Developing a Cancer Therapy: Engineering *Salmonella enterica* Typhimurium to Express and Secrete Interleukin-21

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Background

When most people hear of *Salmonella enterica* Typhimurium, they think of a pathogen that causes the dreaded foodborne illness, gastroenteritis. When most people hear that you are trying to use *S. enterica* as a cancer therapeutic, they think you are a little crazy. *S. enterica* Typhimurium is remarkable in that it can invade and replicate within tumor cells. Tumor to normal tissue ratios of approximately 1000:1 have been observed when measuring the number of *S. enterica* cells in mouse tissues.[1] Cancer immunotherapy is designed to elicit an immune response to inhibit tumor growth and destroy cancerous cells. One major obstacle to using immunotherapies in the clinic is the severe toxicities and negative side effects associated with systemically administering purified immune effector molecules, such as cytokines, to a patient.[2] One approach to limiting these toxicities is tumor-targeted bacterial cancer therapy. Attenuated strains of bacteria, such as *S. enterica* Typhimurium can be used to selectively target tumors and may be engineered to express various immune-stimulating molecules.[3,4]

Previous studies have demonstrated that IL-21 is a promising cytokine for cancer immunotherapy due to its ability to induce the proliferation of T cells and natural killer cells.[5,6,7] Consequently, we are interested in engineering *S. enterica* to express and secrete mouse IL-21 via bacterial expression plasmids. My research has focused on the addition of secretion tags to the mIL-21 protein in order for mIL-21 to be secreted from *S. enterica* into the tumor microenvironment. Once expression of the mIL-21 protein is confirmed and shown to be biologically active, a strain of virulence-attenuated *S. enterica* that secretes mIL-21 will be administered to mice to test its efficacy as a tumor-targeted immunotherapy. If the secreted mIL-21 shows promising results in preliminary experiments in mice, the same secretion tags could be applied to other molecules known to stimulate the immune system.

Materials and Methods

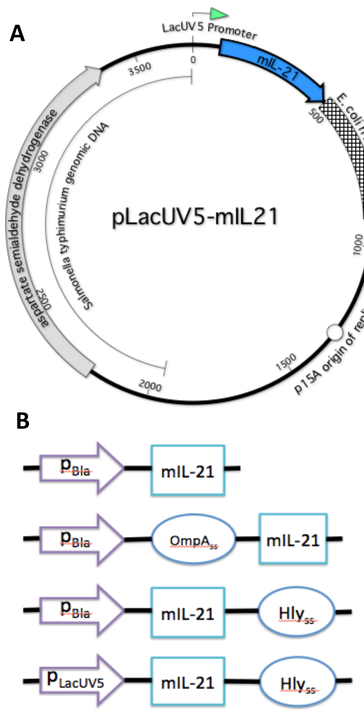

Figure 1. IL-21 Constructs
A. Attenuated χ4550 *S. enterica* Typhimurium was transformed with pLacUV5-mIL21 originating from plasmid pYA292 (see Materials and Methods). pYA292 was modified to contain the mouse IL-21 (mIL-21) cDNA under control of the lacUV5 promoter; this construct was used to make pBla-mIL21, pBla-OmpA-mIL21, pBla-mIL21-Hly, and pLacUV5-mIL21-Hly. B. The pBla-OmpA-mIL21 construct was made by fusing the mIL-21 protein to the N-terminal 21 amino acids of the *E. coli* OmpA protein secretion signal to export mIL-21 into the periplasm. The pBla-mIL21-Hly and pLacUV5-mIL21-Hly constructs were made by fusing the mIL-21 protein to the C-terminal 60 amino acids of the *E. coli* hemolysin A protein to export the mIL-21 fusion protein to the extracellular space. Transcription of the mIL-21 gene was controlled by either the β-lactamase promoter (P_{Bla}) or the *lac*-derived LacUV5 promoter (P_{LacUV5}).

Materials and Methods

Plasmid Construction
Attenuated χ4550 (Δ*crp*-1 Δ*cya*-1 Δ*asd*A1) *S. enterica* Typhimurium and plasmid pYA292 were obtained from Roy Curtiss III (Arizona State University). Plasmid pYA292 contains the aspartate semialdehyde dehydrogenase (*asd*) gene, which is used as a conditional-lethal selection for plasmid maintenance to avoid engineering antibiotic resistance into the bacteria. pYA292 was modified to contain the mouse IL-21 (mIL-21) cDNA under control of the lacUV5 promoter (Figure 1A). Using polymerase chain reaction and restriction enzymes, plasmids were constructed with a secretion sequence fused to the mIL-21 cDNA, resulting in either OmpA-mIL21 or mIL21-Hly, under control of the lacUV5 or bla promoters (Figure 1B). Once the mIL-21 fusions were constructed, they were used to transform *S. enterica* with standard electroporation methodology. Transformants were chosen via selective plating.

Plasmid Isolation & Identification
Transformants were grown in liquid culture, and the plasmids were isolated using Qiagen's Qiaprep Miniprep kit. The plasmids were analyzed by restriction enzyme digest and gel electrophoresis to check the presence and directionality of the cloned DNA. Plasmids that contained the appropriate mIL-21 fusion constructs in the proper orientation were sequenced by the University's Biomedical Genomics Center.

