Engineering Probiotic E. Coli With a Type III Secretion System for Targeted Delivery of Therapeutic VHH

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Engineering Probiotic *E. coli* with a Type III Secretion System for Targeted Delivery of Therapeutic VHH

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A Thesis Submitted to the Faculty of The Harvard Medical School in Partial Fulfillment of the Requirements for the Degree of Master of Medical Sciences in Immunology

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Engineering Probiotic *E. coli* with a Type III Secretion System for Targeted Delivery of Therapeutic VHH

Abstract

To combat severe disease manifestations in the gut from both pathogenic and autoimmune insults, we are engineering probiotic microbes to release therapeutics into the gut as a novel method of treatment delivery. A bacterial protein delivery system was designed based upon the *Shigella flexneri* type III secretion system (T3SS) to secrete therapeutic proteins both inside and in the vicinity of mammalian cells. The T3SS is a nanomachine that traditionally delivers effector proteins into the host cell to mediate pathogenesis. Fusion of a type III effector secretion sequence to therapeutic proteins results in variants that are recognized as secreted substrates. We recently developed probiotic *E. coli* strains that express a T3SS. These probiotic T3SS competent *E. coli* strains can deliver therapeutic proteins modified with type III secretion signals into host cells.

This work describes the development of engineered probiotic T3SS competent *E. coli* that can secrete VHH proteins. VHH are therapeutic antibody fragments that effectively bind and block activity of target antigens. We demonstrate that DH10B *E. coli* can use the T3SS to directly secrete VHH, modified with the OspC3 effector secretion sequence, into mammalian cells. These bacteria successfully deliver a variety of VHHs, including those that bind to inflammasome ASC protein and a heterotetrameric VHH-based neutralizing agent (VNA) designed to neutralize Shiga toxin (Stx). A remaining question is whether VHH or VNA secreted via the T3SS maintain their function. If evidence indicates αASC VHH
inhibit inflammasome activation, we envision this VHH as a potential anti-inflammatory therapeutic. Whereas, a functional secreted αStx VNA could serve as a therapeutic against enterohemorrhagic \textit{E. coli} infections. We envision modifying our T3SS platform to secrete the αStx VNA into the intestinal lumen as well as the cytosol. Finally, we engineered probiotic \textit{E. coli} strains MP, HS and Nissle 1917 for future use in VHH delivery. This research pursues a novel approach of utilizing bacterial type III secretion systems to deliver therapeutic VHH and VNA to the gut.
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Chapter 1: Background

Bacterially delivered therapeutics are emerging as a potential new intervention for the treatment of both infections and autoimmune diseases, particularly those rooted in the gastrointestinal tract [4]. The body of work presented in this thesis describes the development of a bacterial delivery platform to secrete single domain antibodies (VHH) into the intestinal lumen. We have worked to engineer commensal E. coli that express the Shigella flexneri type III secretion system to secrete VHH proteins. This novel delivery method relies on understanding the mechanics of T3SS mediated Shigella pathogenesis, the versatility of VHH in therapy, and the beneficial characteristics of commensal intestinal E. coli.

Shigella Pathogenesis

Shigella flexneri, a Gram-negative bacterium, is the causative agent of bacillary dysentery in humans. Shigella are transmitted by a fecal-oral route, and upon reaching the intestinal lumen, the bacteria infect M cells [5]. After traveling through M cells across the intestinal epithelium, the bacteria are taken up by macrophages [5]. Upon internalization within the macrophage, Shigella rapidly induces pyroptosis and macrophage cell lysis via activation of NLRP3 and NLRC4 inflammasomes releasing the bacteria to subsequently invade nearby epithelial cells [5, 6].

A major Shigella virulence factor is its Type III secretion system (T3SS). T3SS are complex nanomachines, that act as protein delivery systems to often deliver tens of virulence proteins, referred to as effectors, into host cells [7]. Upon contact with a host cell, the outer most portion of the T3SA, the translocon apparatus, inserts into and forms a pore
in the host cell membrane through which the effectors are then delivered (Figure 1). Effectors are recruited to the cytoplasmic platform of the T3SA, unfolded and passed though the needle to reach the cytosol [1]. In the case of *Shigella*, the translocon apparatus is composed of three proteins, IpaB, IpaC, and IpaD [8, 9]. In addition to forming a pore in the host cell membrane, the translocon proteins, like effectors, are also delivered into the host cell cytosol. For example, IpaC acts in concert with some of the secreted effectors to induce actin polymerization resulting in the formation of ruffles that mediate the invasion of bacteria into host cells [5].

![Figure 1: Shigella Type 3 Secretion System.](image)

Figure 1: *Shigella* Type 3 Secretion System. Schematic diagram describes export of effectors from type 3 secretion system into target cell cytosol during a *Shigella* infection. Figure used from Schroeder et al. [1]

Early effectors translocated into the cytosol are focused on manipulating the actin cytoskeleton resulting in the formation of membrane ruffles and facilitate macropinocytosis of the bacterium [10]. In a translocon driven mechanism, *Shigella* escapes from phagosome
into the cytosol [2]. Once cytosolic, *Shigella* polymerizes actin to form actin tails that propel the bacterium throughout cells as well as form protrusions into neighboring epithelial cells that promote cell-to-cell spread [5]. *Shigella* also secretes effectors to modulate the innate immune response. For example, *Shigella* effector IcsB outcompetes binding of autophagy-inducing host protein Atg5 recognition to bacterial proteins [10]. OspF localizes to the nucleus where it inhibits NF-kB activation and transcription of inflammatory response genes [10]. In contrast, IpaH7.8 upregulates macrophage cell death via pyroptosis [11, 12]. There are still many unknown functions of *Shigella* effectors and the mechanisms by which they help facilitate pathogenesis.

**Regulation of expression of the *Shigella* Type III Secretion System and its Effectors**

The T3SS is composed of the cytosolic sorting platform recruits proteins for translocation, a basal body that spans the periplasm between bacterial membranes and finally the needle that extends from the bacteria and makes contact with host cells via the translocation apparatus [13]. The sorting platform controls the order by which effectors are recruited to the type III secretion apparatus (T3SA). Almost all of the effectors and all of the structural components of the T3SA are encoded on the *Shigella* virulence plasmid [14]. The necessary genes required for a functional T3SA are contained within a 31kb region of this plasmid [14]. The transcription of T3SS genes encoded on the virulence plasmid are induced when *Shigella* encounters an environment of 37°C [15]. T3SS gene expression is regulated by two master regulators, transcription factors VirF and VirB [15]. When *Shigella* encounters an environment of 37°C, VirF expression is induced and promotes transcription of VirB, which, in turn, activates transcription of the type 3 secretion operons (Figure 2) [11, 15]. An additional layer of regulation is due to the H-NS protein. H-NS normally represses
both \( \text{virF} \) and \( \text{virB} \) by binding to their promoter regions when environmental conditions are not conducive to Shigella invasion [15].

The secretion of the type III secreted effectors is normally induced when the translocon makes contact with the host cell membrane, a process that can be mimicked \textit{in vitro} by the addition of dye Congo red to the media [16]. Effectors are recruited to the type III secretion apparatus (T3SA) by a secretion sequence encoded within the first 50 amino acids at the N terminus of the effector [3]. A subset of the effectors also requires a chaperone in order to be efficiently secreted through the T3SA [17]. Effectors are unfolded to pass through the type III apparatus and are refolded once delivered into the host cell cytosol.

**Design of the minimal T3SS platform**

In order to reengineer the \textit{Shigella} T3SS to serve as a therapeutic delivery platform, previous research in the Lesser lab focused on constructing a modified version of type III secretion system, known as minimal T3SS (mT3), in order to utilize this nanomachine as a delivery platform in \textit{E. coli}. To do this, the mT3 was built by capturing the 31kb of DNA encoding the genes necessary for a functional T3SA from the \textit{Shigella} virulence plasmid onto an autonomously replicating plasmid [3]. The lab developed variants of this minimal T3SS that include different \textit{Shigella} entry regions. Models of each of these mT3 variants are described in Figure 2. pmT3SS+ (pmT3+) includes T3SS genes \( \text{virB} \) through \textit{orf 163}. pmT3SS (pmT3) includes T3SS genes from \( \text{virB} \) through \textit{spa40}. pmT3SSΔeffectors (pmT3Δeff) includes genes \( \text{virB} \) through \textit{spa40} with deletions of effectors \textit{ipaA}, \textit{ipgB}, \textit{ipgD} and \textit{icsB}. 
Previous research by the Lesser lab also demonstrated that *E. coli* containing only the minimal T3SS plasmid do not express a functional T3SS and are unable to secrete *Shigella* effectors at 37°C when exposed to Congo red [3]. However, upon introduction of a plasmid that expresses either VirB or VirF transcriptional activators, minimal T3SS *E. coli* secretes effectors under the predicted conditions [3]. From these results, the lab created a construct in which arabinose induced *virF* or Isopropyl β-D-1-thiogalactopyranoside (IPTG) induced *virB* can regulate the induction of T3SS genes effectively establishing a switch that generates a functional T3SS on chemical command [3]. Reeves et al also established that the T3SS competent *E. coli* promote the delivery of both chaperone dependent and chaperone independent effectors directly into mammalian cells [3]. In order to secrete

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**Figure 2: Design of mT3SS construct**  Schematic diagram illustrating the *Shigella* virulence plasmid genes that are incorporated into the pmT3SS plasmid. pmT3+ incorporates *virB-orf 167*. pmT3 encompasses *virB-spa40*. pmT3Δeff includes *virB-spa40*, with deletions of effectors. Adapted from Du et al [2].
heterologous proteins, including those of therapeutic value, from mT3SS competent *E. coli*, they developed a screening platform to identify secretion signal sequences (SS) that promote the recognition of heterologous proteins as secreted substrates. These studies established that the first 50 residues from both chaperone dependent and independent effectors are generally sufficient to define a heterologous proteins as secreted substrates [3]. This region is known as the secretion signal sequence. In limited cases, the both function and translocation efficiency of these fusion proteins into mammalian cells was affected based on which effector secretion sequence was fused to the protein [3].

