Enhancement of Cancer Vaccine Therapy by Systemic Delivery of a Tumor Targeting 

Salmonella-based STAT3 shRNA Suppresses the Growth of Established Melanoma Tumors

Edwin R. Manuel¹*, Céline A. Blache¹*, Rebecca Paquette⁴, Teodora I. Kaltcheva¹, Hidenobu Ishizaki¹, Joshua D.I. Ellenhorn², Michael Hensel³, Leonid Metelitsa⁴, and Don J. Diamond¹

¹Division of Translational Vaccine Research, City of Hope, Duarte, California; ²Division of General and Oncologic Surgery, City of Hope, Duarte, California; ³Mikrobiologisches Institut, Universitätsklinikum Erlangen, Erlangen, Germany; ⁴Department of Pediatrics, Baylor College of Medicine, Houston, Texas

* Equal contribution by both authors.

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Corresponding Author: Don J. Diamond, Director, Translational Vaccine Research, City of Hope, 1500 East Duarte Road, Duarte, CA 91010. Phone: (626)-256-4673 Fax: (626)-301-8981 Email: ddiamond@coh.org

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Abstract

Cancer vaccine therapies have only achieved limited success when focusing on effector immunity with the goal of eliciting robust tumor-specific T cell responses. More recently, there is an emerging understanding that effective immunity can only be achieved by coordinate disruption of tumor-derived immune suppression. Towards that goal, we have developed a potent *Salmonella*-based vaccine expressing codon-optimized survivin (CO-SVN) referred to as 3342Max. When used alone as a therapeutic vaccine, 3342Max can attenuate growth of aggressive murine melanomas overexpressing SVN. However, under more immunosuppressive conditions, such as those associated with larger tumor volumes, we found that the vaccine was ineffective. Vaccine efficacy could be rescued if tumor-bearing mice were treated initially with *Salmonella* encoding a shRNA targeting the tolerogenic molecule STAT3 (YS1646-shSTAT3). In vaccinated mice, silencing STAT3 increased the proliferation and granzyme B levels of intratumoral CD4\(^+\) and CD8\(^+\) T cells. The combined strategy also increased apoptosis in tumors of treated mice, enhancing tumor-specific killing of tumor targets. Interestingly, mice treated with YS1646-shSTAT3 or 3342Max alone were similarly unsuccessful in rejecting established tumors, while the combined regimen was highly potent. Our findings establish that a combined strategy of silencing immunosuppressive molecules followed by vaccination can act synergistically to attenuate tumor growth, and they offer a novel translational direction to improve tumor immunotherapy.
Introduction

Survivin (SVN) is a member of the inhibitor of apoptosis protein (IAP) family whose function is involved in prolonging cell survival and cell cycle control (1, 2). SVN is an ideal tumor-associated antigen (TAA) for therapeutic vaccination because it is overexpressed by essentially all solid tumors and is poorly expressed in normal adult tissues (3). Increased expression of SVN is also observed in endothelial cells during angiogenesis, thereby serving as an additional target for therapy (4). In animal tumor models, downregulation or inactivation of SVN has already been shown to inhibit tumor growth (5-7). Therefore, strategies to boost tumor-specific responses, such as using adjuvants or immunogenic vectors, will be critical to the success of therapeutic vaccination (8, 9).

Even when favorable vaccination conditions are discovered that promote robust tumor-specific immunity, these responses can eventually be compromised by expanding numbers of intratumoral regulatory T cells and myeloid-derived suppressor cells (10-13). Signal transducer and activator of transcription 3 (STAT3) has been recognized as an oncogenic transcription factor in myeloid and tumor cells that, when activated, inhibits production of immunostimulatory molecules and promotes expression of immunosuppressive molecules (14-16). A promising approach to inactivating STAT3 is the use of siRNA or shRNA, usually administered intratumorally, alone or conjugated to molecules that target specific cell populations (17, 18).

