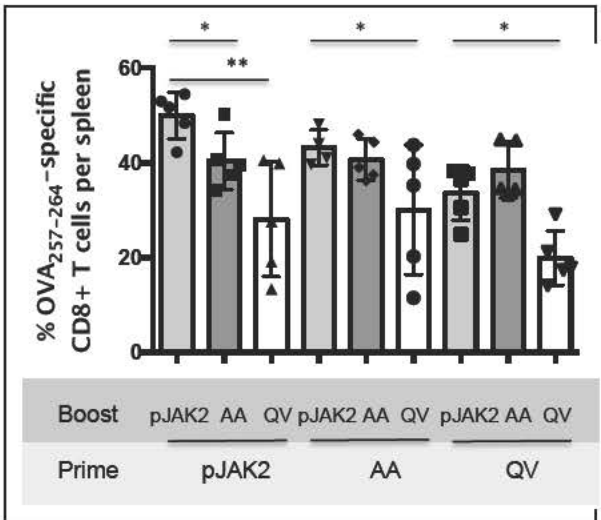

Fig 10. Memory CD8 T cell expansion following boost with ActA-JH1, ActA-JH1-AA or ActA-QVac. Mice primed with *Lm* strains were boosted 21 days later with indicated strain, 5 days post-boost $OVA_{257-264}$-specific CD8+ T cell frequencies were determined by tetramer staining. Experiments were repeated at least twice (mean ± SEM, n = 5. * p<0.05, ** p<0.01).

Summary of Milestone Accomplishments.

Milestone 3.1: Construction and confirmation of ΔactA□inlB-Lm-QV-pJAK2 and ΔactA□inlB-Lm-trp2□V-pJAK2.

Milestone 3.2: Determination of innate and adaptive immune response after vaccination with ΔactA□inlB-Lm-QV-pJAK2.

We achieved these milestones creating 5 novel strains of bacteria that allowed us to characterizing the ability of strains producing the inhibitory peptide attenuating SOCS1 activity versus strains lacking this property to induce innate and adaptive immune responses in vivo in mice. Although our results suggest that the ActA-pJAK2 protein does specifically inhibit the action of the SOCS1 negative regulator on the JAK/STAT pathway in APC cells generating a strong CD8 T cell expansion, the fact that the ActA-pJAK2-AA strain still induces memory CD8 expansion at relatively high levels compared to the ActA-QVac strain would indicate that the double mutation (AA) present in this stroain does not dramatically affect the function of the JH1

domain-containing protein (in contrast to what it was observed with a small peptide) or/and that there are other factors, besides SOCS1, that play a role in the strong CD8 response observed for the ActA-pJAK2 strain. Additional mutations or deletions in the GQM domain of the JH1 portion of the recombinant protein, together with the already analyzed AA mutations, could confirm whether the ActA-pJAK2 protein effectively induces the strong CD8 expansion specifically via inhibition of SOCS1 activity. Moreover, it is possible that our results could be related to the epitope used and may not readily translate to data derived using other epitopes in this system. For example, a different epitope other than SIINFEKL, such as a tumor-associated antigen, could also be engineered in this construct and tested for ability to induce tumor-specific CD8 T cell responses.

PROJECT GENERATED RESOURCES

Although we experienced delays we were finally able to regenerate the original line CD11c-Cre-EGFP Socs^{floxp}. and managed to also establish a second new mouse line of equal importance that will allow us to additionally characterize the role of SOCS1 in macrophages. While the breeding of mouse strains required more resources and time than expected due to unanticipated loss of our original colony, they will nonetheless continue to contribute to the generation of novel data in our laboratory. In addition, novel listeria strains were generated and characterized. These tools provide a positive *in vivo* control for SOCS1 inhibition during vaccination and represent important tools for other research programs examining SOCS1 in the context of immunity related to dendritic cell or macrophage function.

KEY RESEARCH ACCOMPLISHMENTS

- Characterized CD11c.cre.GFP Socs1^{floxp} and Lyzs-creERT2 Socs1^{floxp} animals in the context of vaccination with *L. monocytogenes* revealing that the innate and adaptive immune responses to *L. monocytogenes* in mice lacking expression of *socs1* in CD11c+ cells is attenuated with the exception of TNF-alpha secretion potentially due to altered bacterial clearance in homozygous animals lacking SOCS1 in CD11c+ cells.
- Generation of CD11c.cre.GFP Socs1^{floxp} and Lyzs-creERT2 Socs1^{floxp} animals, representing valuable tools to study the role of SOCS1 in immunological processes involving dendritic cells and macrophages with Listeria-based or other cancer vaccines.
- Construction of a unique strain of *L. monocytogenes* that secretes the ActA-pJAK2 fusion protein capable of inhibiting SOCS1 function *in vitro* and related negative control strain, ActA-pJAK2-AA (Milestone 3.1).
- Novel demonstration of enhanced immunogenicity of Δ*actA*- pJAK2 relative to the Δ*actA* parental strain in vivo (Milestone 3.2)

SIGNIFICANCE AND CONCLUSIONS

The significance of our work can be demonstrated by the generation of novel immunological tools to investigate the impact of inhibiting SOCS1 in DCs or macrophages in the context of cancer vaccines including *L. monocytogenes*. We continue to investigate the role of SOCS1 in the immune response during priming and/or boosting with *Listeria*-based vaccines to evaluate the impact of SOCS1 abrogation on anti-tumor immunity.