**VHH Therapeutics**

A major goal of my work is to develop variants of VHH, single domain antibodies, and VNAs (heteromeric VHH neutralizing agents) that are recognized as type III secreted substrates. VHH, also known as nanobodies, are comprised of the variable domain of heavy-chain-only Camelid antibodies (Figure 3).

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**Figure 3: Comparison of therapeutic antibodies** Schematic diagram demonstrating the differences between traditional human antibodies, heavy chain only Camelid antibodies and the VHH antibody fragment we intend to use as a therapeutic.
We envision that type III secreted VHH or VNA can be translocated into target cells or secreted into the intestinal lumen as therapeutic agents. These proteins have the potential to work as potent therapeutics and have several advantages over traditional antibody-based therapies [18]. First, VHH are effective in binding cognate antigens without the presence of the paired variable domain and the corresponding light chain [18]. Due to their small size, these 15kDa proteins can access antigen sites inaccessible by traditional antibodies [19]. As human antibody size and structure does not allow access to viral and bacterial antigenic sites in cavities, the repertoire of antibodies produced by B cells is restricted to the most accessible sites [19]. In contrast, the complementarity determining region (CDR) loops of Camelid antibodies are longer and therefore much more effective at binding epitopes in cavities in pathogenic proteins and inhibiting their activity [19, 20]. Another advantage of their small size is that these VHH can be secreted by the type III secretion system [18]. Third, their small size allows VHH to penetrate tumor tissues more deeply than traditional antibodies [21]. VHH are also easily cleared easily due to a short half-life in serum and do not contain an Fc domain [21]. Lacking an Fc domain can be beneficial in preventing unwanted downstream immunological responses. However, addition of PEG can help sustain VHH in the serum [21].

Bacterially delivered nanobody therapeutics have proven to be effective and potent in previous research studies. In a breast cancer research study, Impe and colleagues raised a VHH against a capping protein involved in breast cancer metastasis [22]. Researchers were able to deliver this functional VHH from an attenuated strain of EPEC into cancer cells [22]. Another study, done by Vanderbroucke et al, describes a successful murine IBD therapy model using secretion of αTNF nanobody in the gut from orally administered Lactococcus lactis [23]. Bacterially delivered nanobodies have also been utilized to combat
pathogenic intestinal infections. Andersen and colleagues created a system of αTcdB and αTcdA VHH delivery from *Lactobaccillus* to treat *Clostridium difficile* infections in mice [24]. While it is advantageous that bacteria can express and deliver VHH, it must be ensured that these nanobodies maintain their stability and are prevented from clumping which can impair functionality [25]. Based on the characteristics of nanobodies, secretion of functional and stable VHH into the gut by bacteria has incredible therapeutic potential.

**Genetically Tractable Probiotic *E. coli***

I aim to develop T3SS competent probiotic strains of *E. coli* that deliver VHH to the intestinal lumen. Probiotics and commensal bacterial strains that colonize the human intestines have shown a variety of benefits to the host including providing necessary metabolites, preventing pathogenic strains from infecting intestinal epithelial cells by colonization resistance, and regulating the innate immune response [26, 27]. Oral administration of genetically engineered bacteria is not only beneficial to target secreted therapeutics to the intestine, where many commensal bacteria already reside, but some, including Nissle 1917 *E. coli*, also can home to solid tumors [28]. Described here, are three *E. coli* strains that have a symbiotic relationship in the human intestinal environment and provide potential vectors to produce and deposit VHH directly into intestinal cells or the gut lumen.

Discovered during World War I, *E. coli* Nissle 1917 was isolated from a soldier who escaped a severe dysentery outbreak. *E. coli* Nissle 1917, also known as EcN, is a commensal *E. coli* commonly used in Europe and Canada for the treatment of patients with IBD where it is sold under the name Mutaflor® [29, 30]. A meta-analysis in 2015 of EcN treatment in ulcerative colitis yielded results that Nissle treatment was as effective as
treatment with mesalazine, an anti-inflammatory drug, in promoting disease remission [31]. Given promising results from probiotic therapy, research has shown that EcN in the gut does not negatively affect the intestinal immune environment and that EcN has potential to carry therapeutics into the host [32]. In a research study examining EcN migration in the body when administered orally, Loessner observed EcN colonization in the intestine and when administered intravenously, EcN colonization was found in gall bladder and tumors of mice, visualized by bioluminescence from expression of a ‘drug-inducible’ luciferase reporter gene [33]. This work indicates that not only does intravenously administered EcN successfully colonize three different tissue sites, but drug induced gene expression can be activated even when EcN is resident in these remote locations. In another murine model, Zhang and colleagues engineered EcN to constitutively secrete a recombinant azurin protein [34]. When given intravenously to mice carrying 4T1 breast or B16 melanoma tumors, EcN effectively colonized and released azurin at the tumor sites resulting in prevention of tumor growth and metastasis [34]. This study proved that EcN could be engineered to deliver therapeutics to specific tissue sites and resulting in therapeutic benefits.

*E. coli* HS, discovered in 1978, is a common commensal *E. coli* in gastrointestinal microbiota that resides stably in the intestines where is not associated with any disease pathology [35]. HS has not been yet investigated as a potential treatment for symptoms of IBD or as a carrier of therapeutic proteins in the intestine.

MP1 is a commensal strain of *E. coli* discovered by Lasaro and colleagues [36]. While EcN and HS *E.coli* failed to colonize murine intestinal tissue effectively in their experiments, researchers isolated MP1 from healthy mice and were able colonize intestines of mice after treatment with streptomycin [36]. MP1 can reside within the mouse GI tract for
over 2 months without eliciting negative effect [36]. MP1 is genetically tractable and Lasaro et al. have constructed two derivatives of MP1 that express a fluorescent marker (MP7 expresses mCherry while MP13 expresses GFP) [36].

These three commensal *E. coli* strains show potential to work as therapeutic delivery agents and considered commensals of the intestinal microbiota.
Chapter 2: Data

2.1 Introduction

In order to build our mT3SS competent \textit{E. coli} strain and promote functional nanobody delivery, our \textit{E. coli} strains requires a combination of three inter-dependent components (Figure 4).

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\caption{Engineering T3SS competent probiotic \textit{E. coli} strains to secrete VHH}
Schematic diagrams representing 3 plasmids necessary for T3SS competent \textit{E. coli} strain to secrete VHH. This system requires (1) Delivery apparatus: T3SS, (2) Activator: VirF or VirB transcription factors, (3) VHH substrate modified with a secretion signal. Adapted from Reeves et al [14]. A: Describes VirF driven VHH delivery, induced by arabinose or endogenously. B: Describes VirB driven VHH delivery, induced by IPTG.
\end{figure}

First, we need to introduce the genes that express the structural components for the T3SA. As described previously, the lab has developed minimal T3SS (mT3) bacterial strains of \textit{E. coli} that express a functional T3SS in the absence of many \textit{Shigella} effectors [3].
Second, a control switch is required to regulate expression of the T3SS, which is accomplished by introducing a plasmid that expresses one of the two master *Shigella* T3SS transcriptional regulators VirF (Figure 4A) or VirB (Figure 4B). Finally, to secrete VHH from our T3SS competent bacteria, VHH variants modified with a *Shigella* effector type III secretion signal need to be expressed. Engineering the three components (Figure 4) to work in conjunction would yield a successful delivery platform of VHH and potentially other therapeutics. The results that follow describe the development of this organic delivery method and the therapeutic potential of our secreted VHVs.