Advances in the generation of attenuated enteric bacterial vectors, such as *Salmonella typhimurium*, facilitates the highly translational tumor-specific delivery of antigens or plasmids (19-21). The vector itself acts as an adjuvant to elicit innate immunity and aid in generation of adaptive immunity against recombinant antigen. The most common *Salmonella* vaccines employ *Salmonella* pathogenicity I (SPI1) type 3 secretion systems (T3SS), which only produce recombinant antigen in a defined timeframe as the pathogen penetrates the host cell (22). More advanced vaccine designs
utilize SPI2 T3SS, which switches on recombinant antigen production when the *Salmonella* have entered the host cell, allowing for extended antigen production (23). Numerous studies have documented strains that colonize hypoxic regions of solid tumors weeks following intravenous injection, with no detectable bacteria in peripheral organs, making it an ideal delivery vehicle for targeting shRNA therapeutics into solid tumors (24-26).

In this report, we demonstrate a novel strategy utilizing two therapeutic agents delivered systemically that are inadequate to control tumor growth as single agents but succeed as a combined therapy. Specifically, attenuated *Salmonella typhimurium* carrying either a STAT3-specific shRNA plasmid (YS1646-shSTAT3) or an SVN expression plasmid (3342Max) were administered consecutively and observed to function synergistically leading to effective tumor rejection. The combined approach improves the prospects for successful vaccination against cancer by altering the tumor microenvironment to be less antagonistic to tumor infiltrating T cells such as those stimulated by vaccine-encoded TAAs.
Materials and Methods

Animals, tumor lines, and bacterial strains. C57BL/6 mice (Jackson, 6-8 weeks) were obtained from breeding colonies housed at the City of Hope (COH) Animal Research Center (Duarte, CA). The B16F10 murine melanoma line was a kind gift from Drs. Hua Yu and Marcin Kortylewski (COH, Duarte, CA). Cells were maintained in DMEM containing 10% FBS. S. typhimurium strains MVP728 (purD-/htrA-) and YS1646 (ATCC#202165) were cultured by shaking at 37°C in LB or LB-O media.

Salmonella SPI2 expression vectors, shRNA plasmids, and generation of recombinant Salmonella. pWSK29 constructs containing the SPI2 expression cassettes for LisA (2810) or SVN (3342) are described elsewhere (23). For construction of pWSK29 encoding Salmonella codon optimized survivin (CO-SVN), 2810 was digested with XbaI/EcoRV and the gel purified pWSK29 backbone was used to clone the CO-SVN gene (Genscript, Piscataway, NJ) engineered with XbaI/EcoRV sites for in frame fusion with the sseF gene. shRNA constructs against STAT3 (Origene, Rockville, MD) were tested for silencing by stable transfection of B16F10 cells followed by western blot (WB) analysis using polyclonal rabbit antibody against STAT3 (Santa Cruz Biotech, Santa Cruz, CA). The pGFP-V-RS vector containing the 29-mer shRNA sequence ACCTGAAGACCAAGTTCATCTGTGTGACA (ID#GI556360) exhibited >70% STAT3 knockdown and was selected for generation of recombinant YS1646. SPI2 expression vectors and shRNA plasmids were electroporated into MVP728 or YS1646, respectively, with a BTX600 electroporator (BTX, San Diego, CA).

Western blot analysis. WB for Salmonella expression of SVN was carried out as described previously (23). Briefly, 3342 and 3342Max were grown overnight in MOPS based media (Sigma) at 37°C containing either low phosphate (113 uM) to induce SPI2 expression or high phosphate (25
mM). Bacterial pellets were boiled in SDS loading buffer and equal amounts of lysate were loaded. Blots were probed using a monoclonal rabbit antibody (ab76424) against SVN (Abcam, Cambridge, MA).

**Tumor challenge, vaccination, and shRNA therapy.** For tumor challenge, $10^5$ B16F10 cells were injected subcutaneously into C57BL/6 mice. Tumor growth was monitored daily or every other day using a caliper. For testing vaccination alone, MVP728 carrying 2810, 3342 or 3342Max were administrated by gavage twice, 4 days apart, when tumors reached 3.5-4 mm in diameter at $10^8$ cfu. For combined therapy, PBS, YS1646-STAT3 or -scrambled was first injected at $10^7$ cfu in C57BL/6 mice when tumor volumes were $\geq 50$ mm$^3$ (7-8 mm in diameter) followed by gavage with PBS or $10^7$ cfu MVP728-2810 or -3342Max.

