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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

Listeria monocytogenes is a ubiquitous Gram-positive facultative intracellular pathogen typically found in soil and food that is harmless to healthy individuals. We have been working with live-attenuated *L. monocytogenes*-based vaccine platforms for both cancer and infectious disease. *L. monocytogenes* elicits a potent CD8+ T cell response in mice, attributed to the delivery of antigen directly to the host-cell cytosol, as well as an inflammatory cytokine milieu that includes type I IFN, IL-12p70 and IFN-γ. Importantly, these same inflammatory cytokines elicit a negative feedback loop that acts to limit cellular activation.

CD8α+ dendritic cells (DCs) are a subset of antigen presenting cells poised to produce IL-12p70 and prime CD8+ T cells during microbial infection. CD8α+ DCs are directly infected by *L. monocytogenes* and play a critical role in priming *L. monocytogenes*-specific T cells. When the CD8α+ DCs are directly infected they are responsible for priming CD8+ T cells, in consequence we could theoretically modify the bacterium to enhance antigen presentation, maturation and survival of the infected CD8α+ DCs.

The suppressor of cytokine signaling (SOCS) family proteins (SOCS1-7 and CIS) are a group of structurally related proteins characterized by a central SH2 domain; a 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), IFN-α, IFN-γ, 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 also contains a kinase inhibitory region that can directly inhibit JAK tyrosine kinase activity. In addition, SOCS1 regulates MyD88-dependent signaling via the ubiquitylation and degradation of MAL and the p65 subunit of NF-κB. Importantly, silencing SOCS1 in DC enhances antigen presentation, T cell priming, and anti-tumor immunity. Therefore, SOCS1 is an important negative regulator of innate and adaptive immunity, and inhibition of SOCS1 could profoundly augment the potency of specific cancer vaccines. Our primary hypothesis is that SOCS1-mediated negative feedback limits the potency of live-attenuated *L. monocytogenes* based vaccines. While SOCS1 plays a significant role in limiting cytokine and TLR signaling in almost all cell types, the focus of this proposal will be on the role of SOCS1 in DCs and macrophages.

BODY

Aims 1 and 2.

As described in our proposal, we had originally obtained Socs1^{floxp} mice from Dr. Yoshimura. An unfortunate and unavoidable situation within our Institution's vivarium resulted in the loss of this mouse strain at our Institution. We have now obtained new Socs1^{floxp} animals in C57BL/6 background from Dr. Subburaj Ilangumaran at University of Sherbrooke (also originalyy obtained from the Yoshimura lab). These animals are being crossed with B6.CD11c-Cre-EGFP, to obtain mice lacking SOCS1 in CD11c+ cells. Importantly for this proposal, we have already obtained Lyzs Cre-ERT2 from the Institut Clinique de la Souris-ICS, France. These animals will be crossed with Socs1^{floxp} to obtain mice that will show absence of SOCS1 expression in both macrophages when tamoxifen is administrated. This novel strategy will allow us not only to study the role of

SOCS1 in these two important cell types but also to dissect its role at various time points after the priming and/or boost with Listeria-based vaccines.

Aim 3.

To test the hypothesis that the secretion of SOCS1 antagonist proteins by attenuated *L. monocytogenes* can inhibit the interaction between SOCS1 and JAK2 we have constructed multiple strains that are able to secrete a fusion protein containing the JAK2 catalytic domain (JAK homology 1, JH1 residues $_{843\text{-}1132}$). To this end, we amplified the 5' region of the *actA* gene including the promoter region and fused it, by SOE-PCR, with the JH1 encoding sequence. This construct codes for the first 100 aminoacids of the ActA protein that allows for an efficient secretion of the new protein into the host-cell cytosol. The JH1 catalytic domain present in this new protein has been described as essential for the interaction with SOCS1 and SOCS3. It includes 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). In the C-terminus of this ActA-JH1 protein we have added the SIINFEKL ($OVA_{257\text{-}264}$) sequence that will allow us to study the MHC-I presentation for dendritic cells *in vitro* as well as *in vivo* (Fig. 1).

The construct was sequenced, sub-cloned into the pPL2e vector and conjugated into the *L. monocytogenes* strain DPL4029 (*ΔactA*). The gene encoding this ActA-JH1-$OVA_{257\text{-}264}$ (ActA-JH1) protein integrates in the in the chromosome of the mentioned strain. The *ΔactA* deletion allows the bacterium to infect the antigen presenting cells (APC) and escape from the vacuole; however, it is unable to spread to other cells due to its inability to polymerase actin. While its immunogenicity remains intact, the pathogenicity is reduced and for this reason it is an excellent vaccine candidate.