### 2.2 Materials and Methods

**VHH Expression Plasmids**

VHH expression plasmids were created via Gateway site-specific recombination technology (Life Technologies). Florian Schmidt in the laboratory of Hidde Pleogh (MIT) provided Gateway destination plasmids to introduce sequences of interest in-frame and upstream of VHH that recognize PD-L1, CTLA-4, ASC, or NP1 (nuclear influenza epitope), modified with a C-terminal HA tag. The backbone of these plasmids is pDSW206 with an Amp<sup>R</sup> cassette and has a ColE1 origin of replication. VHH genes are regulated via an IPTG-inducible promoter [37]. To introduce *Shigella flexneri* effector type III secretion sequences upstream of the VHH, I conducted LR reactions using the VHH destination plasmids and entry plasmids in the lab that contain the first 150 base pairs of chaperone independent *Shigella* effectors flanked by a 5’ Shine Dalgarno sequence in order to design pDSW206 SS-VHH-HA, Amp<sup>R</sup>. 
VNA Expression Plasmids

For the αStx VNA, I received the open reading frame sequence of a heteromeric αStx1/2 VNA from Dr. Charles Shoemaker. Sewing PCR was used to fuse this ORF to an upstream Shine Dalgarno sequence followed by an OspC3 secretion sequence to the 5' end. AttB sites were also introduced at both ends of the resultant DNA fragment. The OspC3SS- αStx VNA fragment was integrated into pDNR221 by a BP reaction that was sequence verified. I then introduced the OspC3SS- αStx VNA into pDSW206-ccdB-FLAG destination vector via an LR reaction to ultimately generate pDSW206 OspC3SS- αStx VNA-FLAG.

mT3SS Expression Plasmids

The strategy to generate pmT3SS plasmids is described in Reeves et al [3] and Du et al [2]. Using this method, versions of the pmT3SS were generated that contain defined regions of the Shigella virulence plasmid that have been captured onto a pLLx13 plasmid with KanR and TetR genes. As outlined in Figure 2, pmT3+ contains Shigella virB-orf163, pmT3 includes S. flexneri virB-spa40 and pmT3Δeff includes virB-spa40 ΔipaA, ΔlpgB, ΔicsB, ΔlpgD. Generation of IPTG/arabinose-inducible transcriptional activators, virB and virF in pBad18 (AmpR) or pNG162 (SpecR) plasmids had been previously produced in the lab.

Production of mT3SS Competent E. coli Strains

Triparental mating was used to create mT3SS competent E. coli strains. The donor strain was DH10B pLLx13: mT3+ (KanR/TetR/StrepR), mT3 (KanR/TetR/StrepR), or mT3Δeff (TetR/StrepR). Each of these plasmids included an oriT. The recipient strain was either HS or MP E. coli containing either pBad18 virF (AmpR) or pNG162 virB (SpecR). The helper strain was DH5α containing a conjugative plasmid either pRC2.1 (KanR) or pRK2073 (SpecR). All 3 strains were inoculated overnight in appropriate antibiotics then 200 μl of
each overnight culture was added to a nitrocellulose square on an LB plate and incubated at 37°C overnight. The resultant strains were streaked out onto media containing the specific antibiotics to select for pmT3+_pVirF (Kan/Amp/Tet), pmT3_pVirF (Kan/Amp/Tet) or pmT3Δeff_pVirB (Tet/Spec) T3SS competent E. coli strains. Resulting colonies were screened for functional T3SS with a secretion assay probing for IpaC and IpaD.

**VHH and Effector Secretion Assay**

Secretion assay conditions were followed as described by Costa et al [38]. Briefly, the mT3SS competent bacterial strains (including VHH expression plasmid) were inoculated overnight in media containing the appropriate antibiotics in a roller at 37°C, back diluted in the morning at a 1:50 (for *E.coli* background) or 1:100 (for *Shigella* background), and grown for 90 min at 37°C. At 90 min, IPTG to a final concentration of 1 mM was added to each bacterial culture and incubated further at 37°C for 30 min. Next, bacterial cells were harvested by centrifugation (13,000 rpm, 1 min) and resuspended in 2ml PBS Congo red (1uM) at an OD₆₀₀ of ~0.5-0.8. Liquid cultures were incubated for a further 30 minutes at 37°C. Total cell and supernatant fractions were separated by 2 centrifugations (13,000rpm, 1 min). The pellet was removed from the first fraction, diluted in 150μl of protein loading dye (40% glycerol, 240mM Tris/HCl pH 6.8, 8% SDS, 0.04% bromophenol blue, 5% betamercaptoethanol) and 5μl loaded on a 12% SDS-PAGE gel for analysis. Proteins in the supernatant were precipitated with 10% trichloroacetic acid (TCA). Precipitated protein samples were diluted in 50μl protein loading dye and 10μl loaded onto the SDS-PAGE gel. Protein samples were analyzed by Western Blot and probed with mouse anti-HA 11 (clone 16B12, Biolegend) antibodies (1:1,000) for the monomeric VHH and anti-FLAG (clone M2, Fisher Scientific) antibodies (1:10,000) for the αStx VNA and mouse anti-hIL-10 (R&D Systems) antibodies (1:1,000) for IL-10. mT3SS competent *E. coli* protein cell lysate and supernatant samples were probed with rabbit anti-IpaC or anti-IpaD antibodies (1:20,000).
**Cell Culture Conditions**

THP-1 monocytes were maintained in RPMI with Glutamax medium (Life Technologies) supplemented with 10% FBS (Atlanta Biologics) and 50μM betamercaptoethanol. 50ng/ml phorbol 12-myristate 13-acetate (PMA) (Santa Cruz) was added to cell culture to allow for macrophage differentiation 1 day prior to infection assays. Cells were grown at 37°C in a 5% CO2 incubator.

**IL-1β Enzyme-linked Immunosorbent Assay (ELISA)**

Infection: One day prior to infection, THP-1 macrophages were harvested and resuspended in RPMI (with Glutamax, 10% FBS and 50μM betamercaptoethanol) plus 50ng/ml PMA in order to differentiate the cells into macrophages. 100 μl of 1x10^6 cell/ml THP-1 cells were seeded into a single well of a 96 well plate (Corning), resulting in 1x10^5 cells/well. Cells were incubated overnight at 37°C and 5% CO2. Bacterial strains were inoculated overnight in appropriate antibiotics at 37°C on a roller.

On the day of infection, the THP-1 cells were washed 2 times and resuspended in RPMI (with Glutamax, 1% FBS). THP-1 cells were primed with 200ng/ml LPS (Invivogen) for 3 hours in incubator (37°C, 5% CO2).

Bacterial strains were back diluted 1:100 (*Shigella* background) or 1:50 (*E. coli* background), in appropriate antibiotics and 1mM of IPTG and then incubated at 37°C for 90 min. After incubation, all bacterial cultures were resuspended and standardized to OD_{600}=1 in RPMI (with Glutamax, 1% FBS).

After 3 hours of LPS priming, LPS-containing media was removed from THP-1 cells and replaced with 200 μl of an appropriate bacterial culture in RPMI (with Glutamax, 1% FBS, 1mM IPTG) to initiate an infection or with a positive control ATP (5mM). Infected cell cultures were incubated at 37°C and 5% CO2. At 1 hour post infection, ATP was removed.
from uninfected primed wells. At 2 and 4 hours post infection, 200 μl of supernatant from infected wells was removed and frozen.

ELISA: IL-1β ELISA conditions were chosen based on those described by R&D Systems. Briefly, 96 well immunoplates (Nunc) were coated with IL-1β capture antibody diluted to 4μg/ml in PBS. The coated plate was covered and kept overnight at room temperature. Subsequently, ELISA plate was washed with TBST (10% 50mM Tris-HCl pH 7.4, 150mM NaCl, 1% Tween-20) 4 times and blocked in Reagent diluent (1% BSA in PBS) for 1 hour at room temperature. Reagent diluent was filtered through a 0.2μm filter (Corning) prior to use. After 1hour block, 4 washes with TBST were repeated. Next, samples from infection were thawed and diluted 1:10 in Reagent diluent. Then, 100μl of standard or samples were added to the plate and incubated at room temperature for 2 hours. Plate was then washed with 4 times with TBST, detection antibody was added at 200ng/ml and plate was incubated at room temperature for 2 hours. Four washes with TBST were repeated, followed by incubation with streptavidin-HRP for 20 minutes at room temperature. After 20 minutes, plate was washed 5 times with TBST and then 1-Step TMB ELISA Substrate solution (Fisher Scientific) was added. As color saturated in the standards and samples, 2N HCl stop solution was added and plate was read at 450nm on Wallac 1420 Victor2 microplate reader.