**Quantitative PCR for detection of STAT3 levels:** Mice bearing B16F10 tumors ($\geq 50$mm$^3$) were i.v. injected with $10^7$ cfu of YS1646-scrambled, -shSTAT3, or PBS twice, 4 days apart. At days 3, 7, and 10, mice ($n=3$) were sacrificed and RNA was extracted from tumor homogenates for generation of single stranded cDNA (Fermentas, Glen Burnie, MD). To quantitate STAT3 levels, SYBR®-Green qPCR analysis (BD Biosciences, Franklin Lakes, NJ) using primers specific for STAT3 (Forward: 5’-CATGGGCTATAAGATCATGGATGCGAC-3’, Reverse: 5’-AGGGCTCAGCACCCTCCACCGTTATTTTC-3’) was carried out using GAPDH (Forward: 5’-CAAGGTCATCCATGACAACCTTTG-3’, Reverse: 5’-GTCCACCACCCCTGTGCTGTA-3’) for normalization.

**Immunofluorescence staining.** For detection of intracellular SVN expression from recombinant *Salmonella*, RAW264.7 macrophages seeded on coverslips were infected for 30 minutes at an MOI of 10 with wildtype MVP728, 3342, or 3342Max. Cells were incubated overnight in DMEM-10 containing 10 µg/mL gentamicin. Cells were fixed/permeabilized with 1:1 acetone:methanol and
stained with conjugated antibodies FITC-LPS (Santa Cruz Biotech, Santa Cruz, CA) and PE-HA (Covance, Princeton, NJ) overnight at 4°C followed by DAPI. Cells were imaged on an Axiovert 200 using live imaging software (Axiovision, Skokie, IL). Images shown are representative of cells observed within multiple fields.

**Flow cytometry.** Conjugated mAbs directed to PECy7-CD8, PerCP-CD45, and PE-phospho-STAT3 were purchased from BD Pharmingen (San Diego, CA) and mAb to APC-Cy7-CD4, APC-F4/80, FITC-Ki-67, PE-Granzyme B, and FITC-Annexin V were purchased from eBioscience (San Diego, CA). Intracellular phosphor-STAT3, Granzyme B, Ki-67 and Annexin V staining were performed following the manufacturer’s protocol (eBioscience). Samples were run on a FACSCanto (Becton Dickinson, La Jolla, CA) and analyzed using FlowJo™ software (TreeStar, Ashland, OR).

**Cytotoxicity assay.** Cytotoxicity against B16F10 melanoma cells in treated mice was determined using a standard $^{51}$Cr release assay (27). Briefly, effectors were derived from spleens of B16F10-bearing C57BL6 mice ($n=4$) i.v. injected with either PBS, $10^7$ cfu of YS1646-shSTAT3 or -scrambled followed by gavage with PBS or $10^7$ cfu of 3342Max or 2810 4 days later. Mice were sacrificed ~1 week post-gavage and splenocytes were co-incubated with RMA-S cells loaded with human SVN library (27). Effectors were then co-incubated for 4 hours with 5,000 Cr$^{51}$-loaded B16F10 targets in 96-well plates at ratios of 100:1, 20:1, and 4:1 (in triplicate). Radioactivity released into the supernatant was measured using a Cobra Quantum gamma counter (PerkinElmer). Percent specific lysis: (experimental release - spontaneous release)/(maximum release - spontaneous release) x 100.

**Statistical analysis.** Statistical significance for comparisons between two or more groups was calculated with the Graphpad Prism Software v4.03 using the Student’s $t$ test or one-way ANOVA,
respectively. A $p$ value $<0.05$ was considered significant. All experiments were typically performed at least in duplicate, and all data are presented as mean ± SEM. *$p<0.05$, **$p<0.01$, and ***$p<0.001$. 
Results

Construction and evaluation of SVN expression vectors

Previous work using the MVP728 bacterial vector transformed with plasmid 3342, which expresses SVN, demonstrated partial success in rejecting murine models of colon carcinoma and glioblastoma (23). We found that SVN expression from 3342 was suboptimal when compared to LisA expression from 2810 (data not shown). We hypothesized that codon optimization (CO) of SVN to Salmonella preferred codons would allow for increased stability and protein expression leading to greater anti-tumor effects (28, 29). To test this hypothesis, a Salmonella typhimurium CO-SVN sequence was generated using an online algorithm (30) and then synthesized (Genscript). As shown in Fig. 1A, the low copy plasmid pWSK29 was engineered to encode the SPI2 chaperone protein sscB (31) and sseF protein fused to either LisA (2810), SVN (3342), or CO-SVN (3342Max) for expression and secretion by MVP728. Ultimately, expression of these genes would be regulated by the SPI2 specific promoter for sseA.