We tested the expression and presentation of the ActA-JH1 protein by infecting DC2.4 dendritic cells with the new strain constructed as described. By using B3Z reporter cell system we demonstrated that the SIINFEKL peptide is presented by dendritic cells at similar levels as those observed in our positive control (*ΔactA* actAN100-QuadVacc) (Fig. 2).

It has been described that macrophages infected with *L. monocytogene*s show reduced STAT1-Tyr701 phosphorylation following IFN-γ exposure to induce the JAK-STAT pathway. Since this inhibition could be explained by the action of the SOCS1/3 proteins we have tested the functionality of our construct by infecting RAW 264.7 macrophages. To this end, we incubated the macrophages with various *L. monocytogenes* strains for different time points (4, 6 and 12 hours). IFN-γ was then added and the phosphorylation of STAT1 in positions Tyr701 and Ser727 as well as the levels of expression of the newly constructed protein were analyzed by Western blot. It is our hypothesis that the ActA-JH1 protein will bind to SOCS1 inhibiting its interaction with JAK2, and as a consequence, the phosphorylation levels of the STAT-1 protein would be higher in the presence of the new strain. We observed a clear induction of the STAT1-Tyr phosphorylation upon IFN-γ addition and this induction was clearly reduced as described previously in the presence of *L. monocytogenes*. Importantly, although the ActA-JH1 protein could be detected in infected macrophages, the levels of STAT-1 Tyr701 phosphorylation remained unchanged in the presence of ActA-JH1 (Fig. 3).

To determine whether the observed low levels of ActA-JH1 protein (when compared to ActAN100-QuadVac) could be the reason of the absence of response shown in these experiments we designed a new construct and tested it by using a similar approach. This new construct consists of the ActAN100 domain fused to the JH1 domain by a 12 amino-acids "linker" $[(Gly\text{-}Gly\text{-}Ser)_4]$. This new addition would allow the two different domains to potentially move independently of one another thus increasing the probability of interaction between JH1 and SOCS1. We have also added at the C-term of this protein the sequence corresponding to the human influenza hemagglutinin motif (HA) that would allow us to perform immunoprecipitations with macrophages or dendritic cells infected with this strain and confirm the interaction with SOCS1/3 (Fig. 1). Constructs were sequenced, sub-cloned into pPL2e and conjugated into *L. monocytogenes* DPL4029.

When infected RAW 267.4 macrophages were analyzed, higher levels of expression of the newly designed protein (ActA-L-JH1-HA) were observed; however, we were still unable to detect any change in the STAT1-Tyr701 phosphorylation compared to the infected controls (Fig. 4). In addition, the same construct was cloned into the pcDNA3.4 vector and used to transfect in macrophages. After IFN-γ induction STAT1 phosphorylation was analyzed. The levels of pTyr701-STAT1 were similar to those of the non-transfected cells, indicating that the level of the ActA-L-JH1-HA protein secreted during *L. monocytogenes* infection is not a determinant for the observed absence of SOCS1 inhibition.

We are currently performing immunoprecipitation experiments to demonstrate that the ActA-L-JH1-HA protein can interact with SOCS1/SOCS3 *in vivo*. Since we have only analyzed one aspect of SOCS inhibition (STAT-1 Tyr701 phosphorylation), we will also analyze the induction of the GAS-luc promoter, a well-known reporter of the JAK/STAT-1 pathway, during macrophage infection with the new *L. monocytogenes* strain described above.

KEY RESEARCH ACCOMPLISHMENTS.

- Obtained B6.CD11c-Cre-EGFP and Lck-creERT2 animals to be crossed with $Socs1^{floxp}$ to study the role of SOCS1 specifically in dendritic cells and macrophages during priming/boost with Listeria-based vaccines.
- Constructed *L. monocytogenes* strains that secrete the ActA-JH1 fusion protein that will be instrumental to inhibit SOCS1 function *in vivo*.

REPORTABLE OUTCOMES.

Bacterial strains*L. monocytogenes* strains.

Strain name	Genotype
KB007	wt, tRNAArg::pPL2e-ActAN100-JH1-SIINFEKL
KB008	ΔactA, tRNAArg::pPL2e-ActAN100-JH1-SIINFEKL
KB016	ΔactA, tRNAArg::pPL2e-ActAN100-L-JH1-SIINFEKL-HA

Mouse strains.