Immunofluorescence Microscopy

One day prior to infection, a 24 well plate containing UV treated glass coverslips were seeded with 2x10^5 cells/well of THP-1 monocytes resuspended in RPMI (with Glutamax) with 50ng/ml PMA for differentiation. Cells are kept overnight in incubator (37°C, 5% CO₂). Bacterial strains used for infection were inoculated overnight in appropriate antibiotics at 37°C and placed on a roller.
On the day of infection, bacterial cultures were back-diluted 1:50 (E. coli background) or 1:100 (Shigella background), provided with 1mM of IPTG and incubated at 37°C for 90 min on a roller. THP-1 cells are washed 2 times with RPMI (with Glutamax and 1% FBS) incubated in 500 μl of RPMI (with Glutamax). Bacteria were washed and resuspended in RPMI (with Glutamax) at the MOI of 50. Bacterial suspension was added to seeded cells, plate was centrifuged at 2000 rpm for 10 min at room temperature then placed in incubator (37°C, 5% CO₂) for 1 hour. Infected cells were then rinsed 5 times with RPMI and fixed with 3.7% paraformaldehyde (PFA) in PBS for 20 minutes. 3.7% PFA was then removed and replaced with 1% Triton-X-100 and plate was placed on rocker for 20 minutes. Next, the plate was rinsed with 0.1M glycine and then rinsed 5x with FBT (1% FBS, 1% BSA in 20mM Tris HCl pH 8.0, 154 mM NaCl, 0.02% NaN₃). Cellular ASC platform was labeled with αASC primary antibody rabbit anti-ASC (AL177, Adipogen) at 1:100 in FBT for 1 hour followed by goat anti rabbit Alexa Fluor 488 secondary antibody diluted 1:100 for 30 minutes. The αASC VHH-HA was labeled with mouse anti-HA (clone 16B12, Biolegend) diluted 1:100 for 1 hour followed by a goat anti-mouse Alexa Fluor 586 secondary antibody, diluted 1:100, for 30 min. Infected cells were then stained with 100mM DAPI to label DNA of cell and intracellular bacteria. Coverslips were mounted on glass slides, examined with a Nikon TE300 microscope and analyzed in IP LAB.
2.3 Results

Generation of VHH that are recognized as type 3 secreted proteins

In order to secrete VHH from the *Shigella* type 3 secretion system, I first needed to identify sequences that were sufficient to generate variants that are recognized as type III secreted substrates. The lab had previously identified effector secretion signal sequences that when added to the N terminus of heterologous proteins promote their secretion from T3SS competent *E. coli* [3]. This was done by developing a library of plasmids, each of which contains one of 11 different effector secretion sequences flanked by *attL* recombination sites in a Gateway entry clone [3]. We received pDSW206-based Gateway destination vectors designed to insert sequences upstream of αASC-, αPD-L1-, αNP1- and αCTLA-4 VHH-HA from the laboratory of Dr. Hidde Pleog [37]. Using Gateway recombination technology, I created expression plasmids for OspD2\textsuperscript{SS}, OspC3\textsuperscript{SS}, IpaH9.8\textsuperscript{SS} and IcsB\textsuperscript{SS} N terminal effector secretion sequence- αNP1 VHH-HA fusion proteins (Figure 5A). I then tested whether T3SS competent DH10B *E. coli* recognizes these fusion proteins as secreted substrates. In this experiment, I investigated whether VHH could be secreted when fused to either chaperone dependent (OspD2, OspC3, IcsB) as well as chaperone independent (IpaH 9.8) effector secretion sequences [38]. The mT3SS competent *E. coli* used in Figure 5B, was DH10B with pmT3 (*Shigella* genes virB-spa40) integrated into the bacterial chromosome and pVirF\textsuperscript{Endo} to provide the *E. coli* with a functional T3SS, denoted as mT3_pVirF DH10B. Based on the secretion assay results, the immunoblot showed that only the OspC3 effector secretion sequence was consistently successful in secreting the αNP1 VHH into the supernatant. IpaH9.8\textsuperscript{SS}- αNP1 VHH fusion proteins were expressed yet unable to be secreted by T3SS competent *E. coli*. OspD2\textsuperscript{SS}- and IcsB\textsuperscript{SS}- αNP1 VHH fusion proteins were unable to be well expressed. Later it was determined that the OspD2\textsuperscript{SS}
sequence contains a frame-shift, explaining why the OspD2SS fusion protein was not expressed.

Figure 5: Screening secretion sequences that promote recognition of VHH as type 3 secreted proteins

A: Diagram illustrating the secretion signal-αNP1 VHH construct. B: Secretion assay immunoblot showing expression in bacterial lysate (L) and supernatant (S) of αNP1VHH modified at the N terminus with OspD2, OspC3, IpaH 9.8 or IcsB effector secretion signals. Secretion tested in a background of mT3_pVirF DH10B. C: Immunoblot of secretion assay showing expression in lysate and secretion of αPD-L1 VHH, αCTLA-4 VHH, αNP1 VHH and αASC VHH each modified with the OspC3SS. Secretion tested in a background of mT3_pVirF DH10B. GroEL cytosolic protein probed for as a control.
The OspC3 secretion sequence was the only candidate that resulted in expression and secretion when fused to another tested VHH, αPD-L1 VHH (data not shown). Considering these results, I generated variants of each of the four VHH fusion proteins fused to the OspC3 secretion sequence. In a mT3_pVirFEndo DH10B background, all OspC3SS-VHH-HA fusion proteins secreted under traditional secretion assay conditions, were recognized as type 3 secreted substrates (Figure 5C). Thus demonstrating that in T3SS competent E. coli, an N-terminal fusion of the first 50 amino acid residues of Shigella effector OspC3 with a VHH is sufficient to express and secrete a variety of VHH into the supernatant.

Development of mT3 secretion competent commensal E. coli strains

Once I had evidence of VHH secretion from mT3SS competent DH10B E. coli, the next step was to engineer mT3SS competent commensal E. coli strains. DH10B is a common laboratory E. coli strain that could be potentially used in mouse models to assess VHH secretion and function; however, as we eventually want to utilize this therapeutic delivery system in humans, we prefer to deliver VHH from commensal E. coli strains. In particular, I narrowed my focus to engineering three well-described commensal E. coli strains, Nissle 1917, HS, and MP.

As described above, the lab has developed 3 different variants of minimal T3SS plasmids, each of which encodes variable amounts of Shigella virulence plasmid DNA (Figure 2). We wanted to determine which Shigella entry region of our pmT3SS variants is most effective in secreting fusion proteins with minimal Shigella effector expression and function. Each of these pmT3SS variants has proved effective in secretion of proteins in previous experiments [3]. pmT3+ and pmT3 include Shigella effectors, while pmT3Δeff does not. However, lacking these effectors results in bacteria engineered with pmT3Δeff unable to enter epithelial cells as they do not express effectors necessary for invasion.
With these differences in mind, I decided to test each of these T3SS variants, in the commensal strains to determine which is be most efficient at secreting modified VHH. As described in Reeves et al., in order to generate functional mT3SS competent *E. coli*, both plasmids that encode the proteins needed to express a functional T3SA (pmT3+, pmT3, or pmT3Δeff) and one that expresses VirB or VirF need to be introduced [3]. I used triparental mating to transfer pmT3+, pmT3, or pmT3Δeff and pVirF or pVirB into the commensal strains. Triparental mating uses bacterial conjugation to transfer large plasmids, like pmT3SS, from one bacterial strain into the destination bacterial strain. For the triparental mating, I used a donor bacterial strain, recipient bacterial strain and helper bacterial strain. For the donor strain, I used DH10B containing pLLx13 pmT3+, pmT3, or pmT3Δeff (Kan^R/Tet^R), a helper strain DH5α containing a conjugative plasmid (either pRC2.1or pRK2073) and finally my recipient commensal strain containing either pBad18 VirF^Ara^ (Amp^R^) or pNG162 VirB^{iptG} (Spec^R^). Once I generated *E. coli* HS and MP strains carrying pmT3+, pmT3, or pmT3Δeff paired with VirF or VirB plasmids, I tested the ability of each to secrete *Shigella* translocon proteins, IpaC and IpaD. The secretion of these proteins was compared to those secreted by the previously designed pmT3_pVirF Nissle strain as well as WT *Shigella*. As shown in Figure 6A, my mT3SS competent commensal *E. coli* strains exhibited varying degrees of success of levels in expression and secretion the translocon proteins. pmT3+_pVirF^Ara^ MP1 and its derivatives, MP7 and MP13, were unable to consistently reproduce secrete IpaC. This may be a reflection of issues in screening for and identifying T3SS competent MP strains during triparental mating.

Over the course of a series of screens for T3SS competence, I identified T3SS competent HS strains. pmT3+_pVirF^Ara^ HS reliably secreted IpaC as efficiently as the previously established T3SS competent EcN strain. I also tested pmT3_pVirF^Ara^ HS, pmT3_VirF^Ara^ MP, pmT3Δeff_pVirB^{iptG} HS and pmT3Δeff_pVirB^{iptG} MP for secretion of
IpaD (Figure 6B). From Figure 6, it is clear that all the mT3SS competent HS strains were able to secrete IpaD. Conversely, pmT3+ VirF Ara MP7 was the only successful MP strain to exhibit evidence of a functional T3SS during my experiments. Importantly, these results describe that commensal *E. coli* strains can adapt the *Shigella* T3SS and secrete proteins.

![Image](image.png)

**Figure 6: Engineering T3SS competent probiotic *E. coli* strains** A) Secretion assay immunoblot showing expression in bacterial lysate (L) and secretion (S) of IpaC from probiotic bacterial strains MP (1,3 & 7) and HS engineered with pmT3+ pVirF compared to secretion from pmT3 pVirF Nissle and *Shigella*. B) Secretion assay immunoblot showing expression in lysate and secretion of IpaD from probiotic bacterial strains MP (1,3 & 7) and HS engineered with pmT3+, pmT3, or pmT3Δeff and pVirF or pVirB compared to secretion from *Shigella*. 