We determined whether CO actually increased SVN expression by growing the recombinant Salmonella in SPI2-inducing conditions (32) (Fig. 1B). Under non-inducing conditions (PCN+P), we found no significant expression of SVN. Surprisingly, under inducing conditions (PCN-P), we observed much greater SVN expression from 3342Max compared to the non-optimized 3342. To further evaluate SVN expression and secretion by 3342Max, we infected RAW264.7 murine macrophages with either 3342 or 3342Max to determine intracellular expression of SVN by immunofluorescence. As shown in Fig. 1C (HA-SVN panel), we observed greater SVN expression (characterized by more foci) compared to 3342. As expected, mAb staining for both the LPS (LPS-St panel) and HA (HA-survivin panel) localized to the cytoplasm and overlapped in the Merge panel, independent of the nuclear DAPI staining. These data suggest that optimization of SVN
tailored to preferred *Salmonella* codons greatly improves recombinant antigen expression.

**CO-SVN enhances suppression of tumor growth**

We next evaluated whether enhanced expression of SVN by 3342Max translated into a more efficacious vaccine using a B16F10 melanoma, which naturally overexpresses SVN (inset of Fig. 2A). Subcutaneously injected tumor cells were allowed to grow until a palpable tumor was present, generally 3.5-4 mm in diameter (<10mm³). Mice were then gavaged twice (4 days apart) with either PBS or MVP728 harboring 2810, 3342, or 3342Max constructs. As shown in Fig. 2A, 3342Max vaccination was superior to all other experimental treatments in attenuating tumor growth (*p*<0.01). We then determined the lymphocyte subsets that were most responsible for the attenuation by carrying out *in vivo* antibody depletions of CD8⁺, CD4⁺, or NK populations in tumor bearing mice vaccinated with 3342Max as in Fig. 2A. We observed that depletion of CD8⁺ T lymphocytes resulted in significant loss of tumor growth control with an intermediate effect of NK depletion, which has been described previously (17). These data suggest that vaccination with 3342Max elicits superior CD8⁺ T cell responses that limit tumor growth, likely a result of enhanced SVN expression.

**Attenuation of STAT3 mRNA levels using shRNA**

When subcutaneous B16F10 tumors were grown to larger volumes before treatment (7-8 mm in diameter, ≥50mm³), we discovered that 3342Max vaccination had no efficacy to attenuate growth (data not shown). Since we demonstrated that 3342Max works efficiently in less demanding circumstances (Fig. 2), we presumed that failure under more stringent conditions was likely the result of greater levels of tumor-derived immunosuppression (33). To determine if we could rescue the efficacy of the vaccine, we sought to manipulate the tumor microenvironment by silencing the tolerogenic molecule STAT3 (15, 34, 35). We chose to inactivate STAT3 mRNA expression using
an shRNA expression plasmid carried by the tumor-targeting *Salmonella* strain YS1646 (36). We first tested several commercially available shRNA plasmids (Origene) to silence the expression of STAT3 in stably transfected B16F10 tumor lines. As shown in Fig. 3A, shSTAT3#60 showed dramatic silencing (>70%) of endogenous STAT3 when compared to scrambled shRNA control plasmid. Other shSTAT3 plasmids had intermediate to no effect on endogenous STAT3 expression.

**Targeted silencing of STAT3 combined with 3342Max results in significant suppression of tumor growth in a more advanced melanoma tumor model**