Socs1^{floxp} CD11c-Cre-EGFP

Socs1^{floxp} Lyzs Cre-ERT2

CONCLUSIONS.

Although we have to restart our Socs^{floxp} mouse colony, we will be able to generate a new mouse line in addition to the previously described CD11c-Cre-EGFP Socs^{floxp}. These mice will generate novel data about the role of SOCS1 in the immune response during priming and/or boost with Listeria-based vaccines and provide a positive *in vivo* control for SOCS1 inhibition during vaccination.

The construction of novel *L. monocytogenes* strains that are able to secrete the engineered ActA-JH1 proteins (ActA-JH1 and ActA-L-JH1-HA) is an important step towards our main goal of inhibiting SOCS1 function in antigen presenting cells. Future work will be focused on determining (and improving) the levels of interaction between these proteins and the target. In addition, although our preliminary studies only focused on the STAT-1 Tyr701 phosphorylation as a read out for inhibition, we are now analyzing other reporters for this function.

SUPPORTING DATA.

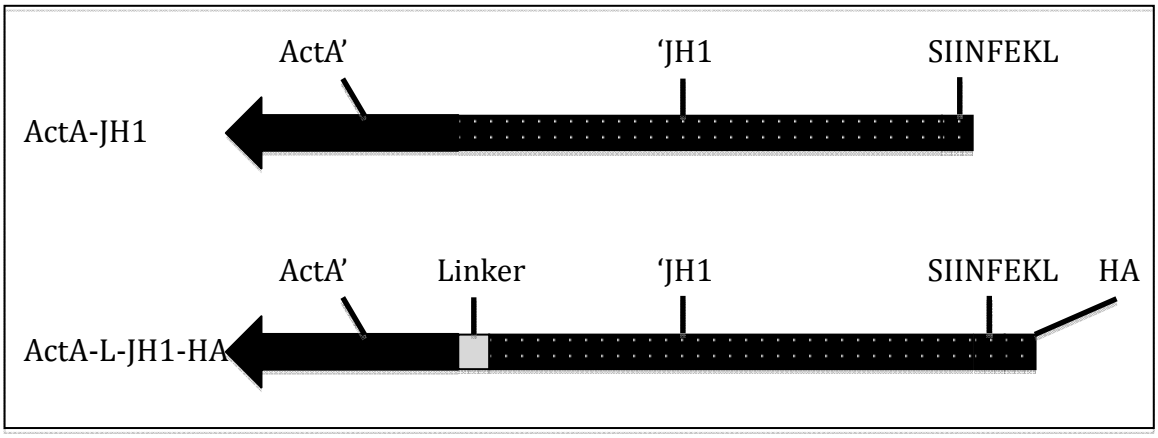

Fig 1. Schematic of the two constructs described in this work.

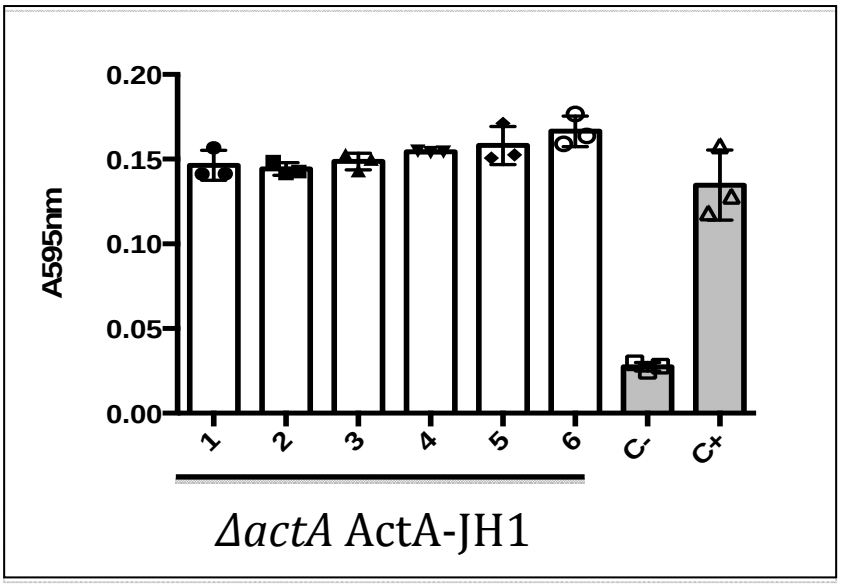

Fig 2. B3Z assay. DC2.4 dendritic cells were infected with various *L. monocytogenes* strains and incubated for 1h at 37C. Extracellular bacteria were removed by washing with PBS and B3Z T-cells added in gentamycin-containing medium. After 16hs of incubation, cells were lysed and β-galactosidase activity measured at A595nm.
Clones 1 to 6 are different clones expressing the ActA-JH1 protein, C-: negative control (*ΔactA*), C+: positive control (*ΔactA* ActA-QuadVac).