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22
Plasmid Stability in mT3 Commensal Strains

Next, I investigated whether MP-derived mT3SS competent *E. coli* strains retain the mT3SS plasmids and continue to secrete proteins when antibiotic selection is removed, an environment akin to the intestine. This was a major concern given that for any therapeutic model in the gut, antibiotic presence would be difficult and problematic to sustain. To assess this, I compared the secretion characteristics of pmT3+ pVirF Ara MP7 passaged for 3 cycles in the presence (A) or absence (LB) of antibiotic selection. For each passage (passage through liquid and restreaked in media) one colony was analyzed through a secretion assay to determine if each strain retained a functional T3SS in the absence of antibiotics. The immunoblot in Figure 7 demonstrates that removal of antibiotics results in loss of a functional T3SS as the strains grown in LB no longer express or secrete IpaD. However, under antibiotics selection, pmT3+ pVirF Ara MP7 maintained its ability to secrete IpaD. Based on these observations, it seems highly likely that mT3SS competent bacterial strains will lose the mT3SS plasmid when administered to patients in the absence of antibiotic selection.

![Figure 7: T3SS retention in commensal E. coli strains](image)

*Figure 7: T3SS retention in commensal E. coli strains* A: Secretion assay immunoblot illustrating retention of a functional type 3 secretion system of mT3+ pVirF MP7. *E. coli* strain underwent 3 passages in either Luria broth (LB) or appropriate antibiotic media (A) to determine if antibiotics were required maintain the T3SS. Results demonstrate expression of IpaD in the bacterial cell lysate (L) as well as secreted protein (S).
Thus, I hypothesize that ultimately for our therapeutic bacterial strains, the *Shigella* virulence plasmid DNA present on the mT3SS plasmids will need to be integrated directly into the *E. coli* chromosome to ensure proper delivery of therapeutics.

**Investigating the functionality of type III secreted αASC VHH**

Once I developed mT3SS competent *E. coli* strains that secrete VHH, I next sought to establish whether the secreted VHH were translocated via the bacteria directly into host cells and remained functional. To investigate VHH functionality, I designed a proof of principle experiment to assess functionality of secreted αASC VHH. ASC is an adaptor protein in many canonical inflammasomes that acts to recruit and cleave caspase 1 [39]. Inflammasomes are multiprotein complexes that arise due to activation of a sensor NLR domain in response to cellular stress or pathogen associated molecular patterns (PAMPS) [39]. Inflammasome activation often results in IL-1β release and pyroptotic cell death [39].

Our collaborator, Dr. Florian Schmidt in the Ploegh lab, observed that when a canonical inflammasome is activated in THP-1 derived macrophages, transfected αASC VHH co-localizes with ASC platforms. In these experiments, the αASC VHH inhibit downstream signaling including caspase 1 cleavage and IL-1β secretion [37]. For these studies, Dr. Schmidt exposed THP-1 derived macrophages, that were transiently transfected to express the αASC VHH, to nigicerin to induce inflammasome activation. Thus, I set out to test whether αASC VHH delivered by bacteria into THP-1 derived macrophages could also inhibit inflammasome activation.

Previous research has indicated that *Shigella* infection of macrophages elicits NLRP3 inflammasome activation in an unknown mechanism and NLRC4 inflammasome activation in recognition of components of the *Shigella* T3SA [6, 12, 40, 41]. Activation of NLRP3 sensors can recruit ASC adaptor forming a large protein platform [41]. Once the ASC platform is formed, it will cleave and activate caspase 1 [41]. Caspase 1 can then
cleave pro-IL-1β transcribed from NF-kB activation [41]. IL-1β is subsequently secreted from the cell. There are conflicting reports as to whether NLRC4 requires ASC recruitment for IL-1β activation [41, 42]. Using this infection model (Figure 8A), I decided to investigate whether otherwise wild type strains of Shigella engineered to secrete αASC VHH would result in decreased inflammasome activation and hence lower induced IL-1β secretion from macrophages. While a Shigella infection should activate NLRP3 and NLRC4, secretion of the αASC VHH into the cell should obstruct ASC platform cleavage of pro-caspase 1 and downstream IL-1β signaling. To evaluate the functionality of αASC VHH, I compared levels of IL-1β secreted from THP-1 derived macrophages infected with Shigella that secrete αASC VHH to those infected with Shigella that secrete a CTLA-4 VHH. I predicted to observe decreased levels of IL-1β secreted from the THP-1 cells infected with Shigella secreting αASC VHH. As a negative control, I infected the THP-1 macrophages with BS103, a strain of Shigella that lacks the virulence plasmid that encodes the T3SS and its effectors. BS103 is unable to escape from phagosomes into the host cell cytosol and thus should not trigger inflammasome activation. THP-1 derived macrophages were LPS primed for 3 hours then infected with each of these strains at an MOI 20. IL-1β secretion was assayed at 2 and 4 hours post infection. The THP-1 macrophage infections with Shigella secreting αASC VHH, shown in Figure 8B, showed no significant decrease in inflammasome activation, as assayed by monitoring IL-1β secretion, in comparison to activation from THP-1 macrophages infected with Shigella secreting αCTLA-4 VHH. The results of the IL-1β ELISA suggest that the secreted αASC VHH does not inhibit ASC activity as assessed by monitoring IL-1β secretion.
Figure 8: Investigating secreted αASC VHH functionality

A: A schematic diagram describing how *Shigella flexneri* infects macrophages and initiates inflammasome activation. B: IL-1β ELISA results illustrating the efficacy of OspC3SS-αASC VHH, secreted from *Shigella*, in inhibiting ASC. IL-1β secretion from inflammasome activation was assessed over a 4 hour time course from a *Shigella* infection (MOI 20) of THP-1 macrophages. C: Florescence images taken after 1 hour infection (MOI 50) of THP-1 macrophages with *Shigella* secreting either αASC VHH or αCTLA-4 VHH. VHH proteins, which contain a C-terminal HA-tag were stained with anti-HA primary antibodies and Alexa Fluor 488 (green) conjugated secondary antibodies. ASC platforms were stained with primary anti-ASC antibodies followed by Alexa Fluor 594 (red) conjugated secondary antibodies. Nuclei were stained with DAPI.
Following this result, I investigated whether the VHH was effectively translocated into the THP-1 cells. Thus, I examined the localization of the αASC VHH within infected macrophages by florescence microscopy. THP-1 derived macrophages were infected with *Shigella* that secrete αASC VHH or αCTLA-4 VHH. The ASC platforms in the cell and the VHH localization were determined by indirect immunofluorescence, 1 hour post-infection. As shown in **Figure 8C**, THP-1 derived macrophages infected with *Shigella* that secrete αASC VHH displayed co-localization of the ASC platform and αASC VHH. This indicates that the αASC VHH is successfully secreted from the *Shigella* T3SS and binds to the ASC platform. In contrast, I observed no co-localization of the αCTLA-4 VHH secreted from *Shigella* with the ASC platform as shown in **Figure 8C**.

The inconsistent data from the microscopy and IL-1β ELISA assays suggested that there might be additional variables that act downstream of the inhibitory αASC VHH or prevent the αASC VHH from blocking caspase 1 cleavage, normally activated upon inflammasome assembly. For example, along with activating inflammasome-mediated responses, *Shigella* secretes many effectors that may regulate immune and cellular response during the context of an infection that may mask the effect the αASC VHH. Another potential issue arises if the kinetics of immune responses occur too early and result in cell death before the secreted αASC VHH can have any effect. To address these potential issues, I constructed mT3SS competent strains (pmT3_pVirB<sup>iptG</sup> BS103 and pmT3Δeff_VirB<sup>iptG</sup> BS103) that secrete only a small subset of *Shigella* effectors during an infection and constructed them to secrete αASC VHH and αCTLA-4 VHH (**Figure 9A**). I then tested if αASC VHH secreted from pmT3_pVirB<sup>iptG</sup> BS103 during an infection of THP-1 derived macrophages would be successful in inhibiting an inflammasome response. As shown in **Figure 9B**, I did not observe any difference in the IL-1β secretion from THP-1 cells infected with pmT3_pVirB<sup>iptG</sup> BS103 secreting αASC VHH compared with THP-1 cells.
infected with pmT3_pVirB^{PTG} BS103 secreting αCTLA-4 VHH. From this ELISA, I concluded that secreted αASC VHH was ineffective in hampering IL-1β secretion from infected THP-1 cells. From our results, we have not yet obtained conclusive data to show whether the secreted αASC VHH is functional.

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**A**

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Probed with αHA

**B**

THP-1 IL-1β Secretion from MOI 20 Infection

- WT Shigella
- BS103 pmT3_pVirB
- BS103 pmT3_pVirB : αASC
- BS103 pmT3_pVirB : αCTLA

IL-1β Secretion (pg/ml)

- 2 hr
- 4 hr

Bacterial Strain
Secretion of a VNA that Inactivates Shiga Toxin

Another VHH based therapy is to modify the type 3 secreting *E. coli* to secrete VHH and VNA directly into the intestinal lumen rather than into cells lining the intestinal tract. In this way, for diseases of the gut, we can deliver therapeutic proteins directly to the site of disease limiting systemic cellular side effects. As proof of concept, I have focused on development of secreted alleles of VNA that bind and inactivate Shiga toxin. Shiga toxin is produced by a group of pathogenic *E. coli* strains (STEC) that often cause foodborne illnesses and deliver Shiga toxin 1 and 2 (Stx1 and Stx2) causing hemorrhagic colitis [43, 44]. Antibiotics treatment of STEC can cause further complications, as antibiotics induce release of phage-encoded Stx1 and Stx2 [45]. The secretion of Stx can cause significant apoptotic cell death resulting in damage that can lead to complications like hemolytic uremic syndrome [46]. Recently, the laboratory of our collaborator, Dr. Charles Shoemaker, has identified 4 different VHH that bind to and inhibit the activity of the B subunits of Stx1 and Stx2 [47]. They have also determined that linking all four VHH as a VNA (VHH neutralizing agent) provides synergistic benefit in inhibiting toxin activity [47].