We next generated YS1646 carrying the shSTAT3#60 plasmid (YS1646-shSTAT3) to test whether systemic delivery of *Salmonella* by i.v. route could silence STAT3 expression *in situ* in the tumor. Mice bearing subcutaneous B16F10 tumors (≥50 mm³) were injected twice i.v. with 10⁷ cfu of YS1646-shSTAT3, -scrambled, or PBS 4 days apart. Post-treatment, no significant attenuation of tumor growth was observed for mice treated with YS1646-shSTAT3 alone compared to control groups (data not shown). These same results were also observed in less stringent conditions where initial tumor volumes were <10mm³ (data not shown). Nonetheless, tumors were isolated, homogenized, and total RNA extracted for quantitative PCR. Surprisingly, there was significant silencing of STAT3 three days after YS1646-shSTAT3 administration compared to mice administered YS1646-scrambled or PBS (Fig. 3B). STAT3 silencing continued to increase on day 7 in the shSTAT3 group, which is consistent with the continued effectiveness of the therapeutic strategy (see below). On day 10, STAT3 silencing moderated, but was still lower than the control groups. Confirmation that YS1646-shSTAT3 succeeded in specifically silencing STAT3 mRNA but failed to reject tumors as a single agent motivated us to combine delivery of shSTAT3 and 3342Max vaccination in mice with significantly larger B16F10 tumors. Therefore, mice bearing B16F10 tumors ≥50 mm³ were i.v. injected with 10⁷ cfu of YS1646-shSTAT3, -scrambled, or PBS.
Four days later, mice were gavaged with $10^7$ cfu of 3342Max, 2810, or PBS. As shown in Fig. 3C, the combination of shSTAT3+3342Max rescues the activity of the vaccination to attenuate tumor growth significantly better than control groups. These results suggest that combining shSTAT3 therapy and SVN vaccination is a powerful synergistic approach to attenuate tumor growth.

**Decreased phospho-STAT3 levels are observed in tumor macrophages following shSTAT3 and 3342Max treatment**

We hypothesized that the success of the combined treatment was in part due to suppression of phospho-STAT3 levels in specific immune populations. Therefore, we used flow cytometry to determine the levels of activated STAT3 in specific immune subsets present in the tumor following treatment. We found no significant changes in phospho-STAT3 levels for CD4+, CD8+, CD11c+, or Gr1+CD11b+ cells in all treatment groups (Supplemental Fig. 1). However, we did observe significantly decreased phospho-STAT3 levels ($p<0.05$) in F4/80+ macrophages for the shSTAT3+3342Max treated group (Fig. 4A). Surprisingly, no significant decreases of phospho-STAT3 were observed for the shSTAT3+2810 group. These results suggest that only the shSTAT3+3342Max treatment is able to prevent activation of STAT3 in the F4/80+ subset, likely a result of early STAT3 silencing followed by tumor growth control, whereas shSTAT3+2810 is unable to do so regardless of early STAT3 silencing due to uncontrolled tumor growth.

**Combined shSTAT3 and 3342Max administration enhances infiltration of T lymphocytes**

Since it was known that ablation of STAT3 increases intratumoral immune function (14, 17, 35), we first examined the frequency and functional status of intratumoral CD4+ and CD8+ T cells in vaccinated mice. The percentage of B16F10 intratumoral CD4+ and CD8+ T cells was statistically greater in mice treated with shSTAT3+3342Max than in the scrambled+3342Max or shSTAT3+2810 treatment groups (Fig. 4B). We next evaluated the proliferative index of these
intratumoral CD4$^+$ and CD8$^+$ T cells by determining Ki67$^+$ expression levels. Both CD4$^+$ and CD8$^+$ populations expressed higher levels of Ki67$^+$ in the shSTAT3+3342Max group compared to control groups (Fig. 4C and D). The markedly higher proliferation potential suggests that the combined shRNA and vaccination treatments allow for a proliferative expansion of intratumoral T cells, and the increased frequency may therefore not solely be explained by a redistribution of existing T cells from other sites.

**YS1646-shSTAT3 enhances tumor-specific cytotoxic responses and tumor cell apoptosis**

We addressed tumor cell death by evaluating the extent of apoptosis using Annexin V staining of gated CD45$^-$ cells, mainly tumor cells (37), from all of the treatment groups. The CD45$^-$ cells revealed significantly higher apoptotic frequencies in mice treated with shSTAT3+3342Max than the control groups (Fig. 5A). The increased apoptosis of tumor cells could either be explained by the cytotoxic activity of immune cells or possibly by a shSTAT3-based mechanism to enhance apoptotic signal transduction. To address immune-based mechanisms, we investigated function of the CD8$^+$ T cell subset by evaluating granzyme B levels in B16F10 tumor-bearing mice ($\geq50\text{mm}^3$) treated with shSTAT3+3342Max versus groups treated with scrambled+3342Max or shSTAT3+2810 (Fig. 5B). The proportion of CD8$^+$ T cells expressing granzyme B in the mouse group treated with shSTAT3+3342Max was dramatically higher than both control groups. These results suggested a potential cytotoxic mechanism for tumor growth control, which we further assessed using a direct *in vitro* cytotoxicity assay.