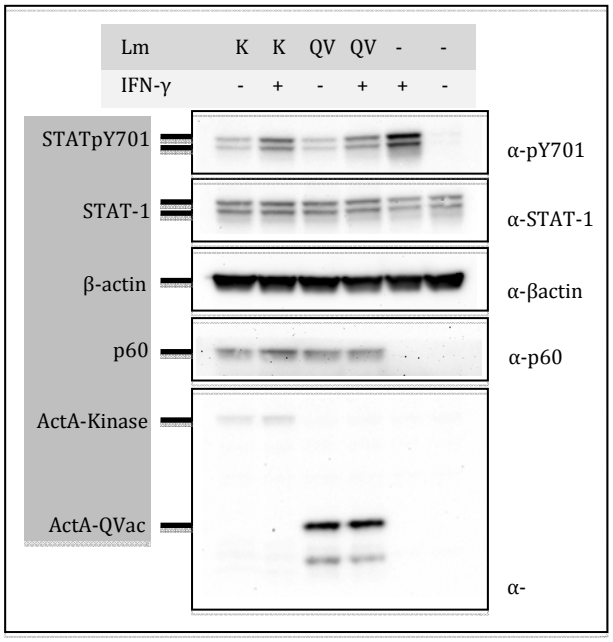

Fig 3. Macrophages were infected for 6hs with the indicated *L. monocytogenes* strain, after which IFN-y 1ng/ml was added for 20min to some of the samples. Cells were harvested, lysed and total proteins extracted. Different proteins expression was determined by Western blot with the indicated antibody. Clone K: *ΔactA* ActA-JH1 strain; QV: *ΔactA* QuadVacc.

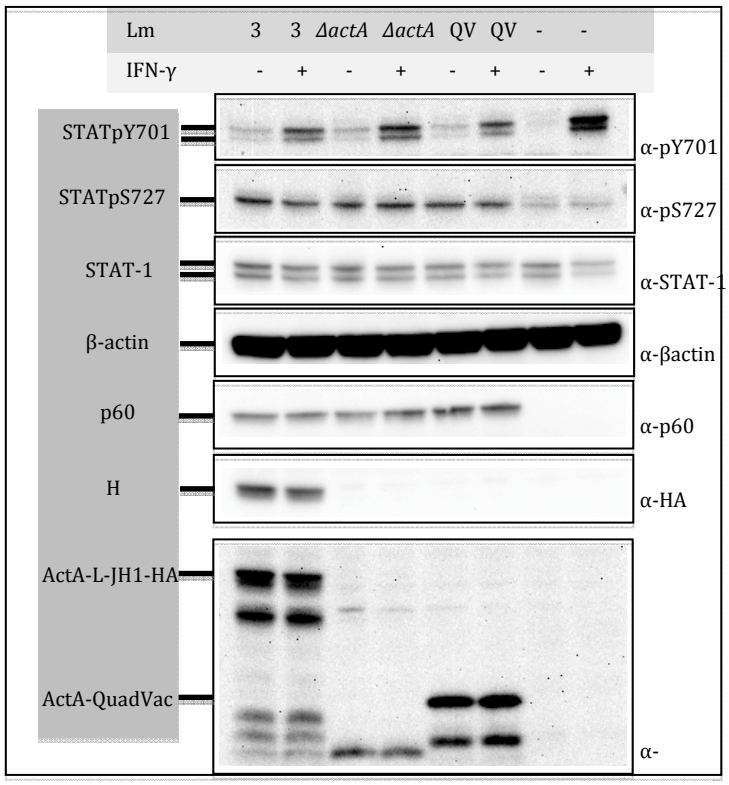

Fig 4. Macrophages were infected for 6hs with the indicated *L. monocytogenes* strain, after which IFN-y 1ng/ml was added for 20min to some of the samples. Cells were harvested, lysed and total proteins extracted. Different proteins expression was determined by Western blot with the indicated antibody. Clone 3: *ΔactA* ActA-L-JH1-HA strain; QV: *ΔactA* QuadVacc.