I first investigated whether this VHH heterotetramer can be modified to be recognized as a type III secreted substrate from our mT3SS competent *E. coli* strains. To accomplish this, I fused the OspC3 type III secretion signal to the heterotetrameric αStx VNA-FLAG (*Figure 10A*) and tested for its secretion from WT *Shigella*, pmT3_pVirB^PTG^ BS103 and mT3_pVirF^endo^ DH10B *E.coli*. The immunoblot in *Figure 10B* demonstrates that...
the 80kDa OspC3^{SS}-\alpha\text{Stx} VNA-FLAG is secreted from *Shigella* and mT3SS competent BS103 and mT3_pVirF_{endo} DH10B. Our T3SS competent HS strains were able to express but did not secrete the OspC3^{SS}-\alpha\text{Stx} VNA. Because the secretion of the OspC3^{SS}-\alpha\text{Stx} VNA is not very efficient in the mT3SS competent *E. coli* background, as seen in Figure 10B, I investigated if increased \alpha\text{Stx} VNA secretion could be detected in a bacterial strain built for constitutive secretion. The lab had previously designed an *E. coli* constitutive secretor strain known as pVPΔleft DH10B. The constitutive secretion from the T3SS was accomplished by deleting the translocon and effector genes, *ipaA-icsB*, from the *Shigella* virulence plasmid, and then introducing this modified virulence plasmid (pVPΔleft) into DH10B. Using this strain, I introduced the plasmid containing the OspC3^{SS}-\alpha\text{Stx} VNA fusion and tested for secretion of the VNA in the supernatant in the presence and absence of Congo red. The western blot, shown in Figure 10C, clearly demonstrates that in the presence and absence of Congo red, the \alpha\text{Stx} VNA was present in the supernatant. Surprisingly, pVPΔleft DH10B secreted higher levels of the \alpha\text{Stx} VNA in the absence of Congo red (overnight culture (O/N) and the –CR conditions) compared with +CR conditions. This data suggests that deletion of the translocon is sufficient to allow the pVPΔleft *E. coli* strain to secrete \alpha\text{Stx} VNA fusion protein constitutively.
Figure 10: Testing secretion of αStx VNA modified with OspC3 secretion sequence
A: Diagram illustrating the OspC3\textsuperscript{SS} - αStx VNA VHH construct. B: Secretion assay immunoblot showing expression in bacterial lysate (L) and secretion (S) of αStx VNA modified at N terminal OspC3 effector secretion signal. Secretion tested in a background of pmT3\_pVirB BS103, pmT3\_pVirB VirB HS, pmT3\_pVirB HS, mT3\_pVirF DH10B and Shigella. C: Secretion assay immunoblot illustrating expression and secretion of αStx VNA from constitutive secretor pVP\textsuperscript{Δ}left DH10B from overnight culture (O/N), and regular secretion assays with Congo red (+CR) and without Congo red (-CR).
The versatility of our delivery system allows for secretion of modified heterologous proteins [3] including VHH and VNA. Another immunomodulatory protein I sought to secrete is the cytokine IL-10. IL-10 is responsible for the differentiation of regulatory T cells and termination of inflammatory responses [48]. Secretion of IL-10 directly into gut lumen has potential therapeutic benefit in combating chronic infections and autoimmune disorders. Using engineered bacteria to secrete IL-10, we could potentially bypass the requirement for activation of a T regulatory cell to release the cytokine [48]. Our method of IL-10 delivery would not require the presence of the regulatory T cell’s cognate antigen to activate IL-10 secretion. Previous research had demonstrated that IL-10 fused to the IpaH9.8 secretion signal sequence is sufficient to result in the secretion of active IL-10 via the Shigella T3SS [49]. Secretion of IL-10 from wild type strains of Shigella exhibited reduced inflammation and mortality in a mouse pulmonary model of infection [49]. I wanted to investigate if I could replicate secretion of IL-10 from our mT3SS competent E. coli. In order to test this, I fused the first 50 residues of IpaH9.8 or OspC3 to IL-10 (Figure 11A). I then tested the secretion of each of these modified IL-10 proteins from mT3_pVirFendo DH10B. I observed that secretion of the IL-10 fusion proteins was extremely poor from mT3SS competent E. coli strains (Figure 11B). While the immunoblot shows IL-10 protein expression evident in the bacterial cell lysate, I observed little evidence of secretion from little OspC3SS fused IL-10 and no secretion for the IpaH9.8SS fused IL-10 candidate.
2.4 Brief Discussion

This body of work describes the development of a bacterial-based therapeutic protein delivery platform, repurposing the *Shigella*’s pathogenic type III secretion system when heterologously expressed in probiotic *E. coli* in order to secrete therapeutic nanobodies into the intestine. The data presented clearly demonstrates that fusion of an OspC3 type III secretion signal to VHH promotes their secretion via the *Shigella* T3SS. I have also shown that delivery through the T3SS is not limited to secreting monomers of VHH, but also trimeric and tetrameric VHH. Our model is not limited to secretion of therapeutics antibody fragments, as I have also investigated the possibility of secreting IL-10, an anti-inflammatory promoting cytokine. In order to deliver these therapeutics, I have engineered several strains of probiotic *E. coli* with a functional T3SS and regulatable switch.
to secrete proteins. We are currently developing a system of constitutive secretion from the type III secretion system. Finally, I have promising evidence that the αASC VHH can translocate into macrophages during an active infection and congregate to the ASC platform indicating that binding is specific. We intend to pursue experiments to determine if the secreted OspC3 SS-αASC VHH and OspC3 SS-αStx VNA are functional and therapeutically beneficial.
Chapter 3: Limitations and Future Directions

The development of a bacterial delivery system secreting Camelid derived antibody fragments does come with significant limitations. However, these limitations have not prevented successful bacterially delivered VHH for breast cancer tumors, *Clostridium difficile* toxin, or TNF as mentioned previously [23, 24, 34]. One specific limitation arises due to the fact that we have a delivery platform that centers around a pathogenic type III secretion system. There is a distinct possibility that T3SS components, specifically rod protein MxiI, will activate the NLRC4 inflammasome and other innate immune responses when injecting therapeutics into host cells [6, 40]. Activating an inflammasome pathway in cells can be ultimately counterproductive and detrimental to overall therapeutic care. However, if the pmT3SS does not contain the translocon components IpaB, IpaC and IpaD, we can effectively build a constitutive secretor strain that secretes VHH extracellularly thus avoiding contact with the host cell cytosol, the site of NLRs that trigger inflammasome activation. We intend to construct a version of the pmT3SS that no longer encodes effectors as well as the translocon apparatus in order to design a constitutive secretion delivery platform. Second, we need to ensure that the commensal *E. coli* strain will reside in the intestine for a short period of time. This will prevent the commensal *E. coli* from altering the microbiome of the intestine and also potentially harming the host. In order to design bacteria with these conditions, the delivery *E. coli* strain could be made auxotrophic or contain an environmentally induced toxin or lysis protein to mediate bacterial cell death [50,51]. However, if a therapeutic application requires the bacterial vector to remain in the intestine for a long period of time we can introduce adhesion proteins to adhere to epithelial cells and promote long term delivery. Third, we need to establish that constitutive secretion of these antibody fragments would not elicit host antibody production against the Camelid derived
VHH proteins. Otherwise, long-term use of the VHH to treat chronic disease could result in an unintended immune response. Studies are currently underway to create humanized versions of VHH antibodies by manipulating the VHH amino acid sequence [52]. Also, because VHH are cleared from serum fairly rapidly, they are less likely for them to aggregate within the body and cause an immune cell response.

The studies described here focused on the development of a functional therapeutic bacterial delivery system has resulted in a few setbacks that inspired future experiments. While the OspC3 effector secretion signal is sufficient in secreting all four nanobodies tested, we were unable to robustly secrete a smaller protein, IL-10 with the OspC3\textsuperscript{SS} modification. We may need to screen other effector secretion signals to optimize secretion of each protein we attempt to secrete in this delivery platform. I would also like to include an IL-10 protein as a positive control in future immunoblots in order to ensure that the detection antibody we are currently using is effective in probing for IL-10. We have sought to resolve this issue by testing two different αIL\textsuperscript{-10} antibodies in our immunoblots, rat αIL\textsuperscript{-10} and mouse αIL\textsuperscript{-10}, to see if the IL-10 secretion was detected better based on the detection antibody used.