**Tumor-specific cytotoxicity contributes to control of established subcutaneous B16F10 tumors**

We determined if T cells obtained from B16F10 tumor-bearing mice treated with shSTAT3+3342Max possessed functional capacity to kill survivin-expressing tumor cells *in vitro* by conducting a chromium release assay (Fig. 5C). Splenocytes harvested from B16F10 tumor-
bearing mice (n=3) treated as in Fig. 5A were in vitro stimulated with a human SVN peptide library, then evaluated for in vitro cytotoxic recognition and killing of chromium-loaded B16F10 tumor targets. Mice treated with either scrambled+3342Max or shSTAT3+2810 could not effectively kill B16F10 tumor cells. In contrast, splenocytes from all mice receiving shSTAT3+3342Max treatment were very effective at killing B16F10 tumor targets (0.001<p<0.01) at all effector ratios (Fig. 5D). These results further suggest that a potential mechanism of tumor growth attenuation is by tumor-specific T cells, stimulated through SVN vaccination or SVN peptide stimulation, directly killing tumor cells, though only when mice are pre-treated with shSTAT3 and then vaccinated with 3342Max.
Discussion

The goal of these studies was to discover a translational approach that would provide durable control of solid tumor growth. Our initial hypothesis was that SVN as a ubiquitously expressed TAA would provide the widest versatility for vaccination. In contrast to Salmonella-based SVN vaccines used in previous studies, which have been relatively ineffective when used alone, the goal was to find a vaccine strategy that would not require additional cytokine or chemokine components for effectiveness (8, 9, 23). A simplified one component regimen based on Salmonella delivery of SVN was the initial goal of this study. Our use of oral systemic administration of Salmonella transformed with SVN expression plasmids was similar to other reports describing Salmonella routes of administration. The advantage to this approach is that Salmonella are efficiently recognized by antigen processing macrophages in the gut or other mucosal sites (38, 39).

Initial trials using 3342 were only partially successful against small tumors that had just a few days to develop vascularization (Fig. 2). By investigating the expression levels of SVN from 3342 and bacterial LisA from 2810, we discovered significantly lower levels of SVN by WB analysis compared to the bacterial LisA protein under identical conditions (data not shown). Since the bacterial LisA protein was so heavily expressed in Salmonella, we theorized that changing the sequence of SVN from human to Salmonella typhimurium preferred codons might achieve the same goal. In preliminary experiments, we found a gradation of effectiveness against growth of established subcutaneous tumors dependent on expression levels (data not shown). The predictability of the increasing effectiveness to reject established tumors made it unnecessary to continue to simultaneously evaluate all forms, and the most effective form (3342Max) was exclusively used in all further comparisons. Ultimately, we found that control of B16F10 tumor growth using 3342Max vaccination only worked shortly after tumor challenge, when tumors
became palpable. Upon treatment of mice with larger B16F10 tumors of volumes ≥50 mm³, the vaccine was unable to attenuate tumor growth. This was not surprising as immunosuppressive mechanisms likely became more established within the growing tumor, and the single modality vaccination had no means to overcome them (11, 40).