Western Blotting
To determine if the *S. enterica* transformants were producing and secreting the mIL-21 fusion proteins, cultures were grown in LB broth. To detect secreted proteins, the culture medium was concentrated using Millipore Amicon Ultra Filters with a 10 kDa cutoff. Periplasmic proteins were isolated by subjecting cells to osmotic shock. Cytoplasmic proteins were isolated from cells by sonication and centrifugation to separate soluble cytoplasmic protein in the supernatant from insoluble protein in the pellet. The protein samples were boiled in loading dye and separated by SDS-PAGE. The proteins were transferred to a PVDF membrane for immunoblotting using antibodies targeting mIL-21 and *E. coli* DnaK.

Results

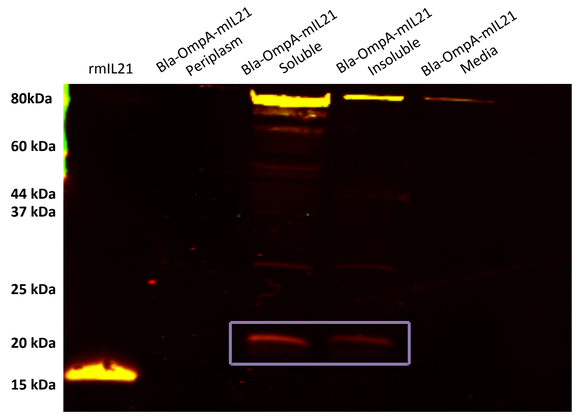

Figure 2. Western Blot of Protein Extracts from *S. enterica* Expressing OmpA-mIL-21.
Overnight cultures of *S. enterica* χ4550 producing OmpA-mIL21 (17.1 kDa) were grown, and the secreted, soluble, and insoluble protein fractions were prepared. Proteins were subjected to SDS-PAGE, transferred to a PVDF membrane, and analyzed by immunoblotting using antibodies against mIL-21 (15.0 kDa) and DnaK (70 kDa). mIL-21 was found in both the soluble and insoluble cytoplasmic protein fractions, which is shown within the purple box on the gel picture above. mIL-21 was not found in the periplasmic or medium fractions. DnaK protein was found mainly in the soluble cell protein fraction as expected, although it was also detected in the insoluble protein fraction and in the culture medium, indicating the presence of unsonicated cells in the insoluble extract and cell lysis in the overnight culture.

Results

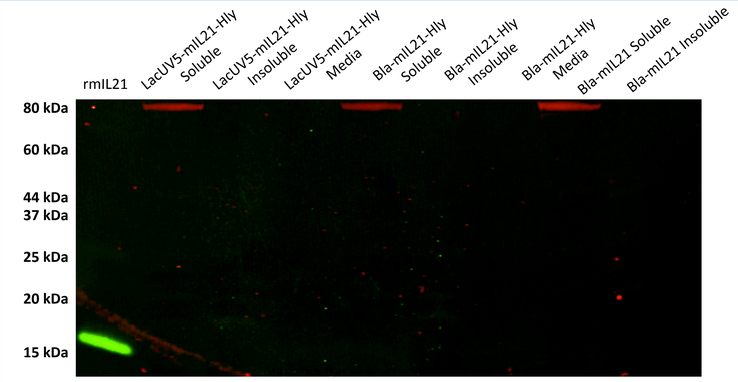

Figure 3. Western Blot of Protein Extracts from *S. enterica* Expressing mIL-21-Hly.
Overnight cultures of *S. enterica* χ4550 carrying either the pBla-mIL21-Hly, pLacUV5-mIL21-Hly, or pBla-mIL2 constructs were grown. Soluble, insoluble, and secreted protein fractions were prepared. Proteins were subjected to SDS-PAGE, transferred to a PVDF membrane, and analyzed by immonoblotting using antibodies against mIL-21 (15.0 kDa) and DnaK (70 kDa). Recombinant mIL-21 (rmIL-21) protein is labeled green and DnaK protein is labeled red on the blot. mIL-21 was not found in the soluble, insoluble, or medium protein samples. DnaK protein was only found in the soluble cytoplasmic protein fraction, indicating no cell lysis in the overnight cultures and no unsonicated cells in the insoluble protein sample.

Conclusions

Four strains of *S. enterica* Typhimurium designed to express mouse IL-21 (pBla-mIL21, pBla-OmpA-mIL21, pLacUV5-mIL21-Hly, & pBla-mIL21-Hly) were successfully engineered. Protein expression was assessed in the media, periplasmic, soluble, and insoluble fractions via Western blot. Addition of the Hly tag to the pBla-mIL21 and pLacUV5-mIL21 constructs, which was designed to cause extracellular secretion of mIL-21, resulted in loss of detectable protein expression. Addition of the OmpA tag, which was designed to cause protein secretion into the periplasm, showed mIL-21 protein in only the soluble and insoluble cytoplasmic fractions. Stronger promoters such as pTrc have been used previously in our laboratory, and a similar lack of mIL-21 expression was seen. These results suggest that the OmpA tag does not effectively direct secretion of mIL-21 into the periplasm, and the Hly tag is deleterious to production of mIL-21. Next steps include testing additional secretion tags such as YebF and SopE in an attempt to construct *S. enterica* Typhimurium strains that secrete mIL-21. Additionally, the mIL-21 cDNA may be codon-optimized for expression in *S. enterica* to help remove mRNA structures or less frequently used codons to improve overall expression of mIL-21.

References

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