Another issue we have faced is freezing and thawing the mT3SS competent commensal \textit{E. coli} strains has affected strain stability. After successful secretion of IpaD in our freshly made mT3SS competent commensal \textit{E. coli} strains, as shown in Figure \textbf{6B}, the strains were retested again after a few months and the secretion of IpaD had severely decreased under the same secretion assay conditions. In order to combat this limitation, we intend to integrate the mT3SS into the \textit{E. coli} chromosome to preserve the secretion efficiency. Integration of mT3SS has proved to work in the pmT3SS:SER2\textsubscript{p}Vir\textsubscript{endo} DH10B strain, which we have found to be a consistent and stable VHH secretor through several passages and freeze-thaw cycles.
We also hope to improve the secretion of T3SS competent MP strains. In the future, we want to select strains not only by antibiotic selection from triparental mating but also confirm that plasmids of interest have been transferred from one strain to another. This will ensure that the both plasmids are intact in MP strains we choose to test for T3SS secretion.

Based on the data presented in this work, it is unclear whether the type III secreted αASC VHH injected into cells is functional. Microscopy of infected macrophages, demonstrated the αASC VHH binding to the ASC inflammasome platform, suggesting the protein is successfully secreted into the host cell cytosol and maintains its ability to bind the targeted epitope. However, the IL-1β ELISAs failed to show an effect of the αASC VHH in preventing inflammasome activation. It is possible that IL-1β ELISA experiments show false negatives if the kinetics of the inflammasome formation occur faster than the secreted αASC VHH could inhibit downstream ASC activity. Also, in our currently VHH delivery model we cannot determine how much αASC VHH is secreted into the host cell. Thus, if the amount of αASC VHH translocated into the cell is not sufficient in obstructing ASC, IL-1β will be secreted. We plan to look at earlier time points in the infection to investigate the functionality of secreted αASC VHH and quantify the amount of nanobody secreted over the course of an infection. Another potential problem with the IL-1β ELISA is that the assay uses an αIL-1β antibody that cross reacts with pro-IL-1β. Therefore, if the macrophages are lysing during the 4 hour experiment, we may not get an accurate read of actual IL-1β secretion. To resolve this possibility, we also plan to assess caspase 1 cleavage during a macrophage infection by fluorescence microscopy and Western blot of the cell lysate. Caspase 1 cleavage may be a more accurate read-out of ASC functionality and inflammasome activation. There is a possibility that the secreted αASC VHH is not functional due to the OspC3 secretion signal modification or improper refolding of the VHH after secretion from the apparatus. In these cases, αASC VHH may not be an appropriate
therapeutic for our delivery system or we will need to test other *Shigella* effector secretion signals that promote αASC VHH secretion. We can test secretion signals from effectors that are secreted early in *Shigella* infection to ensure the αASC VHH is delivered early during the infection model. If the VHH proves to be nonfunctional after secretion, we intend to pursue therapies against cancerous tumors using αCTLA-4 VHH and αPD-L1 VHH secreted by mT3SS competent Nissle strain.

With our success secreting the αStx VNAs from pmT3_pVirF\textsuperscript{endo} DH10B, we plan to purify and quantify the amount of protein secreted in a traditional secretion assay and confirm binding and inhibition of Shiga toxin. Eventually, the αStx VNA will need to be secreted from a constitutive commensal secretor strain. Working with our collaborators, we hope to show neutralization of Shiga toxin with our αStx VNA in an ELISA based experiment. We eventually want to orally administer a probiotic mT3SS *E. coli* strain constitutively secreting the αStx VNA into a STEC mouse infection model to determine if secreted OspC3\textsuperscript{SS}-αStx VNA can neutralize the toxin during an infection *in vivo*.

As evident by the data, IL-10 secreted poorly when fused with the OspC3 or IpaH 9.8 secretion signals. To improve secretion, we can change the conditions of the secretion assay by increasing the concentration of the inducer, Congo red, or secrete IL-10 from a constitutive secreting bacterial strain. We plan to screen more effector secretion signal sequence candidates in order to efficiently secrete IL-10 from an mT3SS competent *E. coli* background and confirm that the secreted cytokine is biologically active.

The potential applications for this delivery platform are boundless. If we are able to secrete functional nanobodies from T3SS competent commensal *E. coli* without significant adverse effects, we can create personalized treatment for a variety of diseases including infections, autoimmune diseases and cancer. These therapies do not only have to be restricted to the gut as commensal microbes exist in the oral cavity, the skin and even home
to tumor microenvironments [4]. In the future, we hope to pursue cancer related immunotherapies by delivering αPD-L1 VHH and αCTLA-4 VHH to tumors to reactive T cells in the tumor microenvironment. We also hope to create a platform to secrete human cytokines into the intestinal lumen. We can even target this therapy to a variety of tissues by adding location specific adhesins to direct the designer bacteria [53].

Other innovative uses of bacterially delivered therapeutics have been developed. A study done by Vooght et. al. applied bacterially delivered VHH to prevent trypanosome growth in Tsetse flies an intervention to prevent transmission of sleeping sickness in humans [54]. In this study, investigators genetically manipulated an endosymbiont of the Tsetse fly to secreted VHH to inhibit trypanosome growth [54]. Another study describes a successful research trial of oral delivery of Lactobacilli producing anchored VHH to treat rotovirus infections in mice [55]. In a diagnostic approach, secreted VHH have also been used as imaging tools to mark tumor growth and cell types. Bacterially delivered nanobodies continue to have wide variety of uses.

Designer bacteria are emerging as novel and organic delivery vectors to administer therapeutics to the intestine and remote tissues in the body. Research has shown the capabilities of designer bacteria to commute to tumor sites to secrete anti-cancer drugs, to deliver antibody fragments to the gut to combat toxins and even to inject cellular reprogramming factors directly into cells [3, 24, 34]. We propose a commensal E. coli driven system that repurposes Shigella’s type III secretion system to be able to deliver VHH both intracellularly and extracellularly as therapeutic agents in the intestine. By engineering a bacterially driven targeted VHH therapeutic platform, we hope to further innovative treatments for disease.
Table S1: Plasmid Constructs

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pmT3(+)</td>
<td>pLLx13 ΔiqaJ::KAN thru orf167, Tet&lt;sup&gt;R&lt;/sup&gt;, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
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<tr>
<td>pmT3</td>
<td>pLLx13 ΔiqaJ::KAN thru spa40, Tet&lt;sup&gt;R&lt;/sup&gt;, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>[3]</td>
</tr>
<tr>
<td>pmT3Δeff</td>
<td>pLLx13 ΔiqaJ::KAN thru spa40ΔipaA, ΔipgB, ΔicsB, ΔipgD, Tet&lt;sup&gt;R&lt;/sup&gt;, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>[2]</td>
</tr>
<tr>
<td>pVirF&lt;sub&gt;Ara&lt;/sub&gt;</td>
<td>pBad18 Arabinose-inducible VirF, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>[3]</td>
</tr>
<tr>
<td>pVirB&lt;sub&gt;IPTG&lt;/sub&gt;</td>
<td>pNG162 IPTG-inducible VirB, Spec&lt;sup&gt;R&lt;/sup&gt;</td>
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<tr>
<td>pVirF&lt;sub&gt;Endo&lt;/sub&gt;</td>
<td>pACYC VirF + 1000 bp upstream sequence, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
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</tr>
<tr>
<td>pOspC&lt;sub&gt;3SS-αNP1 VHH-HA&lt;/sub&gt;</td>
<td>pDSW206 IPTG-inducible OspC&lt;sub&gt;3SS-αNP1&lt;/sub&gt; VHH-HA, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(This study)</td>
</tr>
<tr>
<td>pIpH&lt;sub&gt;9.8SS-αNP1 VHH-HA&lt;/sub&gt;</td>
<td>pDSW206 IPTG-inducible IpH9.8&lt;sub&gt;SS-αNP1&lt;/sub&gt; VHH-HA, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(This study)</td>
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<tr>
<td>pIcsB&lt;sub&gt;SS-αNP1 VHH-HA&lt;/sub&gt;</td>
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<td>pDSW206 IPTG-inducible OspD&lt;sub&gt;2SS-αNP1 VHH-HA, Amp&lt;/sub&gt;</td>
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<td>pOspC&lt;sub&gt;3SS-αASC VHH-HA&lt;/sub&gt;</td>
<td>pDSW206 IPTG-inducible OspC&lt;sub&gt;3SS-αASC VHH-HA, Amp&lt;/sub&gt;</td>
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</tr>
<tr>
<td>pOspC&lt;sub&gt;3SS-αCTLA-4 VHH-HA&lt;/sub&gt;</td>
<td>pDSW206 IPTG-inducible OspC&lt;sub&gt;3SS-αCTLA-4 VHH-HA, Amp&lt;/sub&gt;</td>
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<td>plpaH&lt;sub&gt;9.8SS-IL-10&lt;/sub&gt;</td>
<td>pDSW206 IPTG-inducible IpH9.8&lt;sub&gt;SS-IL-10&lt;/sub&gt; VNA-FLAG, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
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<tr>
<td>pVP&lt;sub&gt;left&lt;/sub&gt;</td>
<td>S. flexneri 2457T VP&lt;sup&gt;1&lt;/sup&gt; ΔipaA-icsB</td>
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<td>pRC2.1</td>
<td>pRC2.1 conjugative plasmid, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pRK2073 conjugative plasmid, Spec&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pDNR221 OspC&lt;sub&gt;3SS-αStx-FLAG&lt;/sub&gt;</td>
<td>Gateway Entry clone, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>[3]</td>
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<tr>
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<td>[3]</td>
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Table S1: Plasmid Constructs Table describes all plasmid constructs developed and tested throughout this work.
Table S2. Bacterial Strains