Despite advances in therapeutic vaccines, there now exists numerous studies that support the idea of tumor-derived immunosuppression contributing to tumor evasion (41). These mechanisms include the secretion of TGF-β or IL-10 leading to Th2 polarization (42-44) or production of IDO by myeloid cells to induce the generation of Tregs and T cell anergy (45, 46). The novel mechanisms by which STAT3 causes immunosuppression are just beginning to be unraveled (14, 15, 47), and with its other roles in tumor progression, has become a multi-faceted target that could potentially attenuate tumor growth on its own or enhance the anti-tumor effects of any immunotherapy. Disrupting tumor-induced immunosuppression has generally been studied in the context of its effects that are independent of antigen-specific vaccination. Only rarely has the combination of disrupting immunosuppressive mechanisms within tumors been combined with vaccination to limit tumor growth. A more recent study has examined tumor-associated stromal cells expressing fibroblast activation protein-α (FAP) as a source of immunosuppression in a model of pancreatic ductal adenocarcinoma (48). Administration of a therapeutic vaccine in the absence of FAP-expressing stromal cells showed significant increases in hypoxia-induced tumor necrosis when compared to FAP⁺ mice. Similarly, we have also shown modest additive effects when vaccination is combined with the drug gemcitabine (27), while others reported increased anti-tumor responses by inhibiting the tolerogenic molecule IDO (49). These studies emphasize that successful outcomes of immunotherapy will likely require overcoming tumor-induced immunosuppression.
In several genetic models of conditional STAT3 deletion, subsequent immune-enhancement enabled dramatic inhibitory effects on tumor growth. Mechanism-based studies revealed changes in cytokine profile, T cell subsets, and signal transduction modifiers that all contributed to the blunting of tumor growth as a result of a reduction or elimination of STAT3 expression (14, 34, 35). These elegant studies have lead to preliminary therapeutic strategies employing small molecule inhibitors and RNA interference by a variety of approaches that have in common direct intratumoral administration. These approaches have shown moderate efficacy, but in every case there is tumor breakthrough within 20-25 days post-administration. An alternative strategy has been the approach of tumor-targeting *Salmonella* delivery of shRNA eukaryotic expression plasmids by i.v. injection. In contrast to our findings, others have found efficacy through intratumoral administration of shSTAT3 alone (18). Nonetheless, the growth attenuation was transient and its translational potential, as an intratumoral therapy, remains in doubt. Although systemic administration of YS1646-shSTAT3 may require more diligent efforts to determine its specific cell targets, this obstacle does not detract from the translational feasibility of the approach for treatment of solid tumors. Our *Salmonella* approach and work published by others was similar in that the STAT3-specific shRNA sequence only had a single target. In contrast, a CpG DNA chimera with an RNAi sequence that was administered intratumorally had multiple off-target sequences >100 in the mouse genome that temper the interpretation of the results (17).

We show for the first time that an intravenously administered shRNA against STAT3 acts synergistically with an oral *Salmonella*-based vaccine against SVN in a therapeutic setting, resulting in suppression of subcutaneous B16F10 melanoma growth. We conclude that the *in vivo* suppression of B16F10 tumor growth is the result of increased tumor cell apoptosis, as determined by Annexin V staining, possibly caused by an increased level of tumor-specific CD8\(^+\) T cells within the tumor. We saw no changes in tumor-expressed SVN during the treatments (Supplemental Figure...
2) that might explain the increase in apoptosis or eventual escape from control (50). The higher Ki67\(^+\) levels also indicated that these intratumoral T cells were actively proliferating, thereby supporting the notion that shRNA against STAT3 attenuated immunosuppression within the tumor microenvironment. Moreover, the fact that neither the vaccine nor shRNA against STAT3 alone was effective to control tumor growth suggests that the combined treatments acted synergistically. These data support that implementing successful immunotherapy may be futile without a receptive tumor microenvironment generated through additional modalities such as shRNA to inhibit immunosuppression.
Figure Legends

**Fig. 1. Construction and validation of SVN expression vectors.** *A*, the expression vectors 2810, 3342, and 3342Max were constructed to encode HA-tagged LisA, SVN, or SVN codon-optimized for *Salmonella* (CO-SVN), respectively, using the low copy plasmid backbone pWSK29. Each of these proteins was fused to the SPI-2 protein sseF and its expression is dependent on the SPI-2 promoter sseA. Each construct was then electroporated into MVP728 (23). *B*, SVN expression from MVP728 harboring 3342 and 3342Max (Max) constructs was detected by western blotting (WB) of bacterial lysates cultured in inducing conditions (low phosphate media, PCN–P) or non-inducing conditions (high phosphate media, PCN+P). Fusion protein was detected using anti-SVN antibody. *C*, RAW264.7 cells infected with MVP728 alone, MVP728-3342, or -3342Max were fixed and permeabilized and then stained with the conjugated antibodies LPS-FITC, HA-PE, and the nuclear stain DAPI. Cells were imaged under 100X oil immersion using an Axiovert 200. *Scale bars*, 5 µm.

**Fig. 2. Codon optimization of SVN enhances suppression of B16F10 melanoma growth.** *Inset of A*, B16F10 melanoma cell lysates were analyzed by WB for SVN expression. *A*, C57BL6 mice (*n*=5) were injected subcutaneously (s.c.) with B16F10 on day 0 and then vaccinated with MVP728-2810, -3342, -3342Max, or PBS on days 3 and 7. Tumor volume was monitored daily. *B*, Following s.c. injection of tumor on day 0, mice bearing palpable B16F10 tumor were vaccinated twice with MVP728-3342Max (days 3 and 7) and then depleted of immune subpopulations (day 5) using 200 ug of anti-CD8 mAb (clone H35), anti-CD4 mAb (clone GK1.5), or anti-NK polyclonal Ab (anti-asialo GM1) with a maintenance dose every 3 days thereafter (27).