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<td><strong>Shigella</strong></td>
<td>S. flexneri 2457T</td>
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<td>DH10B</td>
<td>E. coli DH10B</td>
<td>Life Technologies</td>
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<td>Nissle</td>
<td>E. coli Nissle 1917</td>
<td>Gift from John March, Cornell University</td>
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<td>MP (1,7,13)</td>
<td>E. coli MP1, MP7: mcherry, MP13: gfp</td>
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<td>BS103</td>
<td>S. flexneri 2457T without VP</td>
<td>(This study)</td>
</tr>
<tr>
<td>HS</td>
<td>E. coli HS</td>
<td>Mark Goulian, University of Pennsylvania</td>
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**Minimal T3SS strains + Activators**

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<th>Name</th>
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<td>mT3_pVirF DH10B</td>
<td>atp/gidB::mT3 + pVirF&lt;sup&gt;E&lt;sub&gt;Endo&lt;/sub&gt;&lt;/sup&gt; E. coli DH10B</td>
<td>[3]</td>
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<tr>
<td>pmT3+_pVirF HS</td>
<td>pmT3(+) + pBad18 VirF&lt;sup&gt;Agr&lt;/sup&gt; E. coli HS</td>
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<tr>
<td>pmT3_pVirF HS</td>
<td>pmT3 + pBad18 VirF&lt;sup&gt;Agr&lt;/sup&gt; E. coli HS</td>
<td>(This study)</td>
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<tr>
<td>pmT3Δeff_VirB HS</td>
<td>pmT3Δeff + pNG162 VirB&lt;sup&gt;P&lt;sub&gt;ITG&lt;/sub&gt;&lt;/sup&gt; E. coli HS</td>
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<td>pmT3_pVirF MP1</td>
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<tr>
<td>pmT3Δeff_VirB MP1</td>
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<td>pmT3_pVirF MP7</td>
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<td>pmT3Δeff MP7</td>
<td>pmT3Δeff + pNG162 VirB&lt;sup&gt;P&lt;sub&gt;ITG&lt;/sub&gt;&lt;/sup&gt; E. coli MP7</td>
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<td>pmT3Δeff_VirB MP13</td>
<td>pmT3Δeff + pNG162 VirB&lt;sup&gt;P&lt;sub&gt;ITG&lt;/sub&gt;&lt;/sup&gt; E. coli MP13</td>
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<td>pmT3_VirF Nissle</td>
<td>pmT3 + pBad18 VirF&lt;sup&gt;Agr&lt;/sup&gt; E. coli Nissle</td>
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**Therapeutic Delivery Strains**

**Shigella:**

- pOspC<sup>SS</sup>-αStx VNA-FLAG Shigella
- pOspC<sup>SS</sup>-αCTLA-4 VHH-HA Shigella
- pOspC<sup>SS</sup>-αASC VHH-HA Shigella

**DH10B:**

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<thead>
<tr>
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<tr>
<td>pOspC&lt;sup&gt;SS&lt;/sup&gt;-αCTLA-4 VHH-HA Shigella</td>
<td>pOspC&lt;sup&gt;SS&lt;/sup&gt;-αCTLA-4 VHH-HA S. flexneri 2457T</td>
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<td>pOspC&lt;sup&gt;SS&lt;/sup&gt;-αASC VHH-HA Shigella</td>
<td>pOspC&lt;sup&gt;SS&lt;/sup&gt;-αASC VHH-HA S. flexneri 2457T</td>
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<td>Strain Configuration</td>
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<td>DH10B pVPΔleft pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αStx VNA-FLAG</td>
<td>pVPΔleft + pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αStx VNA-FLAG E. coli DH10B</td>
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<tr>
<td>DH10B pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αNP1 VHH-HA mT3&lt;sub&gt;pVirF&lt;/sub&gt;</td>
<td>mT3&lt;sub&gt;pVirF&lt;/sub&gt; + pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αNP1 VHH-HA E. coli DH10B</td>
<td>(This study)</td>
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<td>DH10B pplaH9.8&lt;sup&gt;SS&lt;/sup&gt;-αNP1 VHH-HA mT3&lt;sub&gt;pVirF&lt;/sub&gt;</td>
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<td>(This study)</td>
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<tr>
<td>pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αNP1 VHH-HA mT3&lt;sub&gt;pVirF&lt;/sub&gt;</td>
<td>mT3&lt;sub&gt;pVirF&lt;/sub&gt; + pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αNP1 VHH-HA E. coli DH10B</td>
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<td>pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αASC VHH-HA mT3&lt;sub&gt;pVirF&lt;/sub&gt; DH10B</td>
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<td>pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αCTLA-4 VHH-HA mT3&lt;sub&gt;pVirF&lt;/sub&gt; DH10B</td>
<td>mT3&lt;sub&gt;pVirF&lt;/sub&gt; + pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αCTLA-4 VHH-HA E. coli DH10B</td>
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<td>pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αPD-L1 VHH-HA mT3&lt;sub&gt;pVirF&lt;/sub&gt; DH10B</td>
<td>mT3&lt;sub&gt;pVirF&lt;/sub&gt; + pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αPD-L1 VHH-HA E. coli DH10B</td>
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<td>pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αStx VNA-FLAG mT3&lt;sub&gt;pVirF&lt;/sub&gt; DH10B</td>
<td>mT3&lt;sub&gt;pVirF&lt;/sub&gt; + pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αStx VNA-FLAG E. coli DH10B</td>
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<td>(This study)</td>
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<td>pplaH9.8&lt;sup&gt;SS&lt;/sup&gt;-IL-10 mT3&lt;sub&gt;pVirF&lt;/sub&gt; DH10B</td>
<td>mT3&lt;sub&gt;pVirF&lt;/sub&gt; + pplaH9.8&lt;sup&gt;SS&lt;/sup&gt;-hIL-10 E. coli DH10B</td>
<td>(This study)</td>
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<tr>
<td>BS103: pmT3&lt;sub&gt;pVirB&lt;/sub&gt; + pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αASC VHH-HA BS103</td>
<td>pmT3&lt;sub&gt;pVirB&lt;sup&gt; IPTG&lt;/sup&gt;&lt;/sub&gt; + pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αASC VHH-HA BS103</td>
<td>(This study)</td>
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<tr>
<td>BS103 pmT3&lt;sub&gt;pVirB&lt;/sub&gt; + pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αCTLA-4 VHH-HA BS103</td>
<td>pmT3&lt;sub&gt;pVirB&lt;sup&gt; IPTG&lt;/sup&gt;&lt;/sub&gt; + pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αCTLA-4 VHH-HA BS103</td>
<td>(This study)</td>
</tr>
<tr>
<td>BS103 pmT3&lt;sub&gt;Δeff&lt;/sub&gt;_pVirB + pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αASC VHH-HA BS103</td>
<td>pmT3&lt;sub&gt;Δeff&lt;/sub&gt;_pVirB&lt;sup&gt; IPTG&lt;/sup&gt; + pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αASC VHH-HA BS103</td>
<td>(This study)</td>
</tr>
<tr>
<td>BS103 pmT3&lt;sub&gt;Δeff&lt;/sub&gt;_pVirB + pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αCTLA-4 VHH-HA BS103</td>
<td>pmT3&lt;sub&gt;Δeff&lt;/sub&gt;_pVirB&lt;sup&gt; IPTG&lt;/sup&gt; + pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αCTLA-4 VHH-HA BS103</td>
<td>(This study)</td>
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<tr>
<td>BS103 pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αStx VNA-FLAG BS103</td>
<td>pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αStx VNA-FLAG BS103</td>
<td>(This study)</td>
</tr>
<tr>
<td>HS: pmT3&lt;sub&gt;Δeff&lt;/sub&gt;_pVirB + pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αStx VNA-FLAG HS</td>
<td>pmT3&lt;sub&gt;Δeff&lt;/sub&gt;_pVirB&lt;sup&gt; IPTG&lt;/sup&gt; + pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αStx VNA-FLAG E.coli HS</td>
<td>(This study)</td>
</tr>
<tr>
<td>pmT3&lt;sub&gt;pVirB&lt;/sub&gt; + pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αStx VNA-FLAG HS</td>
<td>pmT3&lt;sub&gt;pVirB&lt;sup&gt; IPTG&lt;/sup&gt;&lt;/sub&gt; + pOspC3&lt;sup&gt;SS&lt;/sup&gt;-αStx VNA-FLAG E.coli HS</td>
<td>(This study)</td>
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<sup>1</sup>Vp, Virulence plasmid

<sup>2</sup>SS denotes the first 150 bp of the N-terminal open reading frame sequence for each gene

**Table S2: Bacterial Strains** Table describes all bacterial strains developed and tested throughout this work.