**Fig. 3. Targeted silencing of STAT3 using YS1646-shSTAT3 results in significant suppression of tumor growth when combined with 3342Max.** *A*, WB of STAT3 protein expression from B16F10 lysates after stable transfection of shRNA contracts (#58-61) with potential for silencing
STAT3. β-tubulin is used as a loading control. B, silencing of STAT3 expression in B16F10 tumor following i.v. injection of YS1646-shSTAT3. Mice bearing palpable B16F10 tumors were i.v. injected with $10^7$ cfu of YS1646-shSTAT3 twice, 4 days apart. Mice ($n=3$) were sacrificed on d3, d7, or d10 after first injection and tumor lysates were subjected to RNA extraction for qPCR analysis of STAT3 transcripts. C, YS1646-shSTAT3 rescues anti-tumor effects of MVP728-3342Max in B16F10 model. C57BL/6 mice bearing B16F10 tumors ($\geq 50$ mm$^3$) were treated with either PBS, YS1646-shSTAT3 or -scrambled by intravenous injection. Four days following treatment, mice were then vaccinated with either PBS, MVP728-2810, or -3342Max and then monitored for tumor growth.

**Fig. 4. YS1646-shSTAT3 treatment followed by 3342Max vaccination attenuates STAT3 activation in resident tumor macrophages and enhances infiltration of T lymphocytes.** B16F10 tumor-bearing mice ($\geq 50$ mm$^3$, $n=5$) were injected i.v. with $10^7$ cfu of YS1646-scrambled, -shSTAT3, or PBS. Four days later, mice were then gavaged with $10^7$ cfu of MVP728-3342Max, -2810, or PBS. B16F10 tumors were excised from mice seven days after vaccination and then homogenized for staining and flow cytometry. A, Comparison of phospho-STAT3 levels in F4/80$^+$ macrophage for each treatment group. Phospho-STAT3 expression is presented as mean fluorescence intensity (MFI) and error bars represent standard error of the mean (SEM). B, Frequency of CD4$^+$ and CD8$^+$ cells found in the tumor for each treatment group. Data represent absolute number of cells/mm$^3$ tumor. CD4$^+$ ($C$) and CD8$^+$ ($D$) T cells were also analyzed for the expression of the proliferation marker Ki-67.

**Fig. 5. YS1646-shSTAT3 enhances tumor-specific cytotoxic responses.** B16F10 tumor-bearing mice ($n=5$) received combined treatment as described in Fig. 4. A, Individual histograms of FITC-Annexin V stained tumor homogenates for a representative mouse from each treatment group. B,
tumor homogenates (n=5) from each group were stained with FITC-Annexin V and analyzed by flow cytometry. Mean fluorescence intensity (MFI) of Annexin V represents cells gated from total tumor CD45- cells. Error bars represent SEM. C, tumor homogenates (used in A) were stained with PE-Granzyme B and PeCy7-CD8 and then analyzed by flow cytometry. Data represent mean percentages of Granzyme B+CD8+ cells out of total CD8+ cells. D, splenocytes from mice in A (n=4) were isolated to generate effectors for use in a chromium release assay against B16F10 targets. To generate effectors, splenocytes were incubated for 7 days with RMA-S cells initially loaded with total human SVN library (15mers, overlapping by 11). Effectors were then incubated in a 4-hour Cr51 release assay with Cr51-loaded B16F10 targets at E:T ratios of 100:1, 20:1, and 4:1, in triplicate. Percent specific lysis: (experimental release-spontaneous release)/(maximal release-spontaneous release) x 100%.
References


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Figure 1
Figure 2
Figure 3
**Figure 4**
Figure 5
Enhancement of Cancer Vaccine Therapy by Systemic Delivery of a Tumor Targeting Salmonella-based STAT3 shRNA Suppresses the Growth of Established Melanoma Tumors

Edwin R Manuel, Céline A Blache, Rebecca Paquette, et al.

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